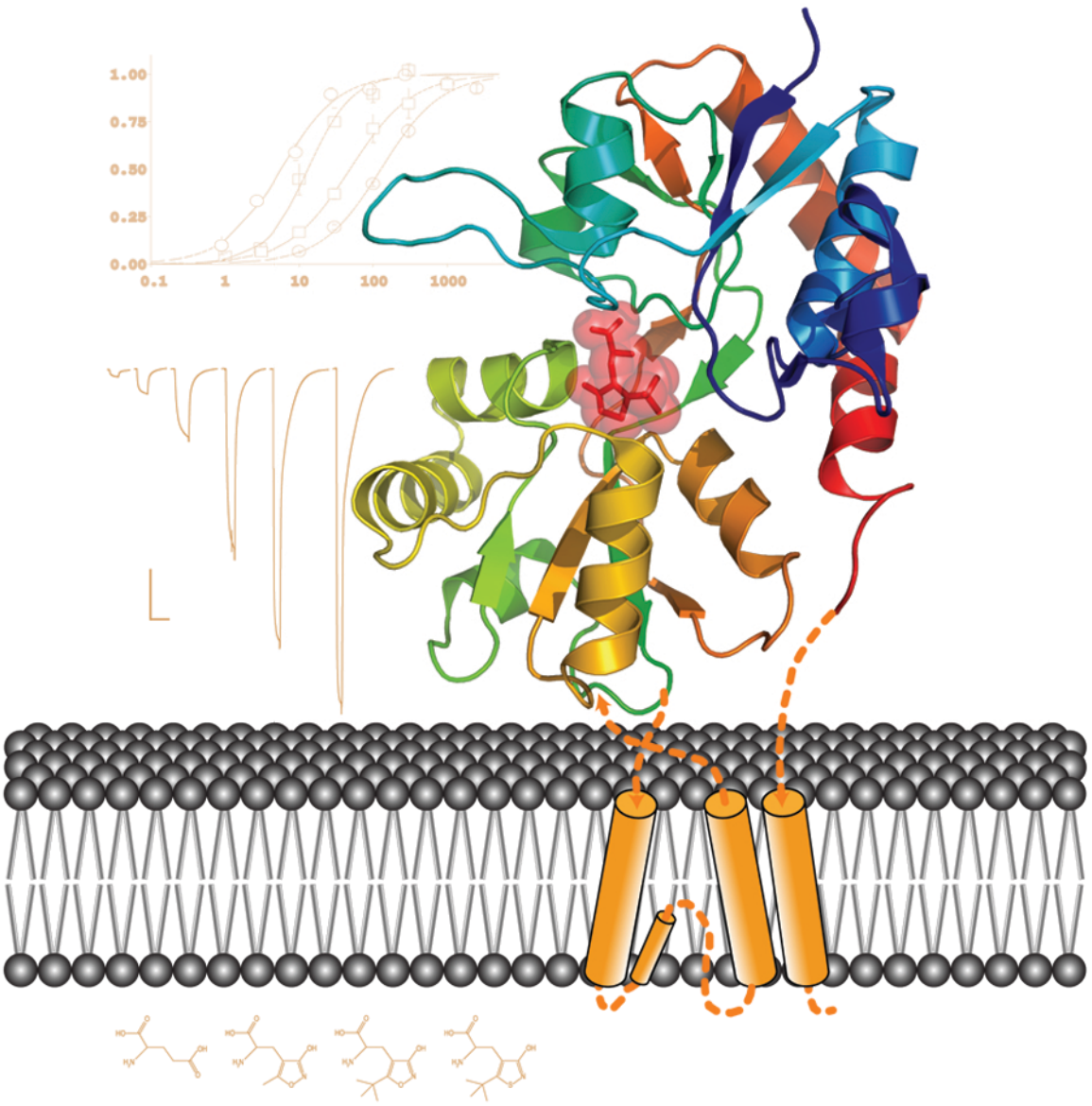
Amino Acid Receptor Research



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Editors

NOVA

AMINO ACID RECEPTOR RESEARCH

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Contents

Preface

Amino acid receptors and cell surface proteins that bind amino acids and trigger changes which influence the behavior of cells. Glutamate receptors are the most common receptors for fast excitatory synaptic transmission in the vertebrate central nervous system, and GAMMA-AMINOBUTYRIC ACID and glycine receptors are the most common receptors for fast inhibition. This new book presents the latest research in the field.

Expert Commentary - Transmitter-gated ion channels are integral membrane protein complexes that modulate synaptic neurotransmission directly through the binding of a transmitter and the opening of a pore permeable to specific ions. Ligand-gated channels can be divided in three major families: the cys loop receptors including nicotinic, GABA, glycine and 5-HT3A receptors, glutamate-gated channels (AMPA, NMDA and kainate) and ATP P2X receptor-channels. In view of the clear structural differences between ligand-gated channel families, it has been assumed that each receptor type acts independently of the other. However, recent studies have challenged this principle of independence by showing that the co-activation of P2X and nicotinic receptors induces a current that is less than the sum of currents induced by applying the two transmitters separately. Activity-dependent cross-inhibition was also observed between $P2X_2$ and 5-HT3A or between several P2X and ionotropic GABA receptors. The close proximity of $P2X_2$ and $\alpha4\beta2$ nicotinic channels, the physical association between $P2X_2$ and 5-HT3 or GABA gated-channels as well as the involvement of the intracellular domain of both receptors strongly suggested that a molecular coupling underlies their activity-dependent cross-inhibition. In addition, the interaction between ATP and GABA-gated channels may also regulate receptor trafficking and targeting. However, the functional interaction between distinct ligand-gated channels appears to be a complex molecular process and the identification of the precise underlying mechanisms and regulatory factors requires further studies. Asymetrical cross-inhibition has also been observed between AMPA and NMDA receptors and between $GABA_A$ and glycine receptors, although in the latter case, the interaction was dependent on intracellular phosphorylation pathways triggered by glycine receptor activation. Therefore, interactions between distinct ligand-gated channels represent a new mechanism for receptor regulation, which may be essential for the integration of fast synaptic signaling.

Short Communication - The N-methyl-D-aspartate subtype of glutamate receptor (NR) is a ligand-gated ionic channel that plays an important role in synaptic plasticity, memory and spatial learning and is also implicated in the sensitization of pain pathways. For these properties, it represents a prominent drug target. Research on NR ligands and modulators has

progressed enormously by the use of heterologous cell systems, in which NR complexes of known composition are selectively expressed. All functional NRs are tetrameric complexes and contain two copies of the essential subunit NR1 and two copies of any of the four different NR2s (A, B, C and D); so the heterologous expression of NR channels is complicated by the requirement for two types of subunit. The expression system of first choice, RNA-injected *Xenopus laevis* oocytes, has some drawbacks mainly correlated with the big dimensions of the cells and their endogenous currents. On the other hand, transfected mammalian cells represent an ideal model to study biophysical and pharmacological properties of NR. As permanent transfection is hardly feasible, reliable and efficient protocols for transient transfection are greatly required. The authors describe here an optimized protocol we developed for HEK293 cells and that involves the use of an electroporator and CD8 antibody coated beads to identify transfected cells without the need of a specific assay or equipment. Their method allows to produce NR expressing cells on a daily basis suitable for direct electrophysiological investigation and other functional assays.

Chapter 1 - Glutamate is present in several cell types in the retina, including photoreceptors, bipolar and ganglion cells, constituting a major excitatory pathway in the tissue. The presence of different classes of metabotropic and ionotropic receptors was detected since the early stages of development. The chick retina is an excellent model for the study of neurochemical events during CNS development since many neurotransmitters, neuromodulators and neuroactive substances found in the brain are also found in this tissue playing different roles in the mature as in the developing tissue. Moreover, early developing chick retinal cells can be dissociated and mantained in culture for relatively long periods where they differentiate many properties of the intact retina. In the present work, the authors review neurochemical and developmental aspects of glutamate receptors in the chick retina. Emphasis is given to the following aspects: i) glutamate-induced release of neurotransmitters and neuroactive substances such as GABA, acetylcholine, adenosine and ascorbate in cultures of retinal cells; ii) glutamate excitotoxicity in purified cultures of retinal neurons and its regulation by adenosine and nitric oxide; iii) regulation of protein synthesis and nitric oxide production by NMDA receptors; iv) transduction pathways involved in glutamate signaling. The results reveal a complex neurochemical network regulated by glutamate in the developing and mature retina.

Chapter 2 - The concept of an intricate network of immuno-neuro-endocrine interactions has evolved in the last 30 years. The scientific field investigating these interactions has been termed “psychoneuroimmunology”, and it was proposed that the immune system plays a sensory role, as a “sixth sense”, that detects invaders such as pathogens, antigens, allergens and tumors with great sensitivity and specificity. In addition, the fact that each of the systems (i) can produce neurotransmitters, -peptides, hormones, and cytokines, (ii) expresses a common receptor repertoire, and (iii) shares signal transduction pathways, leads to the conclusion that the systems use a common chemical language for intra- and inter-systemic communications. In the last years it became apparent that there are also intimate interactions between the immune system and glutamate receptor-mediated signalling mechanisms. Immune cells have been shown to express different types of glutamate receptors as well as subtypes of the high-affinity glutamate transporter family, similar to observations in other peripheral tissues. Glutamate and its receptors were found to modulate specific and non-specific immune functions, as well as neural activity associated with pain conditions. Furthermore, it was suggested that glutamate may serve as a fast-acting signalling agent

between nerval and immune tissues, providing the basis for a multidirectional flow of information among the different organ systems within the body. Taken together, these results indicate that glutamate, besides acting as the major neurotransmitter in the central nervous system, is also involved in peripheral neuroimmunomodulation, working in a receptor/transporter-mediated manner. In summary, new insights into these communication circuits should provide a better understanding of mind-body medicine – a priority for medicine and human life.

Chapter 3 - The first high resolution structure of an ionotropic glutamate receptor ligand-binding core was published a decade ago (Armstrong et al., 1998). This discovery was a breakthrough within the glutamate receptor field and has enabled a cascade of structure-function studies on the mechanism of action of this receptor family. Insights from these studies have contributed significantly to the understanding of the series of events coupling ligand-binding to channel motions for the glutamate receptors, but may also add valuable input to researchers working with other ligand activated receptors.

This chapter focuses on a line of studies where X-ray analysis has been combined with electrophysiological studies of a range of ligand-receptor combinations. Here the agonist 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) has been employed as an important pharmacological tool due to the large number of substituents, which have been introduced on the isoxazole ring. This has revealed important information on ligand selectivity and kinetic properties of the receptor complex. Given that more than fifty structures of the AMPA receptor ligand-binding domain have been determined, our centre of attention is the AMPA receptor family, however where appropriate, the authors will correlate the results to the closely related kainate receptors.

Earlier studies explored the central ligand-binding pocket in order to gain agonist selectivity. In this chapter the authors describe how recent efforts resulted in ligands that protruded out from the AMPA binding site and reached into a novel sub-domain to obtain highly subunit selective agonists.

Important information has also been obtained on the desensitization mechanism. It was recently revealed that desensitization results from a disruption of the interactions between the upper parts of the ligand-binding core. The authors speculate that a novel interdomain twist observed in some crystal structures might facilitate these movements. In addition, they will discuss the high-resolution structures of AMPA-derived competitive antagonists bound to the ligand-binding site of the AMPA receptor GluR2.

In this chapter the authors first present a basic introduction to the AMPA receptors followed by a brief description of the agonist and antagonist induced changes in the receptor complex. Subsequently, they discuss selected structure-function studies grouped after position of the substituent in the AMPA isoxazole ring.

Chapter 4 - Many cellular elements of the skin appear to involve glutamate for signalling. This has been most extensively studied in nociceptive nerve endings (supplied by C and Aδ fibres). Both ionotropic and metabotropic glutamate receptors are involved in inflammation, and modulate hyperalgesia and allodynia. Less work has been on low threshold mechanoreceptors in the skin (supplied by Aβ fibres), although there is clear evidence for a modulatory role here too. There is evidence that other structural cellular elements, such as keratinocytes use glutamate for signaling, and may be important for skin growth and wound healing. Finally, excessive release of glutamate and/or aberrant expression of metabotropic glutamate receptors may be contributory factors in the development of skin melanomas.

There appears to be significant clinical potential in the development of topically applied glutamate receptor active compounds to treat a range of different skin disorders.

Chapter 5 - The neuronal activity modulates the efficacy of synaptic transmission at both excitatory and inhibitory synapses in the central nervous system. Such an activity-dependent synaptic plasticity has been regarded as a cellular basis for learning and memory. Since balanced regulation of excitation and inhibition is critical for the information processing, clarification of detailed mechanism to control inhibitory synaptic plasticity is important. Fast neuronal inhibition is mainly mediated by gamma-amino butyric acid receptor type A ($GABA_AR$). In this article, the authors summarize recent advances regarding the regulatory molecular mechanism of plasticity at $GABA_AR$-mediated synapses. They shortly review several forms of plasticity at GABAergic synapses in various brain regions, and then focus on synaptic plasticity in the cerebellum. At the GABAergic synapses on a Purkinje neuron, four forms of synaptic plasticity have been reported. They are short-term potentiation or depression of presynaptic release probability of synaptic vesicles, long-term potentiation of postsynaptic $GABA_AR$ responsiveness and long-term depression. They discuss how different patterns of neuronal activity leads to different forms of synaptic plasticity, what kind of intracellular signaling cascades including protein kinases/phosphatases control the induction of plasticity, and how $GABA_AR$ binding proteins are implicated in modification of $GABA_AR$ function.

Chapter 6 - The mammalian central nervous system (CNS) is a highly complex integrative system, processing a vast number of positive and negative inputs. Proper functioning of the brain depends on a delicate interplay between excitatory and inhibitory neurotransmission among neurons. The major inhibitory neurotransmitter in the vertebrate brain is gamma-aminobutyric acid (GABA). GABA mediates its inhibitory effect through its interaction with a variety of receptors in all areas of the central nervous system. Two major types of GABA receptors have been well described: Type A GABA receptors ($GABA_AR$) and type B GABA receptors ($GABA_BR$). These receptors are quite different, not only from a pharmacological point of view but also structurally and functionally. From the therapeutic point of view the relevance of $GABA_AR$ is determined by the fact that its function is regulated by pharmaceutically significant drugs. The variety of genes discovered to encode the different subunits and subunit isoforms of the $GABA_AR$ and the pentameric stoichiometry imply a tremendous heterogeneity of $GABA_AR$ subunit composition. This raises the question as to why there is such a structural diversity of $GABA_AR$ subunits and why this exceeds the currently known functional diversity of GABA-mediated currents in neurons. It is conceivable that $GABA_AR$ heterogeneity has evolved so as to meet the specific requirements of distinct neuronal cells. The clustering of $GABA_ARs$ at discrete and functionally significant domains on the nerve surface is an important determinant in the integration of synaptic inputs. The large structural heterogeneity of $GABA_AR$ led to the hypothesis that there could be a link between $GABA_AR$ gene diversity and the targeting properties of the receptor complex. $GABA_AR$ lateral mobility could be a mechanism by which neurons recruit and maintained specific receptors at specific synaptic sites. In fact evidence suggests that the subunit composition of the receptors determines not only different targeting characteristics but also differences in the lateral mobility of the complexes.

The types of subunit isoforms that compose the protein complex could determine the different ways in which these receptors are routed to specific synaptic locations. This might represent an exquisite form of synaptic plasticity.

Chapter 7 - Pain enables the specialized nervous system to detect sources of bodily harm and trigger responses which can prevent or mitigate potential damage. Chronic or intractable pain is the most common reason for a visit to a physician in the United States. Recent studies demonstrate that plastic changes in neurons of the central nervous system (CNS) may contribute to the mechanisms that underlie pain hypersensitivity. The neurotransmitter glutamate and its membrane receptors play significant roles in generating and maintaining a variety of sustained neuronal responses. Activation of NMDA receptors and the consequent influx of calcium are major events that contribute to pain processing. For this reason, antagonists of NMDA receptors, such as ketamine and phencyclidine are commonly used as anesthetics in clinical practice. However, the disturbance of glutamate systems may cause a variety of neuronal toxicities, such as abnormal neuronal development, abnormal synaptic plasticity and neurodegeneration. This review focuses on the role of glutamate receptors, especially NMDA receptors, in pain processing. As tools for clinical interventions, antagonists of NMDA receptors will be discussed with particular emphasis on their ability to induce neurodegeneration during development.

Chapter 8 - Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system. This amino acid activates both metabotropic and ionotropic glutamate receptors. Among the latter class of receptors is the *N*-methyl-D-aspartate (NMDA) receptor, which activation at the level of the spinal cord increases neuronal responsiveness resulting in central sensitisation. This is manifested clinically as altered pain in which non-noxious stimulation is perceived as painful (allodynia) and normally painful stimuli are felt as even more painful (hyperalgesia). The increased nociceptive response is believed to be due to the entry of Ca^{2+} into the neurons, which triggers different metabolic pathways activating a number of enzymes and ultimately immediate-early genes. The increase in intracellular Ca^{2+} concentration after NMDA receptor stimulation activates phospholipases. Activation of phospholipase A_2 hydrolyses membrane phospholipids increasing the level of free arachidonic acid. Arachidonate, in turn, may be converted by cyclooxygenases into prostaglandins, which activate specific prostanoid receptors. In addition to activating phospolipases, Ca^{2+} entering through NMDA receptors activates calcineurin and binds to calmodulin forming the Ca^{2+}-calmodulin (CaM) complex. Calcineurin desphosphorylates the constitutive nitric oxide synthase (NOS) enzymes, which catalytic activity is usually reduced by protein kinase C-induced phosphorylation, and the CaM complex further activates the enzyme. NOS catalyze the conversion of L-arginine into L-citrulline and NO, and this gas activates guanylate cylases to increase cGMP production. Apart from interacting with each other, retrograde second-messengers resulting from these cascades cause presynaptic and interneuron augmentation of neurotransmitter and/or neuropeptide release, as well as the formation and expression of new receptors. All these complex mechanisms involved in the amplification and prolongation of neural responses, which underlie many forms of spinal sensitisation, are discussed in this chapter.

Chapter 9 - The γ-aminobutyric acid type A ($GABA_A$) receptors are ligand gated chloride ion channels in the central nervous system (CNS). These receptors are hetero-oligomeric proteins composed of the different subunit peptides, which are differentially expressed in the different regions of the brain and are responsible for the major fast inhibitory signal transmission in the neuronal circuits, which in turn prevent the brain from overwhelm in stressful situations. Enhancement of inhibitory signals through $GABA_A$ receptors is a potential therapeutic strategy for the treatment of anxiety disorders, sleep disturbances,

seizure disorders and muscle spasms. Mood-defining drugs such as benzodiazepine derivatives, barbiturates, neuro-steroids, and general anesthetics act on $GABA_A$ receptors. Various components of foods and beverages such as ethanol in liquors potentiate the responses of both synaptic and extra-synaptic $GABA_A$ receptors. Reportedly, behavioral consumptions of alcohol as low as 3 mM potentiate the response of extra-synaptic $GABA_A$ receptors. However, synaptic $GABA_A$ receptors, which consist of γ-subunit with combination of other subunits, are being potentiated only at high concentration of ethanol (>50 mM). Various fragrant compounds in foods and beverages, i.e., cineol, eugenol, geraniol, linalool, *cis*-jasmone, methyl jasmonate, 1-octen-3-ol, myrcenol, terpinen-4-ol, ethyl phenylpropanoate may have calming effect on mood or consciousness because they also strongly enhance the inhibitory signal transmission through $GABA_A$ receptors similar to ethanol, and mood-defining drugs. It has already been revealed that alcohols with long carbon chain, esters with short carbon chain, and phenol derivatives with low numbers of phenolic groups strongly enhance $GABA_A$ receptor responses. These fragrant compounds also increase the pentobarbital-induced sleeping time or delay pentetrazole-induced convulsions in mice when they are injected to mice prior to intraperitoneal administration of pentobarbital or pentetrazole. Moreover, fragrant compounds also decrease restriction-stress-induced increases in the plasma adrenocorticotropic hormone (ACTH) levels in rats, as does diazepam, a benzodiazepine derivative. Furthermore, behavioral studies, i.e., elevated plus maze, vogel's anti-conflict test, ambulatory activity in mammals proved that fragrant compounds have anxiolytic effects, which is mediated through $GABA_A$ receptors. Thus such fragrant compounds in foods and beverages may have calming effects on the brain, affecting moods or consciousness.

Chapter 10 - AMPA receptor-mediated excitotoxicity has been proposed to play a major role in death of motor neurons in amyotrophic lateral sclerosis (ALS), the most common motor neuron disease. The authors demonstrated that RNA editing of GluR2 mRNA at the Q/R site was decreased in autopsy-obtained spinal motor neurons of patients with sporadic ALS. This molecular change did not occur in the other regions of central nervous system (CNS), and has been demonstrated as a molecular change inducing neuronal death in mutant mice, hence may be highly relevant to ALS pathogenesis. They found that the extent of GluR2 Q/R site-editing was reduced to various extents in motor neurons of all the sporadic ALS cases irrespective of the phenotype. On the contrary, although AMPA receptor-mediated neurotoxicity plays a role in SOD1-associated familial ALS (ALS1), the underlying molecular mechanism is an up-regulation of GluR2-lacking Ca^{2+}-permeable AMPA receptors but not GluR2 Q/R site-underediting as seen in motor neurons of sporadic ALS. The molecular mechanism increasing the GluR2-lacking AMPA receptors in ALS1 model rats may likely result from up-regulation of GluR3 mRNA as occurred in kainic acid-induced ALS model rats that we recently developed, hence over-activation of AMPA receptors might participate in death of motor neurons in ALS1. On the contrary, AMPA receptor-mediated neuronal death mechanism did not seem play a role in dying motor neurons of spinal and bulbar muscular atrophy (SBMA).

Because GluR2 Q/R site-editing is specifically catalyzed by adenosine deaminase acting on RNA type 2 (ADAR2), it is likely that regulatory mechanism of ADAR2 activity does not work well in the motor neurons of sporadic ALS. Although ADAR2-null mice die young from status epilepticus, conditional ADAR2 knockout mice that the authors newly developed exhibited slow progressive death of motor neurons as seen in transgenic mice for artificial

Ca^{2+}-permeable GluR2 (GluR-B(N)). Therefore, it is likely that deficient ADAR2 activity induces neuronal death via underediting of GluR2 Q/R site. Indeed, ADAR2 expression level was significantly decreased in the spinal ventral gray matter of sporadic ALS as compared to normal control subjects. Taken together, it is likely that ADAR2 underactivity selective in motor neurons induced induces deficient GluR2 Q/R site-editing and resulting neuronal death in sporadic ALS.

Thus, there seem to be multiple different molecular mechanisms underlying death of motor neurons in various motor neuron diseases, and the molecular mechanism generating Ca^{2+}-permeable AMPA receptors that mediated neuronal death may not be uniform among death of motor neurons; an increase of GluR2-lacking Ca^{2+}-permeable AMPA receptors plays a role in ALS1, whereas an increase of Q/R site-unedited GluR2-contatining Ca^{2+}-permeable AMPA receptors in sporadic ALS.

Chapter 11 - Anatomical evidence has shown that glutamate receptors are localized on the peripheral terminals of primary afferent neurons. Electrical stimulation of primary afferents results in release of glutamate peripherally. This raises the question whether glutamate and its receptors enable autocrine and paracrine signaling between nerve terminals in the periphery. The authors found that spontaneous nerve discharge increases in a cutaneous branch from a spinal dorsal ramus in the thorax following antidromic stimulation of a cutaneous branch from an adjacent spinal segment. This enhanced activity can be blocked by local application of NMDA or non-NMDA receptor antagonists into the receptive field of the recorded nerve. It is well known that the dorsal cutaneous branches of the selected thoracic nerves run parallel to each other and no synaptic contacts at the periphery are known to exist. In addition, because each of the branches are transected distal to the spinal cord, it provides a new model by which they can abolish central interactions and investigate the role of glutamate and its receptors in non-synaptic communication between peripheral nerve terminals arising from different spinal segments. The preparation has provided evidence that glutamate released from peripheral nerves may contribute to the occurrence of secondary hyperalgesia and allodynia outside the area of initial injury. The excitatory neurotransmitters such as substance P (SP) and adenosine triphosphate (ATP) may have functions analogous to that of glutamate in peripheral tissues. Some inhibitory neurotransmitters such as opioid and somatostatin appear capable of antagonizing glutamate's effect in peripheral tissues. The functional balance between excitatory and inhibitory neurotransmitters may help maintain normal physiological conditions. This review will focus on the role of glutamate receptors in the peripheral nervous system. It will provide a basis for understanding that glutamate receptors in peripheral tissues could serve as a new target for drug delivery to relieve pain of peripheral origin and mechanosensitivity disorder in gastrointestinal tract.

Chapter 12 - Glutamate is the most abundant excitatory neurotransmitter in the mammalian nervous system. It acts through ionotropic and metabotropic receptors which mediate fast excitatory and second messenger-evoked transmission, respectively. It is well known that glutamate can be neurotoxic causing neurodegeneration in many acute and chronic disorders such as ischemic stroke, epilepsy and Parkinson's disease through overactivation of glutamate receptors. In addition to neuronal death, damage to the white matter in a spectrum of clinical conditions including stroke, multiple sclerosis and other neurodegenerative disorders may be related to glutamate receptor-mediated injury. Presence of excess extracelluar glutamate resulting in excitotoxicity has also been put forward as a predominant mechanism of injury to developing cerebral white matter. Activation of

glutamate receptors is thought to initiate damage in the adult and developing brain by a cascade of events such as increased calcium influx and enhanced production of cytokines which are known to cause oligodendrocyte death resulting in destruction of myelin and dysfunction of axons. Glutamate receptor antagonists have proven to be promising therapeutic agents in attenuating white matter damage in the developing and adult brain.

Chapter 13 - The concentration of glutamate in brain extracellular fluid is presumed to be biologically negligible under nonpathological conditions. But this is unlikely to be true. In fact, measurements suggest that ambient extracellular glutamate concentrations are high enough to cause significant steady-state desensitization of glutamate receptors, implying that ambient extracellular glutamate may be a critical regulator of synaptic transmission and brain function. Unfortunately, in vivo measurements of ambient extracellular glutamate have serious caveats, and new less invasive techniques for measuring extracellular composition have yet be developed. It's also unclear what controls ambient extracellular glutamate; virtually nothing is known about the molecular regulation of this glutamate pool. Finally, there are significant challenges posed by the idea that brain function can only really be understood in the context of a three-dimensionally-intact and volumetrically-perfused structure. Nevertheless, tackling these difficulties promises significant reward.

Chapter 14 - Glutamate is the major excitatory neurotransmitter of the brain, which acts at specific receptors located on the post-synaptic membrane of most CNS neurons. These receptors then exert their effect either *via* a G-protein-linked signalling cascade (metabotropic) or though the opening of an intrinsic ion channel (ionotropic).

The ionotropic glutamate receptors can be sub-divided into classes based on their affinity for selective agonists. The *N*-methyl-D-aspartate (NMDA) class of receptors ($GluR_{NMDA}$) are heteromeric receptors that are both ligand- and voltage-gated. The opening of the $GluR_{NMDA}$ ion channel is dependent on the binding of both glutamate and the co-agonist glycine, together with attenuation of the plasma-membrane potential to remove the Mg^{2+} block. Activation of $GluR_{NMDA}$ receptors leads to an influx of Na^{+} and Ca^{2+} ions into the cell, altering its membrane potential further toward the threshold for the generation of an action potential. The increase in intracellular Ca^{2+} also affects several Ca^{2+}-dependent signaling pathways.

Amino-acid transmission and the action of ethanol in the human brain are closely linked. Ethanol acts at the $GluR_{NMDA}$ to specifically alter its activity, especially by reducing Ca^{2+} influx. This interaction is influenced by the subunit composition of the receptor. Ethanol is thought to bind to the NR1 subunit, but this binding is altered by the identity of the NR2 subunits that make up the complex.

Under conditions of persistent ethanol exposure, such as in the alcoholic brain, $GluR_{NMDA}$ undergoes a process of adaptation. This adaptation is brought about by the regulation of the expression of the various subunits, leading to an alteration in the number and type of subunits that comprise the receptor complex. This process is notably influenced by the genotype and co-morbid pathology of the individual.

Here the authors review the effects of acute ethanol administration and long-term ethanol exposure on $GluR_{NMDA}$ sites. They also present recent research on the quantification of expression of $GluR_{NMDA}$ subunits.

Chapter 15 - Umami is a human taste quality, in most cases produced by some amino acids, such as glutamic acid and aspartic acid. This chapter briefly introduces to the concept of umami taste, presents current status of the research on umami in mammals and in larvae of

Lepidoptera (caterpillars), speculates whether or not umami is a separate taste modality in caterpillars as it is in humans or rodents, and (using the case of the codling moth, *Cydia pomonella* L., as an example) delineates the directions for using comparative taste physiology in rational plant protection.

Chapter 16 - The amino acid glutamate is the major excitatory neurotransmitter in the brain. Neuronal interactions and signaling in the olfactory system are mediated by a variety of neurotransmitters, principally including glutamate. In the main olfactory bulb (MOB) of the brain, glutamate acts at multiple levels of synaptic processing. At the level of sensory input to the MOB glutamate is released from olfactory nerve terminals into olfactory glomeruli. Glomeruli are structural and functional odorant receptor-specific units that serve as the initial sites of synaptic processing in the olfactory system. They house the dendrites of projection neurons and local interneurons, juxtaglomerular cells. Projection neurons of the MOB, mitral and tufted cells, are glutamatergic themselves, and glutamate released from their dendrites acts to mutually excite and couple the activity of output cells within the same glomerular unit. Glutamate released from these neurons also mediates dendrodendritic transmission at synapses with juxtaglomerular cells and with GABAergic granule cells deeper in the MOB, controlling MOB output to higher centers of the brain. At this level of processing, axons of centrifugal fibers originating in olfactory cortical areas in turn provide spatially segregated glutamatergic input to the MOB.

New experiments in the MOB have used tract tracing, recordings of field potentials, patch-clamp recordings, microdissection, flash photolysis, two-photon-guided local stimulation, and optical-imaging techniques. These experiments describe novel cellular and network olfactory mechanisms that process odor-specific activity patterns elicited by activation of glutamatergic inputs to MOB neurons. Neuronal activity in the MOB is controlled by glutamate acting at ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, kainate receptors, and N-methyl-D-aspartate (NMDA) receptors, as well as metabotropic glutamate receptors coupled to intracellular second messengers. Glutamate activates serial excitatory and inhibitory neural circuits in the MOB such that olfactory processing is regulated through interplay of responses to excitatory glutamate receptors and inhibitory neurotransmitter receptors. Olfactory coding is both spatially and temporally dynamic: glutamate receptors are a key element generating the rhythmic, correlated activity within the MOB that underlies the neural encoding of odors.

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Expert Commentary

Interactions between Ligand-Gated Ion Channels: A New Regulation Mechanism for Fast Synaptic Signaling?

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Abstract

Transmitter-gated ion channels are integral membrane protein complexes that modulate synaptic neurotransmission directly through the binding of a transmitter and the opening of a pore permeable to specific ions. Ligand-gated channels can be divided in three major families: the cys loop receptors including nicotinic, GABA, glycine and 5-HT3A receptors, glutamate-gated channels (AMPA, NMDA and kainate) and ATP P2X receptor-channels. In view of the clear structural differences between ligand-gated channel families, it has been assumed that each receptor type acts independently of the other. However, recent studies have challenged this principle of independence by showing that the co-activation of P2X and nicotinic receptors induces a current that is less than the sum of currents induced by applying the two transmitters separately. Activity-dependent cross-inhibition was also observed between $P2X_2$ and 5-HT3A or between several P2X and ionotropic GABA receptors. The close proximity of $P2X_2$ and $\alpha4\beta2$ nicotinic channels, the physical association between $P2X_2$ and 5-HT3 or GABA gated-channels as well as the involvement of the intracellular domain of both receptors strongly suggested that a molecular coupling underlies their activity-dependent cross-inhibition. In addition, the interaction between ATP and GABA-gated channels may also regulate receptor trafficking and targeting. However, the functional interaction between distinct ligand-gated channels appears to be a complex molecular process and the identification of the precise underlying mechanisms and regulatory factors requires further studies. Asymetrical cross-inhibition has also been observed between AMPA and NMDA

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receptors and between $GABA_A$ and glycine receptors, although in the latter case, the interaction was dependent on intracellular phosphorylation pathways triggered by glycine receptor activation. Therefore, interactions between distinct ligand-gated channels represent a new mechanism for receptor regulation, which may be essential for the integration of fast synaptic signaling.

Synaptic transmission between neurons is achieved through the release of one or more neurotransmitters or modulators from the same presynaptic terminal, resulting in the activation of different classes of receptors co-localized at the same post-synaptic site.

Receptors are classified by their transduction mechanisms into two main families: G protein-coupled receptors (GPCRs) and ligand-gated ion channels (LGICs). In response to the binding of the ligand, GPCRs mediate their activation through the activation of G proteins which engage second messenger pathways; whereas ligand-gated channels lead to the opening of a central pore permeable to selected ions. GPCRs are typically monomeric proteins with seven transmembrane domains, an extracellular N-terminal domain and an intracellular COOH-terminal domain. Genome and cDNA sequencing analysis has revealed that there is three major families of ligand-gated channels, each with an unique architecture. Members of the nicotinic acetylcholine receptor family - also called “cys loop” receptors - include cationic receptor-channels for acetylcholine (Ach), serotonin (5-HT) and anionic receptor-channels for γ-aminobutyric acid (GABA) and glycine (Sine and Engel, 2006). These receptors are heteropentamers of subunits with four transmembrane domains (TM), a large extracellular domain, an intracellular loop between TM3 and TM4, and a short C terminal tail. Glutamate receptors are cation-selective channels divided into AMPA, NMDA and kainate receptors formed by a tetrameric association of subunits with three transmembrane domains and a reentrant loop between TM1 and TM2 (Mayer, 2005). ATP- and proton-gated-channels constitute the recently discovered receptor family. Although these are unrelated in terms of sequences, both channel families consist of trimeric homo- or heteromeric association of subunits with two transmembrane domains, a large extracellular domain, and both intracellular NH_2 and COOH termini (Khakh et al., 2001)(Fig. 1).

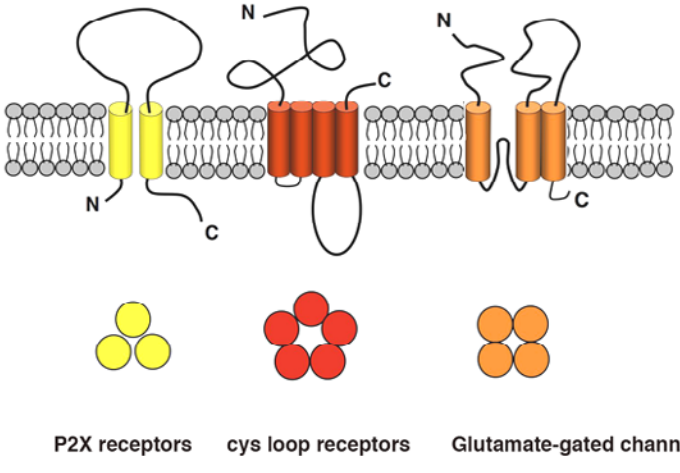

Figure 1. Schematic representations of subunit topology and stoichiometry of the different ionotropic receptors.

In view of the clear structural differences between neurotransmitter-gated channels, it was assumed that each receptor type acts independently of the other. However the principle of independence has now been challenged by several studies that provide strong evidence for functional interactions between several distinct ligand-gated channels.

Nakazawa et al first observed in rat phaeochromocytoma (PC12) cells, that the combined effect of ATP and nicotine was less than the linear sum of the individual ATP and nicotinic currents and initially proposed that ATP and nicotine activated the same channels formed by an association of P2X and the nicotinic subunit around the same pore (Nakazawa et al., 1991). Cross-inhibition between P2X and the nicotinic receptor was confirmed by several groups studying sympathetic, myenteric neurons and in oocytes co-expressing $P2X_2$ and $\alpha3\beta4$ nicotinic receptors (Barajas-Lopez et al., 1998; Khakh et al., 2000; Searl et al., 1998; Zhou and Galligan, 1998). Together these studies clearly demonstrated that $P2X_2$ and nicotinic receptors form separate channels and that the co-activation of both receptors results in non-additive responses owing to an inhibition of both channel types. The authors also found that the current inhibition was bidirectional and did not involve ligand binding sites, a calcium-dependent mechanism, a change in the driving force, or a cytoplasmic signaling mechanism. In addition, decreasing the level of expression of $P2X_2$ and $\alpha4\beta4$ nicotinic receptors resulted in additive responses suggesting that a close proximity of the receptors is required for the inhibition (Khakh et al., 2000). Similar inhibitory cross-talk was also demonstrated between $P2X_2$ and 5-HT3, and between $P2X_2$ and $GABA_{A/C}$ receptor-channels through co-expression studies and in native myenteric neurons (Fig. 2) (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a; Boue-Grabot et al., 2004b). The investigation of $P2X_2$ and $GABA_A$ receptor co-activation revealed that direction of the inhibition was dependent on a specific $GABA_A$ subunit composition (Boue-Grabot et al., 2004b). In cells expressing GABA receptors containing α and β subunits, the channels showed reciprocal inhibition, i.e. non-additive responses were recorded when ATP and GABA were co-applied, when GABA was administered during exposure to ATP, or when ATP was administered during exposure to GABA. In oocytes expressing α, β and γ $GABA_A$ receptor subunits, GABA inhibited the response to ATP whereas ATP did not inhibit the response to GABA. Regardless of the GABA receptor composition, current occlusion between $P2X_2$ and $GABA_{A/C}$ receptors was independent of the ion flow direction, calcium or voltage, thereby suggesting that a molecular process was involved.

Co-purification experiments indicated that $P2X_2$ interacts physically with 5-HT3 and $\rho1$ GABAc receptor channels (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a). In addition, the co-transfection of $\rho1$ and $P2X_2$ receptors revealed a co-clustering of these receptors in transfected hippocampal neurons and that $P2X_2$ receptors modified the addressing of $GABA_C$ receptors in a dominant way by inducing their translocation from an internal vesicular compartment to surface clusters common to both receptors. The close spatial arrangement between $P2X_2$ and $\alpha4\beta2$ nicotinic receptors in transfected hippocampal neurons was also demonstrated by fluorescence resonance energy transfer analysis using fluorescent-tagged subunits (Khakh et al., 2005), suggesting that activity-dependent cross-inhibition between $P2X_2$ and "cys loop" receptors including nicotinic, 5-HT3 or GABA receptors may arise from molecular interactions.

Indeed, it was recently discovered that G-protein coupled receptors can interact directly with ligand-gated channels leading to a functional and reciprocal modulation of each receptor

type. Such a direct interaction was first demonstrated between dopamine D5 and $GABA_A$ receptors. Through a series of biochemical approaches, Liu et al. convincingly demonstrated that the intracellular COOH-terminal tail of the D5 receptor specifically binds directly to the main intracellular loop of the $GABA_A$ γ2 subunit (Liu et al., 2000). D5 receptor activity was downregulated upon GABA receptor activation and the reciprocal activation of D5 receptors reduced the amplitude of GABA-induced currents. The reduction was abolished with the intracellular administration of peptides corresponding either to the D5 receptor or γ2 interacting domains showing that their physical coupling underlied the functional cross-modulation. A direct interaction was also demonstrated between D1 and NMDA receptors (Lee et al., 2002; Pei et al., 2004). In this case, the authors identified not one but two distinct sites of interaction: one between the C-terminal domain of the D1 and NR1 subunits and a second between the C-terminal domain of D1 and NR2A with different consequences for NMDA receptor function. By similar approaches, theses authors have also shown that the interaction between D1 and NR2A leads to an inhibition of the NMDA-induced current by D1 stimulation, whereas the interaction between D1 and NR1 seems to suppress NMDA receptor-mediated cell death upon D1 activation. Reciprocally, NMDA receptor activation increased the number of D1 receptors on the plasma membrane surface, and consequently enhanced the response to dopamine.

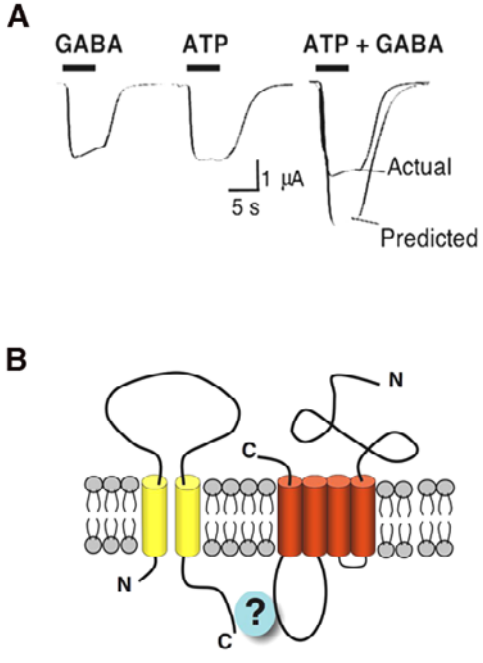

Figure 2. Functional and molecular interactions between $P2X_2$ and $GABA_A$-gated channels. A. Whole-cell current responses recorded in oocytes co-expressing α2β3 $GABA_A$ and $P2X_2$ receptors. Co-application of ATP + GABA (100 µM each) induced currents (Actual) significantly smaller than the sum (Predicted) of the individual ATP and GABA responses (from Boue-Grabot et al., 2004). B. Direct or indirect interactions between the C-terminal tail of P2X subunits and the main intracellular loop of cys loop receptor subunit underly the current inhibition.

The first evidence that molecular interactions lead to the functional cross-talk between ligand-gated channels came from experiments using a C-terminal truncated form of $P2X_2$ subunits. Truncation of this domain abolishes the functional cross-talk between $P2X_2$ and 5-HT3, nicotinic or GABA receptor-channels (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a). Likewise, expression of a minigene encoding either the distal C-terminal portion of the $P2X_2$ receptor or the main intracellular loop located between the third and fourth transmembrane domains of 5-HT3 or ρ1 subunits reduced the current inhibition between $P2X_2$ and 5-HT3 or ρ1 GABA receptor-channels, respectively. These results indicated that an interaction between the intracellular domains of each receptor is required for the functional cross-talk. For $GABA_A$ receptors which comprise heteromeric associations of α,β or α,β and γ subunits, it has been problematic to identify the actual subunits involved in the molecular coupling. This problem has been overcome by using chimeric ρ1-$GABA_A$ receptors with α2, β3 or γ2 sequences downstream from the third transmembrane domain (Boue-Grabot et al., 2004b). Each ρ1-$GABA_A$ receptor forms a functional homomeric receptor similar to the homomeric wild-type ρ1. Non-additive responses and co-clustering were observed between P2X2 and ρ1-β3 chimeras, but not with ρ1-α2 or ρ1-γ2, indicating that $P2X_2$ interacts mainly with β subunits of $GABA_A$ receptors. This is consistent with FRET experiments between $P2X_2$ and α4β2 nicotinic receptors which showed a higher fluorescence transfer efficiency between $P2X_2$ and β2 subunits, rather than α4 subunits (Khakh et al., 2005).

Although, most research has focused on the interaction of native and recombinant $P2X_2$ subtypes, several P2X channels have the ability to interact functionally with distinct ion channels. A cross-inhibition between $P2X_3$ or $P2X_{2/3}$ and $GABA_A$ was described in dorsal root ganglia neurons but, in contrast to other studies, the authors suggested an ion-dependent mechanism (Sokolova et al., 2001). It was proposed that chloride efflux through GABA-gated channels inhibits P2X channels and conversely, calcium influx though P2X inhibits $GABA_A$ receptors. This was in contrast to studies involving $P2X_2$ and "cys loop" receptors in particular with experiments showing that cation-selective $GABA_A$ receptor-channels, generated by a mutation of β3 subunits, did not prevent functional cross-inhibition with $P2X_2$ (Boue-Grabot et al., 2004b). These findings clearly demonstrated that chloride is not involved in this coupling, although it could be argued that $P2X_2$ or $P2X_3$ interact with other channels by distinct mechanisms. Recently Toulmé et al. (2007) provide convincing evidence for a molecular interaction between $P2X_3$ and $GABA_A$ receptors that was also responsible for the ion-independent cross-inhibition in both dorsal root ganglia neurons and a recombinant expression system (Toulme et al., 2007). The authors identified a specific intracellular motif of three consecutive amino-acids QST at positions 386-388 in the C-terminal tail of $P2X_3$ subunits required for the functional coupling with $GABA_A$ receptors. The current occlusion between native $P2X_3$ and $GABA_A$ receptors in DRG neurons was abolished either by infusion of a peptide containing the QST motif or a viral infection of the main intracellular loop of $GABA_A$ β3 subunits.

Together, these results strongly suggest that a molecular interaction between P2X and members of "cys loop" receptors leads to an activity-dependent cross-inhibition by a conformational spread mechanism in which the motion triggered by the gating of one channel type is communicated to the other channels and induces its closure (Bray and Duke, 2004).

However, presently, there is no clear evidence of a direct interaction between the intracellular domains of individual channels, and several findings also argue in favor of the

involvement of protein complexes to promote an association between distinct ligand-gated channels (Fig. 2).

The fact that ATP and ACh were shown to operate through independent receptors with an additive response in visceral ganglia neurons (Reyes et al., 2006) or that nicotinic and $P2X_2$ receptors were closely localized in hippocampal neurons but not in ventral midbrain neurons (Khakh et al., 2005), indicates that interactions between distinct ligand-gated channels may involve regulory factors.

It is also unlikely that such an interaction is direct, due to the fact that the interacting region of "cys loop" receptors (nicotinic, β or ρ1 GABA and 5-HT3 subunits) shows no primary sequence homology. Similarly, the COOH-terminal sequence of P2X subunits are of varying length and sequence. Indeed the QST interacting motif within the tail of $P2X_3$ subunits is absent from other P2X subtypes including receptor-interacting $P2X_2$.

It is now clear that neurotransmitter receptors are involved in a complex interplay with numerous submembranal protein networks that regulate their function or trafficking (Collins and Grant, 2007), this therefore raises the possibility that interactions between receptor-associated proteins promotes the physical and functional coupling between distinct ligand-gated channels.

Recent studies have also provide evidence for activity-dependent cross-talk between two different members of the "cys loop" receptor family -$GABA_A$ and glycine receptors- in spinal cord neurons or $GABA_A$ and 5-HT3 receptors in myenteric neurons, and between two different ionotropic glutamate receptors -AMPA and NMDA receptors- in hippocampal neurons (Bai et al., 2002; Li et al., 2003; Miranda-Morales et al., 2007). Inhibition of GABA-evoked currents by glycine receptor activation, as well as inhibition of NMDA receptors by activation of AMPA receptors, are both mediated by non-ionotropic mechanisms. The mechanism of the interaction between GABA and glycine is however different and involves the state of phosphorylation of the $GABA_A$ receptor and/or mediator proteins. Cross-talk between distinct glutamate receptors has been found G-protein or protein kinase independent, and so it would be interesting to determine if direct or indirect interactions occur between these channels.

Taken together, these data demonstrate that the concept of functional independence might not be true for many receptors and that there is a complex interplay between fast neurotransmitter receptors in the nervous system. The physiological relevance of this new level of regulation at the synaptic level remains to be established. However, because fast neurotransmitters such as GABA/glycine (Jonas et al., 1998), ATP/acetylcholine (Galligan and Bertrand, 1994; Redman and Silinsky, 1994) or ATP/GABA are co-released in the nervous system (Jo and Role, 2002; Jo and Schlichter, 1999), interactions between their respective receptor-channels may exert powerful and rapid receptor modulation which may be essential for the integration of synaptic signals.

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Short Communication

A Rapid and Efficient Protocol for Transfecting NMDA Receptor Channels into Mammalian Cells

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Abstract

The N-methyl-D-aspartate subtype of glutamate receptor (NR) is a ligand-gated ionic channel that plays an important role in synaptic plasticity, memory and spatial learning and is also implicated in the sensitization of pain pathways. For these properties, it represents a prominent drug target. Research on NR ligands and modulators has progressed enormously by the use of heterologous cell systems, in which NR complexes of known composition are selectively expressed. All functional NRs are tetrameric complexes and contain two copies of the essential subunit NR1 and two copies of any of the four different NR2s (A, B, C and D); so the heterologous expression of NR channels is complicated by the requirement for two types of subunit. The expression system of first choice, RNA-injected *Xenopus laevis* oocytes, has some drawbacks mainly correlated with the big dimensions of the cells and their endogenous currents. On the other hand, transfected mammalian cells represent an ideal model to study biophysical and pharmacological properties of NR. As permanent transfection is hardly feasible, reliable and efficient protocols for transient transfection are greatly required. We describe here an optimized protocol we developed for HEK293 cells and that involves the use of an electroporator and CD8 antibody coated beads to identify transfected cells without the need of a specific assay or equipment. Our method allows to produce NR expressing cells on a daily basis suitable for direct electrophysiological investigation and other functional assays.

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Introduction

The N-methyl-D-aspartate subtype of glutamate receptor (NR) is a ligand-gated ion channel that plays a prominent role in many forms of synaptic plasticity and in high brain functions, such memory and spatial learning, cognition and behavior. Excessive activation of these receptors is a critical factor in neurological diseases, including stroke, head trauma and epilepsy, while abnormal expression, function and regulation of NRs may be implicated in schizophrenia and in some chronic neurodegenerative pathologies. Recent studies also suggest a significant involvement of NMDA receptors in sensitization of pain pathways. All these features make NR a prominent drug target and the requirement for effective NR ligands that are able to modulate the receptor function in a pharmacological significant range and at the different subunit level is an outstanding clinical challenge.

All functional NRs are heteromeric complex (tetramers) and the most common varieties contain two different types of subunits, the essential subunit NR1 and at least one of the four different NR2 types (named A, B, C and D; Yamakura & Shimoji, 1999). Both subunits need to be expressed and take part to the protein complex in a most likely stoichiometry of 2NR1:2NR2x (Paoletti & Neyton, 2007).

The study of NR ligands and modulators has progressed enormously with the use of heterologous model systems in which NR ensembles of known composition are selectively expressed. In these systems, it is possible to study the receptor complex in isolation or in a controlled protein environment and obtain a detailed description of the mechanicistic action of different substances. The expression system of first choice to study membrane ionic channels is the RNA-injected *Xenopus laevis* oocyte. However, and in particular in the case of NR channels, this system has significant drawbacks. First, due to the large size of the cell, it is difficult to control the solution exchange adequately and to stimulate the entire membrane surface evenly. Secondly, *Xenopus* oocytes have prominent endogenous currents that are triggered by Ca^{2+} influx through the NR channel (Leonard & Kelso, 1990), making it often advisable to use a Ba^{2+}- instead of Ca^{2+}-containing bathing solution, a non-physiological condition.

In contrast, mammalian epitelial cell lines, such as transformed human embryonic kidney cells HEK293, are easily subjected to fast solution exchange and express very few endogenous channels. Because functional NR complexes are assembled from at least two different subunits, which need to be expressed in the same cells, the successful coexpression of all target subunits in a single cell is a key prerequisite. However, in general, only a fraction of transfected cells expresses all subunits present during transfection (Ma et al., 2007), resulting in a reduced functional expression rate. While it would be desirable to design standardized procedures that yield an enriched population of NR expressing cells, permanent transfection protocols have been reported only occasionally for NR receptors (Steinmetz et al., 2002) and frequently are regarded as unreliable. Instead, transient transfection remains the most popular procedure. The method of choice for transient transfection of cultured mammalian cells with NRs is either the calcium phosphate (Cik et al., 1993; Vicini et al., 1998) or cationic lipid carriers (Collett & Collingridge, 2004; Qiu et al., 2005; Domingues et al., 2007).

Instead, we have used the method of electroporation for the insertion of NR cDNAs into HEK293 cells (Marchetti & Gavazzo, 2003). This technique is based on the ability of a rapid

voltage shock to disrupt areas of the phospholipid bilayer membrane temporarily, allowing polar molecules to pass, with subsequent quick and efficient reseal. Among the major faults of this method is the sizeable mortality, with up to 50% of the cells that can be destroyed in the process, and the requirement for a large quantity of DNA. Despite these problems, electroporation is frequently considered the best method for transfecting mammalian cells, because it is simple, fast, and extremely reliable. Here, we describe a protocol that minimize electroporation caused cell death and maximize receptor expression, allowing to produce NR expressing cells on a daily basis suitable for direct electrophysiological investigation and other functional assays.

Methods

Maintenance and transfection of HEK293 cells - HEK293 cells (CRL 1573; ATCC, Rockville, Maryland) were maintained in DMEM/F12 medium (Sigma D8062), containing 2 mM glutamine, and supplemented with 10% fetal bovine serum, at 37°C and in a 5% CO_2 enriched atmosphere. DNA clones for eucaryotic cell transfection, encoding the rat NR1a (GenBank accession number X63255; formely NR1-1a, see Hollmann et al., 1993), NR2A (GenBank AF001423) and NR2B (GenBank U11419) subunits were a generous gift of Stefano Vicini (Georgetown University, Washington, USA). Human CD8 clone was kindly provided by J. Barhanin (CNRS UPR 411, Valbonne, France). cDNAs were purified by Nucleobond PC100 Midi kit (Macherey-Nagel, Düren, FRG) every two weeks and samples were used only if the absorbance ratio A_{260}/A_{280} was higher than 1.8. The DNA mixture was prepared in the electroporation cuvette adding an equal amount of NR1a and NR2 (A or B) and 0.6 - 1 µg of CD8 (see results) in 500µL phosphate buffered saline (PBS; Sigma D8537). HEK293 cells were detached from the flask by mild trypsin treatment and resuspended in PBS. A volume of 200 µL containing roughly 0.35 million cells was transferred in the cuvette (0.4 cm Zeus Electroporation Cuvettes, Genlantis, San Diego, CA, USA) and subjected to the electroporation treatment by an Easyject Plus Electroporation System (EquiBio, UK). Cells were immediately transferred in 3 mL of pre-warmed complete medium and centrifuged. The pellet was resuspended in complete medium containing 100 µM (±)-2-amino-5-phosphopentanoic acid (D-AP5), a NMDA-receptor antagonist, at a final concentration of 0.23 million cells/ml, and seeded on poly-L-lysine coated glass coverslips for both electrophysiological and immunofluorescence applications. Channel expression was obtained from 18 to 36 hours after transfection. Successfully transfected cells were identified by a brief incubation with CD8 antibody coated polystyrene beads (Dynabeads M-450, Dynal Biotech, Oslo, Norway; Jurman et al., 1994). The coverslip was thoroughly washed to remove loosely attached beads before further experiments.

Electrophysiology - Total membrane currents were measured in whole-cell clamp configuration as in our previous works (Gavazzo et al., 2001; Marchetti and Gavazzo, 2003, 2005). Cells were continuously superfused by gravity flow (10 ml/min) with a Mg^{2+}-free solution containing (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, Hepes 5 and glycine 30 µM. The pH was 7.4 with NaOH. The pipette solution contained (in mM) CsCl 50, CsF 80, EGTA 11, $CaCl_2$ 1, $MgCl_2$ 1, Hepes 10; the pH was 7.3 with Trizma base. Agonist (glutamic acid, 50 µM) was applied by bath solution exchange. Currents

were recorded by an Axopatch amplifier (Molecular Devices, Union City, CA 94587). Voltage stimulation and data acquisition were performed by a PC through a Digidata 1440A interface and Pclamp-10 software (Molecular Devices). Cells were voltage-clamped at – 60 mV and challenged with 50μM glutamate. Current traces were low-pass filtered at 2 kHz and digitized at 5 kHz. For single channel recordings, currents activated by glutamate were measured in outside-out patch configuration. The experimental conditions were as in whole-cell recordings (Marchetti & Gavazzo, 2005).

Immunofluorescence - After 18 – 22 hours from transfection, cells were fixed by treatment in cold methanol solution for 5 minutes. Then they were incubated at room temperature in PBS supplemented with 3% BSA for one hour for blocking reaction and subsequently exposed to the primary antibody against either NR1a or NR2B subunit at 1:100 dilution for one hour. The following primary antibodies were used: rabbit polyclonal AB1516 for NR1 subunit, recognizing the C-terminus of the common NR1 subunit from rat and polyclonal rabbit AB1557P for NR2B subunit, recognizing C-terminus of the NR2B (amino acids 984-1104), both from Chemicon-Millipore, (Billerica, MA, USA), and rabbit polyclonal for the extracellular domain of NR2B (amino acids 984-1104) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies were revealed by using anti-rabbit Alexa Fluor 488 secondary antibody from Molecular Probe (Invitrogen, San Giuliano Milanese, Italy). Coverslips were treated with this secondary antibody for one hour in the dark at room temperature, washed and mounted with Mowiol® 4-88 (Sigma Aldrich). On the following day, coverslips were observed by a fluorescence microscopy (Zeiss Axiovert 100) and photographs were collected by a digital camera (Nikon Coolpix 995).

Results

HEK293 cells were transfected with cDNA encoding the NR1a subunit in combination with cDNA encoding either the NR2A or the NR2B subunit. We used the same amount of cDNA for each subunit, but varied the total amount to maximize transfection efficiency. Best results were obtained with a relatively high quantity of total DNA, 14.5 μgr of each cDNA in 0.7 ml (corresponding to roughly 43μgr/ml total DNA). Other factors were found of significant importance. As actively dividing cells are more suitable for transfection, only cultures at $\leq$ 70% confluency were used. The electroporation process was carried out in PBS and this significantly improved cell survival following the voltage shock. In contrast, when performed in culture medium, electroporation may cause up to 50% cell mortality, as frequently reported for other systems. With PBS, the mortality rate was reduced to roughly 20% and there was no need to change medium to remove dead cells after ~ 6 hours, when viable cells had attached to the substrate. As previously reported (Cik et al., 1993), a NMDA antagonist, D-AP5, was required in the post-transfection culture medium to ensure survival of successfully transfected cells.

The electroporation protocol was also optimized. We used a double pulse OptiPulse® sequence (Equibio Easyject Plus manual of operation) that consists in a double pulse discharge where the second pulse is released before the first is completely dissipated. We verified that this stimulus pattern contributes to enhance the method performance. The parameters that gave best results in terms of efficiency and survival were: for the first pulse,

high voltage mode with V = 700 V, C = 25 μF, R= 99 Ω; for the second pulse, low voltage mode, V=150 V, C= 1500 μF and R= 99 Ω.

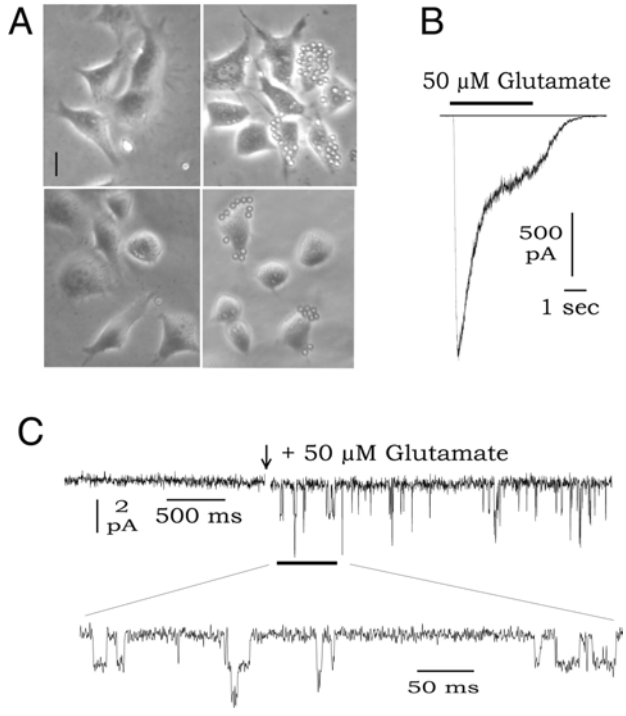

Figure 1. Transiently transfected HEK293 cells with cDNAs encoding the NR1a, NR2A subunit and CD8. (A) Pictures of transfected cells incubated in the presence of CD8-coated beads 18 hours after transfection. The left column shows control cells that were subjected to electroporation in the absence of endogenous DNA. The right column shows positively transfected cells 'decorated' by beads. Bar : 10 μM (B) Glutamate-activated current recorded from a transfected cell that was identified by its ability to bind beads. The cell was voltage-clamped at –60 mV and challenged by 50 μM glutamic acid, applied by bath solution exchange in the presence of 30 μM glycine. At this potential, the current was totally inhibited by 1 mM $MgCl_2$ in the external bath (not shown). (C) Glutamate-activated single channel currents recorded in a cell-free outside –out patch from a bead-binding transfected cell. The cell was voltage-clamped at –60 mV and challenged by 50 μM glutamic acid in the presence of 30 μM glycine. The lower trace shows a portion of the upper trace on an expanded scale.

To maximize the rate of successful recording, it is important to use a valuable method to recognize properly transfected cells. We used CD8 cotransfection and CD8 antibody coated beads (Jurman et al., 1994). This approach has several advantages: it is fast and simple, and does not require reagent preparation or the use of specific equipment, such as a fluorescence microscope. The amount of CD8 cDNA was kept as low as possible to avoid the production of a large population of cells expressing CD8 only, or CD8 in concurrence with only a single NR type subunit. Best results were obtained with 0.8 μgr in 0.7 ml (approximately 1.14 μgr/ml cDNA). On average, when the transfection was carried out in PBS, roughly 40% of total cells bound the microspheres (fig.1A). Whole cell recording from bead-binding cells revealed evidence of glutamate-activated current in nearly 100% of cases and allowed satisfactory recordings of such a current in 75% of cases (fig.1B). In practice, when testing

bead- binding cells, only one out of four was discarded, even if some current was present in almost every cell. Similar results were obtained when recording single NR channels from outside-out patches (fig.1C).

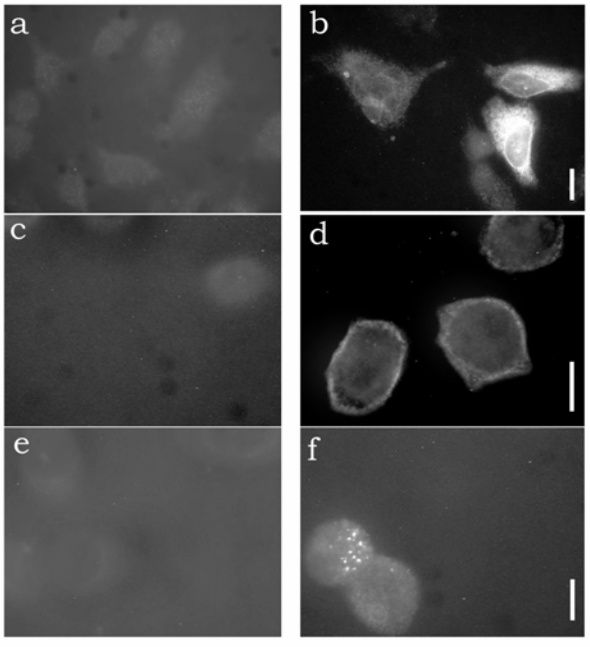

Figure 2. Representative immunofluorescent images of HEK293 cells that were subjected to electroporation without DNA (left column, a, c and e) and positively transfected cells by cDNA encoding the NR1a, NR2B subunit and CD8 (right column, b, d and f). From top to bottom, cells were treated with a primary antibody against the NR1a (b), the extracellular domain of NR2B (d) and the C-terminal domain of NR2B (f). Bar : 10 μM.

The percentage of successfully transfected cells was further verified by immunofluorescence staining. Appreciable staining was obtained with three commercially available antibodies, one against NR1a and two against NR2B subunit (see Methods). Although we did not perform a detailed quantitative analysis, the number of stained cells was in good agreement with the prediction of approximately 40% of successfully transfected cells (fig.2). This approach proved fruitful also for the task of characterizing antibody specificity for subsequent use in primary culture.

Conclusion

Our study demonstrates that electroporation can be employed successfully to study NRs at single cell level. Although an initial investment is required to buy the electroporator unit, after that the procedure is very inexpensive and reproducible because the involved variables are easily controlled. In particular, the use of a physiological saline during the voltage pulse instead of culture medium contributed a significant enhancement in cell survival, while identification of double transfected cells was improved by minimizing the concentration of

the control CD8 DNA. The use of PBS represented a significant improvement not only for cell survival, but also for the expression rate, which was more than doubled with respect to the same procedure carried out in a culture medium. We have not a single and definitive explanation for this fact. It is possible that inorganic salts facilitate, or at least minimize hindrance for DNA permeation across the membrane, whereas other components present in the medium, such as amino acids and vitamins, may hamper DNA penetration and increase cell suffering.

The success of this protocol for a relatively difficult sample, as NMDA receptor channel, suggests the strategy can be recommended for most transfection experiments. Our procedure yields an experimental system ideally suitable for testing pharmacologically active ligands.

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Chapter 1

Glutamate Receptors in the Retina: Neurochemical and Developmental Aspects

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Abstract

Glutamate is present in several cell types in the retina, including photoreceptors, bipolar and ganglion cells, constituting a major excitatory pathway in the tissue. The presence of different classes of metabotropic and ionotropic receptors was detected since the early stages of development. The chick retina is an excellent model for the study of neurochemical events during CNS development since many neurotransmitters, neuromodulators and neuroactive substances found in the brain are also found in this tissue playing different roles in the mature as in the developing tissue. Moreover, early developing chick retinal cells can be dissociated and mantained in culture for relatively long periods where they differentiate many properties of the intact retina. In the present work, we review neurochemical and developmental aspects of glutamate receptors in the chick retina. Emphasis is given to the following aspects: i) glutamate-induced release of neurotransmitters and neuroactive substances such as GABA, acetylcholine, adenosine and ascorbate in cultures of retinal cells; ii) glutamate excitotoxicity in purified cultures of retinal neurons and its regulation by adenosine and nitric oxide; iii) regulation of protein synthesis and nitric oxide production by NMDA receptors; iv) transduction pathways involved in glutamate signaling. The results reveal a complex neurochemical network regulated by glutamate in the developing and mature retina.

1. Introduction

In the 1970´s, a number of autoradiographic, immunohistochemical, biochemical and electrophysiological evidence suggested that the amino acids L-glutamate (L-Glu) and γ-amino butyric acid (GABA) were neurotransmitters in the retina (Lam & Steinman, 1971; Graham, 1972; Bruun & Ehinger, 1974; Murakami et al., 1975; Kennedy et al., 1977; Marc et al., 1978; Lam et al., 1979; Yazulla & Brecha, 1980; Pourcho et al., 1984). Nowadays it is well established that GABA and L-Glu are, respectively, the major inhibitory and excitatory neurotransmitters in the central nervous system (CNS), including the retina of most species (Mosinger et al., 1986; Yazulla, 1986; Massey & Redburn, 1987; Thorenson & Witkovsky, 1999; Yang, 2004). L-Glu is localized in photoreceptors, bipolar, horizontal, and ganglion cells (Barnstable, 1993; Kalloniatis & Fletcher, 1993; Sun & Crossland, 2000) and is involved with the processing of information from photoreceptors to ganglion cells along the direct pathway served by bipolar cells (Mosinger et al., 1986; Agardh et al., 1987; Barnstable, 1993; Massey & Maguire, 1995; Thorenson & Witkovsky, 1999). This flow of information is modulated by GABAergic inputs from horizontal and amacrine cells in the outer and inner plexiform layers, respectively (Yazulla, 1986; Tachibana & Kaneko, 1988; Barnstable, 1993; Sun & Crossland, 2000), which constitute the intrinsic circuitries of the retina.

Finally, since GABA and L-Glu are the major inhibitory and excitatory neurotransmitters in the retina, they control retinal excitability as has been characterized in neuronal circuitries of the brain.

1.1. Glutamate Receptors in the Retina – General Aspects

L-Glu is the major excitatory neurotransmitter in the CNS, and its actions are mediated through L-Glu receptors, classified as metabotropic or ionotropic. Metabotropic L-Glu receptors (mGluRs) are G protein-coupled receptors linked to second messenger systems, while ionotropic L-Glu receptors (iGluRs) are ligand-gated cation channels, grouped as N-methyl-D-aspartate (NMDA) and non-NMDA receptors subgrouped as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. The binding of L-Glu to iGluRs increases membrane permeability, leading to several events including neurotransmitter release (Ampe et al,2007; Clow and Jhamandas, 1989) and activation of gene programs (Lee et al, 2005). These molecular changes underlie physiological roles played by L-Glu such as long-term potentiation and synaptogenesis, although L-Glu has also been implicated in pathogenesis of many neurological diseases (Coyle and Puttfarcken, 1993) due to its Ca^{++}-linked excitotoxicity in neurons (Liu et al, 2004). There is evidence that the transduction mechanisms of L-Glu signaling are far-off, since L-Glu receptor-like proteins were found in plants (Lam et al, 1998) and sponge (Perovic et al, 1999). Indeed, broad knowledge of glutamatergic synapses were obtained from studies developed in *Aplysia californica* (Ezzeddine and Glanzman, 2003) and *C. elegans* (Brockie and Maricq, 2006; Rongo and Kaplan, 1999). Arthropods and vertebrates use L-Glu in visual pathways (Bicker et al, 1988; Thorenson and Witkovsky et al, 1999), acquiring more complex patterns of architecture and physiology along evolution. However, in this review we are going to focus on the role of L-Glu in the vertebrate retina.

1.2. The Retina

The retina is a nervous structure specialized in transducing luminous stimuli. The vertebrate retina contains six neuronal cell types (Livesey and Cepko, 2001), organized in layers of cell bodies and cell processes. The predominant glial cell type in the neural retina is the Müller cell (Newman and Reichenbach, 1996). Cone or rod photoreceptors release L-Glu in the dark and when light stimuli hyperpolarize these cells L-Glu release drops in the synaptic cleft. Photoreceptors connect two classes of bipolar cells identified by light divergent responses evoked through different L-Glu receptors. Off-bipolar cells express AMPA/Kainate receptors and depolarize in darkness. On-bipolar cells are depolarized by light, and this effect is mediated by mGluR6 metabotropic receptors present at the photoreceptor-bipolar synapse (Nawi, 1999; Nakajima et al, 1993). Excitatory transmission from bipolar to ganglion cells is also mediated by L-Glu, forming the vertical pathway in the retina. Additionally, horizontal and amacrine interneurons modulate the flow of information along the retina. Horizontal cells receive glutamatergic inputs from photoreceptors (Sarantis et al, 1988), and negative feedback of horizontal cells to cones involves L-Glu-gated receptors (Fahrenfort et al, 2005). Amacrine cells express mGluRs and iGluRs that modulate the feedback to bipolar cells through diverse neurotansmitters or electrical synapses (reviewed by Yang, 2004).

Many other neurotransmitter systems are present in the retina (Karten and Brecha, 1983). These neurotransmitters interact and are also able to modulate L-Glu neurochemistry, as dopamine (Castro et al, 1999) and acetylcholine (Sernagor et at, 2000).

1.2.1. The Chick Retina as a Model

The development of the chick visual system starts approximately at the second embryonic day (reviewed by Mey and Thanos, 1999). The eye arises from an evagination of the prosencephalon to form the optic vesicle which invaginates and forms the eye cup. The internal epithelium of optical vesicles originates the neural retina which is fully mature at egg hatching.

The chick retina has been largely utilized to study developmental and neurochemical aspects of the nervous system (Magalhães et al, 2006; Sampaio and Paes-de-Carvalho, 1998; Fujita and Horii, 1963). The retina shares the embryonic origin with the brain, presenting a rich neurochemical pattern in a well known histology accomplished by relatively few cell types, about six neuronal and one glial cell type.

During most embryonic stages, it is possible to access the retinal tissue (Coulombre,1955) making this tissue a strong candidate for developmental studies of the nervous system. The patterns of receptor expression, neurotransmitter synthesis, transduction pathways and transcription factors in the chick retina are very similar to other vertebrates, as well as the homology between diverse signaling proteins (Zarain-Herzberg et al, 2005; Odani et al, 2007).

In early stages of development, chick retinal cells can be dissociated and cultivated using different culture systems: i) mixed cultures of neurons and glial cells (de Mello, 1978), ii) purified cultures of neurons (Adler et al., 1984) and iii) purified cultures of glial cells (Cossenza and Paes-de-Carvalho, 2000). There is also the possibility of culturing retinal cells conserving its laminated pattern of cell bodies and processes using small pieces of retinal tissue, the explant culture (Zhang et al, 2003; Yamagata and Sanes, 1995), or aggregate cultures, also called retinospheroid cultures (Layer and Willbold, 1993). The dissection

procedure is reliable and does not cause extensive damage to cells, since the chick retina is a non-vascularized tissue and conserve a high degree of glicolytic metabolism. The main advantage in using the cultures is the possibility to control the medium composition and study several aspects of neuronal pharmacology and physiology which would be impossible using *in vivo* conditions. Indeed, culture preparations reproduce biological phenomena that occur *in vivo*, such as expression of neurotransmitter phenotypes (de Mello et al, 1982), neuron-glia interactions (Cossenza and Paes-de-Carvalho, 2000), and synapse formation (Puro et al, 1977).

1.3. Glutamate Receptors in the Chick Retina

1.3.1. Metabotropic Glutamate Receptors (mGluRs)

L-Glu responses were believed to be mediated only by ionotropic receptors until mid-1980's, when L-Glu was reported to stimulate phospholipase C (PLC) in striatal neurons by a mechanism independent on NMDA, AMPA or kainate receptors (Sladeczek et al 1985). Similar effects were described in hippocampal slices and cultured astrocytes (Nicoleti et all 1986, Pearce et al 1986). Today, it is well known eight distinct subtypes of mGluR (1-8) classified in agreement with sequence homology, pharmacological profiles and associated transduction systems (Pin and Duvoisin, 1994). Group I include mGluR1 and mGluR5, positively coupled to PLC. Group II includes mGluRs2-3, and group III includes mGluR4 and mGluRs6-8, all negatively coupled to adenylyl cyclase (for review Gerber, 2003).

Several reports pointed out the existence of mGluR members in the retina, as well as its broad contribution to retinal physiology. mGluRs can be directly involved in synaptic transmission. For instance, relay of photoreceptor signals from On-bipolar cells to ganglion cells is directly mediated via cyclic GMP-gated cation channels negatively regulated by G_0-coupled mGluR6 in the darkness (Nawy, 1999). mGluRs were implicated in the process of information flow in photoreceptors, and mGluR8 was identified in mammalian photoreceptors (Koulen and Brandstatter, 2002), working as an autoreceptor down-regulating internal calcium concentrations (koulen et al 1999). Application of group II or III mGluR antagonists blocks the response of luminosity-type horizontal cells to green and red cones in the carp retina (Luo and Liang 2003).

mGluR 1 and 5 were immunodetected in post-hatching (PH) chick retina at both outer (OPL) and inner plexiform layers (IPL) (Kreimborg et al,2001). In a subsequent study, these receptors were found to be localized in a similar pattern at the synaptic level at the OPL and IPL, indicating a colocalization of these receptors and cross talking between mGluR 1 and 5 (Sen and Gleason, 2006). These data provide strong evidence that mGluRs are involved in refinement of light information by lateral elements as well as in information flow from photoreceptors to ganglion cells. In a biochemical approach, group II and III mGluRs agonists were reported to inhibit cyclic AMP accumulation stimulated by forskonin along embryonic development of the chick retina (Sampaio and Paes-de-Carvalho, 1998). This study also shows the ontogenesis of groups II and III mGluRs in the chick retina. The inhibitory effect of group III agonist decreases with age, while group II agonist seems to increase the inhibition of cyclic AMP accumulation in PH retinas, suggesting physiological roles by different mGluRs since early development and in mature chick retina (Sampaio and Paes-de-Carvalho, 1998).

1.3.2. Ionotropic Glutamate Receptors (iGluRs)

The first characterized L-Glu receptors were ligand-gated cation channels, also called ionotropic L-Glu receptors (iGluRs). These receptors were classified according to agonist affinity as NMDA and non-NMDA, the latter separated as AMPA and Kainate receptors. In a general view, the functional iGluR consists of a macromolecular complex of four subunits (Rosenmund et al, 1998). Each iGluR subunit contains three transmembrane domains named M1, M3 and M4, and an incomplete loop named M2 in the intracellular side, which forms the channel pore (Wollmuth and Sobolevsky, 2004). The amino terminal portion and the region between M3 and M4 lies in the extracellular side of the plasma membrane and together they form binding sites for L-Glu (Armstrong et al, 1998). The carboxy terminal domain in the intracellular face of plasma membrane contains several motifs of interaction with signal transduction and scaffold proteins, implicated in localization, traffic and function of iGluRs (reviewed in Chen and Wyllie, 2006).

1.3.3. AMPA and Kainate Receptors

AMPA and kainate receptors mediate the majority of fast excitatory synaptic currents in the nervous system. Although there is evidence for separated AMPA and kainate receptors, these receptors are activated by the same agonists with different affinities (Bettler and Muller, 1994). AMPA receptors are formed by the subunits GluR1 to 4, while Kainate receptors are formed by GluR5-7 and Kai1-2 subunits. Different assemblies of subunits render these receptors with distinct functional properties. For example, AMPA receptors which lack GluR2 subunits are permeable to Ca^{++} ions, and additionally, a splicing edition in the M2 region of this subunit, switching a glutamine to an arginine, confers Ca^{++} permeability to assemblies containing GluR2 (Cull-Candy et al, 2006).

In the retina, the role of AMPA and kainate receptors becomes evident at Off-bipolar cell synapses (Brandstätter and Hack, 2001), where L-Glu induces depolarization via these receptors. However, AMPA and kainate subunits are expressed in the outer retina, including On-bipolar cells (Morigiwa and Vardi, 1999), implicating the contribution of different class of L-Glu receptors in light perception. Neurite retraction of fish horizontal cells related to dark adaptation is mediated by calcium-permeable AMPA receptors (Okada et al, 1999).

In the chick retina AMPA/kainate receptors were identified in the inner nuclear layer, ganglion cell layer and plexiforms layers, but the distribution of subunits was not uniform (Bredariol and Hamassaki-Brito, 2001). Studies performed with radioligands during chick retinal development revealed that AMPA receptors reach a peak at E7 and showed an important change in affinity during ontogeny, in addition to other maturational changes (Somohano et al, 1988). Development of AMPA/kainate receptors was determined in cultures of retinal cells, mimicking the ontogenetic pattern of increasing expression of GluR1-4 and 6/7 subunits in the chick retina (Cristóvão et al, 2002).

1.3.4. NMDA Receptors

NMDA receptors are functionally expressed as heteromers composed of four subunits, two NR1, which form the ionic channel, one or more NR2 (A-D) subunits, which confer distinct biophysical and pharmacological properties to the receptor, and possibly NR3 (A-B) subunits. The diversified array of NMDA receptors is increased by eight alternative splicings of the NR1 subunit (for recent reviews see Kohr, 2006 and Paoletti & Neyton, 2007). The activation of NMDA receptors by L-Glu requires the binding of the coagonist glycine to a

strychnine-insensitive site located within the NR1 subunit, whereas the L-Glu recognition sites are formed by NR2 subunit interactions (Kessler et al., 1989; Smothers & Woodward, 2007). Physiological concentrations of magnesium blocks the channel pore at resting potential and ion flow through NMDA receptors depends on pre- and postsynaptic depolarization, suggesting that the NMDA receptor can work as a coincidence detector.

NMDA receptors are expressed preferentially in ganglion and amacrine cells in the mammalian retina (Jakobs et al, 2007; Kalloniates et al, 2004), but few recent findings showed the presence of NMDA subunits in rat photoreceptors and horizontal cells (Fletcher et al, 2002; Gründer et al, 2000). Moreover, evidence of functional NMDA receptors was described in cone-driven horizontal cells of the carp retina (Shen et al, 2006).

Signaling by NMDA receptors has been strongly linked to the nitric oxide (NO) pathway, classically induced by calcium currents and subsequent nitric oxide synthase (NOS) activation. This signaling pathway was broadly described in the nervous system producing plastic changes and also excitotoxicity (Radenovic and Selakovic, 2005; Yamada and Nabeshima, 1997; Garthwaite et al 1988). Our previous report showed that calcium influx evoked by L-Glu was responsible for NOS activation in chick embryo retinas as early as at eight days of development (E8), and this effect was fully blocked by the NMDA antagonist MK801 (Paes-de-Carvalho and Mattos, 1996). Likewise, NMDA incubation increases NOS activity in E8 retinas (Ientile et al, 1996). Expression of NMDA receptor subunits was associated with synapse formation during chick retina development, and these receptors attain maximal amounts at E15, and stimulation with light induces down regulation of NR2a subunits (Cristóvão et al, 2002).

1.4. Expression of Glutamate Receptors in Cultured Retinal Cells

1.4.1. NMDA Receptors

Relevant findings on the neurochemistry of NMDA receptors have been achieved using retinal cells in culture. Application of NMDA to mixed cultures of chick retinal cells is able to increase the release of purines (Paes-de-Carvalho et al, 2005). In a similar culture system, activation of NMDA receptors inhibits protein synthesis increasing free L-arginine and increasing NO synthesis (Cossenza et al, 2006) (see bellow). Incubation with L-Glu increases secretion of neurotrophins in a dose dependent manner in cultured rat Müller cells (Taylor et al, 2003). This finding correlates with the increase in the expression of L-Glu transporter EAAT1 and down-regulation of NR1 subunits in these glial cells, corroborating a trophic and homeostatic role for L-Glu in the retina. Chick Müller cells appear to express NMDA receptors (López et al, 1997), and a recent work shows that the kinetic and pharmacologic properties of glycine interaction with the NMDA receptor coagonist site differ in Müller cells and retinal neurons (Lamas et al, 2005). Chick retinospheroid cultures also present functional NMDA receptors, and the pattern of expression of NR1, NR2a/b and NR2c is very similar to that in the intact tissue during embryonic development (Cristóvão et al, 2002).

1.4.2. AMPA/Kainate Receptors

The presence of AMPA/kainate receptors in cultured retinal cells has been detected through several approaches, and takes account of a myriad of functional interactions involving L-Glu in this tissue. For instance, in cultured fish horizontal cells, the affinity of L-

Glu receptors is affected by NO and dopamine, by a mechanism independent of desensitization (McMahon and Schimidt, 1999). Freshly isolated horizontal cells from rabbit retinas respond to L-Glu application with an increase of cation conductance via AMPA receptors (Blanco and De La Vila, 1999). An increase of phosphorylation and surface expression of GluR4 was observed after PKC stimulation in cultured retinal neurons (Gomes et al, 2007).

Studies carried out in culture systems are also of higher significance for understanding aspects of retinal development. The activation of calcium-permeable AMPA receptors in explant or dissociated chick retina cultures causes cell death in the late development, while continuous application of high doses of kainate since early periods as E6 is not excitotoxic (Allcorn et al, 1996). Moreover, neurite outgrowth in cultures of embryonic chick retinal cells decreases when calcium-permeable AMPA receptor is activated (Catsicas et al, 2001). The permeability to calcium ions demonstrated in the latter reports could indicate a link of AMPA receptors with signaling pathways involved in gene transcription, cell death and neuronal survival (Santos et al, 2001). Furthermore, the expression of GluR1-4 (AMPA) and 6-7 (kainate) subunits was shown in chick retinospheroid cultures at different stages of development, but however no functional AMPA receptors can be found in early cultures (Cristóvão et al, 2002).

1.4.3. Metabotropic L-Glu Receptors (mGluRs)

Function and expression of mGluRs in cultured cells is of great importance to understand the mechanisms of signal transduction employed by this receptor class, and the chick retinal culture is a good model to reach this goal. In mixed cultures of embryonic chick retinal cells, activation of group III mGluRs with L-AP4 is able to inhibit forskolin-induced cyclic AMP increases (Sampaio and Paes-de-Carvalho, 1998). [^{3}H]acetylcholine release evoked by high K^+ in cultured chick amacrine cells is also reduced by L-AP4, an effect reverted by group I and II mGluR agonists (Caramelo et al. 1999), indicating the presence and interaction of the three mGluR classes in amacrine neurons. In a similar culture system, treatment with the mGluR5 agonist CHPG enhanced $GABA_A$-evoked current involving calcium ions and PKC (Hoffpauir and Gleason, 2002), and interaction of mGluR5 with PKA was also reported modulating Ca^{++} voltage-dependent currents (Sosa and Gleason, 2004). In addition, class II mGluRs are involved in GluR4 AMPA receptor subunit phosphorylation and activity (Gomes et al., 2004).

2. Glutamate and the Release of Neurotransmitters and Neuromodulators in the Retina

2.1. Release of Neurotransmitters in Chick Retina

Several authors investigated the pattern of neuronal content of neurotransmitters and the mechanisms of L-Glu-evoked release in the chick retina. These studies evaluated neurotransmitter release either through a perfusion technique using radiolabeled neurotransmitter, HPLC or, more recently, endogenous GABA detected by

immunohistochemistry from different cell populations *in situ* after retinal stimulation (Andrade da Costa et al., 2000; 2001; Calaza et al., 2001; 2003; 2006).

L-Glu and to a less extent aspartate (asp) are the main excitatory neurotransmitters in the retina. Thus, L-Glu seems to affect several neurochemical cell subpopulations. Here we are going to focus on the interactions of L-Glu with GABA, acetylcholine, dopamine, purines and ascorbate containing retinal cells.

2.2. GABA Release

GABA is the major inhibitory neurotransmitter in the chick retina and the GABAergic cells in this tissue comprise horizontal, amacrine neurons and cells in the GCL, but in other species, they can also be detected in ganglion cells and also Müller cells that can at least take up GABA released in the extracelular medium (Lam & Steinman, 1971; Graham, 1972; Kennedy et al., 1977; Marc et al., 1978; Yazulla & Brecha, 1980; Hampton et al., 1983; Moran et al., 1986; Mosinger et al., 1986; Yazulla, 1986; Osborne et al., 1986; Massey & Redburn, 1987; Agardh et al., 1987; Hokoç et al., 1990; Barnstable, 1993; Kallomiatis & Fletcher, 1993; Andrade da Costa, 1997). This seems not to be the case for Müller cells of the chick retina (Marshall & Voaden, 1974; Morán et al., 1986; Calaza et al., 2001, 2003).

The influence of L-Glu on radiolabeled-GABA release was previously studied by several authors using the isolated chick retina (Tapia & Arias, 1982; Moran et al, 1986; Calaza et al., 2003), either in three-dimensional organotypic cultures of dissociated retina from chick embryos- so called aggregates (do Nascimento et al., 1996) or in monolayer cultures of chick retinal cells (do Nascimento et al., 1985, 1998a; de Mello et al., 1988; Kubrusly et al., 1998; Duarte et al., 1992; 1993; Carvalho et al., 1995).

L-Glu-evoked [^{3}H]-GABA release can be detected quite before synaptic circuits have been established in the retina (Calaza et al., 2006). Furthermore, [^{3}H]-GABA release evoked by L-Glu, L- and D-asp, but not D-Glu, also occurs in embryonic chick retina cells either isolated or maintained in vitro (Tapia & Arias, 1982; do Nascimento & de Mello, 1985, 1998a; Moran et al, 1986; de Mello et al., 1988; Kubrusly et al., 1998; Duarte et al., 1992; 1993; Carvalho et al., 1995; Calaza et al., 2003).

The presence of magnesium ions decreases the release of [^{3}H]-GABA induced by L-Glu in monolayer cultures of retinal cells (Kubrusly et al., 1998; do Nascimento et al., 1998a), suggesting the involvement of NMDA receptors. Indeed, the release of GABA induced by L-Glu is due to the activation of both NMDA or non-NMDA receptors (de Mello et al., 1993; Kubrusly et al., 1998; do Nascimento et al., 1998a) and it is also activated by their agonists, NMDA and kainate, respectively.

In all cases, the L-Glu effect is calcium-independent and notably sodium-dependent, features that lead to the idea that [^{3}H]-GABA release might be through a sodium-dependent, carrier-mediated mechanism and not by the classical exocytotic mechanism. In fact, [^{3}H]-GABA release promoted by NMDA or kainate is inhibited by the non-competitive GABA carrier blocker NNC-711, indicating that in both cases most of the release was mediated by reversal of the GABA transporter (do Nascimento et al., 1998a). This mechanism of neurotransmitter release was first suggested by Haycock and collaborators (1978). In cultures of chick retina cells, the neurochemical features of GABA transport were investigated by do Nascimento and collaborators (1998b). The authors have suggested that sodium-dependent

GABA release from cultured chick retina cells is mediated by a GAT 1- like transporter which shows some, but not all, of the pharmacological properties of the GAT 1 carrier (do Nascimento et al., 1998b). The results of several investigators using autoradiographic methods to analyze [^{3}H]-GABA content of specific cell populations of fish and amphibian retinas reinforced the idea that most of the excitatory amino acids (EAAs)-evoked [^{3}H]-GABA release in retinas of different species occurs via a sodium-dependent, carrier-mediated process (Yazulla, 1983; Yazulla & Kleinschmidt, 1982; Schwartz, 1982; Tapias & Arias, 1982; Moran & Pasantes-Morales, 1983; Yazulla & Kleinschmidt, 1983; Ayoub & Lam, 1984).

The hypothesis considered by Kubrusly and co-workers (1998) for the mechanism of GABA release by D- and L-aspartate (but not D-glutamate) is that since these amino acids are efficiently taken up by EAA transporters, through an electrogenic mechanism (Nicholls & Attwell, 1990), the eventual uptake of aspartate in the neighborhood of NMDA receptors could promote a focal change in membrane polarity, leading the receptor to a state of lower affinity for Mg^{2+}. Thus, the NMDA receptor activation and aspartate uptake could create the conditions to influence the outflow of GABA. Therefore, besides acting on the ligand site of the NMDA receptor, L-aspartate uptake seems to be important to allow functional activation of the NMDA channel, independent of non-NMDA receptor activation.

A small amount of [^{3}H]-GABA released by L-Glu agonists NMDA or kainate appears to occur via the vesicular mechanism in primary cultures of embryonic chick retinal cells (Ferreira et al., 1994; Carvalho et al., 1995; Santos et al., 1998). Moreover, calcium influx occurs through L type voltage-gated calcium channels (Duarte et al., 1992; 1993; Carvalho et al., 1995).

The major source of GABA in mature nervous system is glutamic acid that is converted into GABA after its decarboxylation by glutamic acid decarboxylase (GAD). However, in early embryonic stages a significant amount of GABA is synthesized from putrescine, a compound which is abundant in the undifferentiated tissue (de Mello et al., 1976). In fact, GABA can be detected in avian retina before the expression of GAD (Hokoç et al., 1990). L-Glu stimulation also increases 5-7-fold the release of [^{3}H]-GABA derived from [^{3}H]-putrescine by activation of ionotropic receptors through mechanisms similar to those utilized by neurons in the mature nervous system (de Mello et al., 1993). The data suggest that the mechanism of GABA release, provenient from putrescine, is similar to that involved with the release of GABA pools labeled by [^{3}H]-GABA taken up by cultured retinal cells (Tapia and Arias, 1982; Moran and Pasantes-Morales, 1983; Lam and Ayoub, 1983; Yazulla, 1983; do Nascimento and de Mello, 1985; Schwartz, 1987; de Mello et al., 1988; Hofmann and Möckel, 1991). As for the retinal tissue, calcium-independent mechanism for GABA release seems to be a common phenomenon in several parts of the CNS (Adam-Vizi, 1992, Schoffelmeer et al., 2000).

Despite the fact that studies using [^{3}H]-GABA release gave substantial information regarding the mechanisms of release, the behavior and identification of different GABAergic cell populations involved with GABAergic activity in the intact retina remained elusive. Therefore, an alternative to answer some of these contradictions consists of measuring the GABA content of different retinal cell types by an immunohistochemical approach, using specific anti-GABA antibodies (Osborne and Herrera, 1994). Thus, if significant changes in GABA content of cells or neurite regions of tissue segments exposed to excitatory stimuli occurs, then one would be able to detect these changes by comparing the intensity of

immunolabeled GABA remaining in the cells after stimulation, with the immunostaining of untreated tissue. Using this approach it was possible to identify GABAergic cell populations that responded to excitatory amino acids in the chick retina (Calaza et al., 2001, 2003).

The exposure of retinal segments to L-Glu or kainate promotes a considerable reduction in the number of GABA immunoreactive cell bodies and a significant decrease of immunolabeling of cell processes projecting to the plexiform layers, when compared to control, untreated retinas. Qualitatively, the decrease of GABA immunoreactivity (GABA-IR) induced by L-Glu is similar to that observed with kainate, except that the number of GABA positive cells is always smaller after kainate treatment (Calaza et al., 2001; 2003). The more potent effect of kainate could reflect a difference in the affinity for the binding sites and/or the active cellular uptake of L-Glu which would reduce its effective concentration in the extracelular space.

GABAergic amacrine cells are always affected by excitatory amino acids (EAAs), L-Glu or its ionotropic receptor agonists and L-, D-aspartate (Calaza et al., 2001, 2003). This is in agreement with data showing EAAs depolarizing effects on amacrine cells of mudpuppy and rabbit retinas (Slaughter and Miller, 1983; Bloomfield and Dowling 1985).

Stimulation with NMDA results in 50% reduction in the number of GABA immunoreactive amacrine cells (Calaza et al., 2001). As NMDA, L- or D-aspartate (100μM) treatment also results in 50% decrease in the number of GABA immunoreactive amacrine cells. NMDA, L- or D-aspartate are unable to stimulate GABA efflux in horizontal cells, as opposed to kainate that significantly reduces (50%) GABA-immunoreactive horizontal cells. Thus, chick retina GABAergic horizontal cells respond to glutamate via the exclusive activation of non-NMDA receptors. This is in agreement with the observed depolarization of horizontal cells by non-NMDA receptors agonists and not by NMDA in rat retina (Hankins & Ikeda, 1991).

The effects of EAAs on GABA-containing cells follow the classic activation of specific receptors, since MK-801 and DNQX, respectively antagonists of NMDA and non-NMDA receptors, totally prevents GABA release.

As described in cultured retinal cells, GABA release induced by glutamate agonists kainate, NMDA and aspartate occurs predominantly by the reversal of the sodium-dependent GABA transporter in both amacrine and horizontal cells. Therefore, this form of release is an important mechanism through which GABA signaling occurs in the retina. (Kamada et al., 1981; Schwartz, 1982; Yazulla and Kleinshmidt, 1983; do Nascimento and de Mello, 1985; Johnson et al., 1986; Hofmann and Möckel, 1991; do Nascimento et al., 1992, 1998).

2.3. Acetylcholine Release

The chick retina also contains a proeminent cholinergic system, which includes three populations of cholinergic amacrine cells (Millar et al., 1985; Spira et al., 1987). Acetylcholine (Ach) plays an important role, among other processes, in neurite outgrowth of ganglion cells, dendritic filopodia motility, remodeling during synaptogenesis, as well as in the development of spontaneous rhythmic activity in retinal ganglion cells during the period in which their connectivity pattern is shaped and the selective-direction function of some ganglion cells is stablished (Wong et al., 1998, 2001; Sernagor et al., 2000; Wong and Wong, 2000; Yoshida et al., 2001; Vaney and Taylor, 2002, Wässle, 2004). There is a good

colocalization of GABA and choline acetyltransferase, the synthetic enzyme of acetylcholine, and a co-release of both neurotransmitters (Vaney and Young, 1988; Brecha et al., 1988; O´Malley & Masland, 1989; Wulle and Wagner, 1990; O´Malley et al., 1992). Based on all these facts, some authors studied the effect of glutamate on acetylcholine release as in the case of GABA.

[^{3}H]-Ach release can be induced by depolarization by KCl due to calcium entry through L- and N-type voltage-gated calcium channels probably via an exocytotic mechanism (Thompson, 1982; Campochiaro et al., 1985; Agardh, 1986; Santos et al., 1998a, b). L-Glu also evokes the release of [^{3}H]-Ach via the classical exocytotic process, both in isolated or retinal cells maintained in vitro (Thompson, 1982; de Mello et al., 1988; do Nascimento et al., 1996). Neither D-asp nor D-glutamate influences the efflux of [^{3}H]-Ach (de Mello et al., 1988).

The ionotropic L-Glu receptor agonist kainate also induces [^{3}H]-Ach release in isolated chick retina (Campochiaro et al., 1985). [^{3}H]-Ach exhibits kainate-evoked release greater than that elicited by KCl and it is inhibited by 83% in absence of calcium. Quisqualate is nearly as effective as kainate in evoking [^{3}H]-Ach release, an effect partially calcium-dependent (Campochiaro et al., 1985). In addition, unlike quisqualate and kainate, NMDA is ineffective in eliciting release of [^{3}H]-Ach (Campochiaro et al., 1985).

Finally, activation of group III metabotropic glutamate receptors (mGluRs) inhibits the KCl-evoked release of [^{3}H]-Ach (Caramelo et al., 1999). Although activation of group I and II mGluRs does not affect the release of [^{3}H]-Ach evoked by KCl, they reverse the inhibitory effect of stimulation of group III mGluRs (Caramelo et al., 1999).

Some authors claimed that most, if not all, of the toxic effects on neurons promoted by Amiloid β peptide (Aβ) appear to be mediated by L_Glu excitotoxicity (Louzada et al., 2004). Both L-Glu system and Aβ seem to decrease the activity of the ACh system through several mechanism including the decrease of [^{3}H]-Ach release (Loureiro-dos-Santos et al., 2002; Melo et al., 2002, 2003). Thus, it seems that the L-Glu effect on Ach release, increasing or decreasing it, must depend on the L-Glu receptor subtype expressed by the neuronal type.

2.4. Dopamine Release

Dopamine is a neurotransmitter involved in adaptation to a photopic condition. Thus, light induces dopamine release that uncouples gap-junctions of glycinergic AII amacrine cells inhibiting glycine release and facilitating transmission in the cone pathway (Hampson et al., 1992; Witkovsky and Dearry, 1992; He et al., 2000). Moreover, it has been shown that the GABAergic system is modulated by dopamine (Calaza et al., 2001; 2003), at least in some amacrine subpopulations, through a direct inhibition of NMDA receptors (Castro et al., 1999; Calaza et al., 2001; 2003). In addition to neurotransmission, dopamine is involved in many other functions such as neurogenesis, neurite extension of dopaminergic amacrine cells, neuronal differentiation, rythmic events, neuroprotection and decreased retina pigmented epithelium phagocytosis (Lankford et al., 1988; Nguyen-Legros et al., 1999; Guimarães et al., 2001; Reis et al., 2007).

Although dopamine plays several important functions in retinal development and physiology very few studies have been performed to evaluated dopamine release in the retina (Pasantes-Morales et al., 1980; Tapias & Arias, 1982). Depolarization induced by high KCl

increases [^{3}H]-dopamine release (100%) which is completely prevented in the absence of calcium (Pasantes-Morales et al., 1980; Tapias & Arias, 1982). On the other hand, L-Glu and kainate does not affect dopamine release (Tapias & Arias, 1982).

2.5. Purine Release

Adenosine is an important neuroactive nucleoside in the brain and exerts its activity through different G protein-coupled receptors named A1, A2a, A2b and A3 receptors (Fredholm et al., 2005; Ribeiro et al., 2002). The effects of adenosine on neuronal activity are predominantly inhibitory, and it is believed that this is mediated by inhibition of excitatory transmitter release (Goodman et al., 1983; reviewed in Ribeiro, 1995). The presence of adenosine and its receptors was described in retinas from different vertebrate species, including chick, rat and human retinas (Blazynski, 1990; Blazynski and Perez, 1991; Braas et al., 1987; Paes-de-Carvalho, 1990; Zhang et al., 2006). Adenosine promotes cyclic AMP accumulation in the chick retina and this effect is detected since the 13th day of embryonic development (E13), attaining maximal levels at E17 and decreasing to lower levels at the post-hatching tissue (Paes-de-Carvalho and de Mello, 1982). This effect is also observed in cultures of chick retinal cells (de Mello et al., 1982). Interestingly, adenosine is also able to inhibit cyclic AMP accumulation induced by dopamine, and this effect is mediated by A1 receptors and present in early periods of development such as E10 (Paes-de-Carvalho and de Mello, 1985). A1 receptors, as measured by binding of (^{3}H)cyclohexyladenosine, is detected at E10, increases up to E17 and then decreases in the post-hatching period (Paes-de-Carvalho, 1990). A3 receptors were also studied in mammalian retinas, and found to have important neuroprotective properties (Zhang et al., 2006b).

Adenosine uptake and metabolism were also studied in the brain as well as in the retina (Geiger et al. 1988; Johnston and Geiger, 1989; Blazynski et al., 1989; Paes-de-Carvalho et al., 1990; 1992). Adenosine uptake is mediated by concentrative or equilibrative nucleoside transporters, which are sodium-dependent or independent (King et al., 2006; Ritzel et al., 2001). The main transporter expressed in chick retinal neuronal cultures is the NBMPR-sensitive equilibrative transporter, since the uptake is almost completely inhibited by NBMPR at low micromolar doses (Paes-de-Carvalho et al., 1990). The autoradiographic analysis of these neuronal cells in culture after incubation with (^{3}H) adenosine reveals that approximately 50% of neurons take up adenosine while all photoreceptors take up the nucleoside. Accordingly, immunocytochemistry using an antibody against adenosine gave similar results (Paes-de-Carvalho et al., 1990). Autoradiographic analysis of (^{3}H) NBMPR bindng sites as well as of A1 receptors were also performed in the intact developing chick retina. While the A1 receptors are detected in the plexiform layers since E10 and in older tissues, NBMPR binding sites are detected since E8, when the plexiform layers are not already developed. As soon as the plexiform layers appear, these sites are concentrated in these structures where most synapses are localized (Paes-de-Carvalho et al., 1992), strongly suggesting that the adenosine system is closely related to synaptic functions in the retina.

The enzymes adenosine kinase and adenosine deaminase are important in the nucleoside catabolism. The kinase is often considered as more important due to its lower Km (Gu et al., 1993). Indeed, when the intracellular metabolites are analysed after adenosine uptake in mixed cultures of retinal cells, more than 95% of intracellular radioactivity is present as

nucleotides and very little is found as inosine, hypoxanthine or adenosine itself (Paes-de-Carvalho et al., 2005). In contrast, most of the released radioactivity is detected as inosine (see bellow). Adenosine deaminase but not adenosine kinase was previously shown by immunocytochemistry in retina (Senba et al., 1986).

Adenosine is also released from cells in different conditions and stimuli, and the equilibrative nucleoside transporter appears to have an important participation in this process (Pinto-Duarte et al., 2005; Parkinson et al., 2002; Parkinson et al., 2006). Adenosine behaves as a retaliatory metabolite increasing its intracellular concentration in conditions of hypoxia and ischemia (Bouma et al., 1997; Perez-Pinzon et al., 1993). The consequence is an increase of extracellular adenosine due to its efflux through the equilibrative transporter down its concentration gradient (Laghi-Passini et al., 2000). In cultures of chick retinal neurons incubated with (^{3}H) adenosine, depolarization with high potassium concentration induces the release of radioactivity in a calcium-dependent manner (Paes-de-Carvalho et al., 1990). The analysis of this radioactivity shows that inosine is the major component, with smaller amounts of hypoxanthine, adenosine and nucleotides (Paes-de-Carvalho et al., 1990). L-Glu also promotes the release of purines from chick retinal cultures and this effect is blocked by either the NMDA receptor antagonist MK801 or the AMPA/kainate antagonists CNQX or NBQX (Paes-de-Carvalho et al., 2005). Interestingly, the effect of L-Glu is completely blocked by either one of the antagonists alone, suggesting that the cell releasing purines contains both NMDA and AMPA/kainate receptors (see figure XXX). This is different to what happens with the release of GABA by L-Glu that is only partially inhibited by NMDA or AMPA/kainate antagonists and completely inhibited only when both antagonists are present (Kubrusly et al., 1998). This suggests the existence of at least two GABAergic cells in the cultures, one containing NMDA receptors and other AMPA/kainate receptors (figure XXX). However, in the intact retina kainate promotes a larger release of GABA than L-Glu, probably refelcting the intense uptake of this amino acid by glial cells (Calaza et al., 2001;2003).

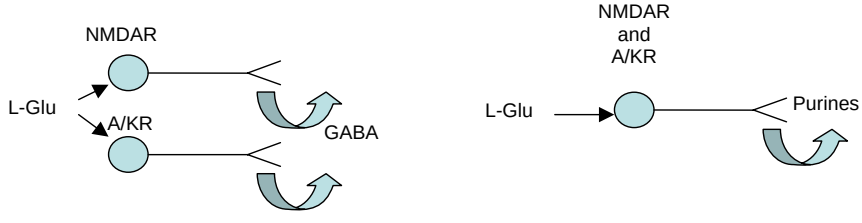

Figure 1. Schematic diagrams showing GABAergic and purinergic neurons displaying different sets of L-Glu receptors in chick retinal cultures. NMDAR, NMDA receptors; A/KR, AMPA/Kainate receptors.

The release of purines stimulated by L-Glu or kainate in chick retinal cells in culture is completely blocked by calcium removal from the extracellular medium or by NBMPR, an adenosine uptake blocker, indicating that the effect is mediated by the nucleoside tranporter in a calcium-dependent manner (Paes-de-Carvalho et al., 2005). The release of purines by L-Glu or the uptake of adenosine is also completely blocked by preincubation with the calcium/calmodulin-dependent kinase (CAMK) inhibitors KN62, KN93 or the myristoylated autocantide-2-related inhibitory peptide, indicating that the nucleoside transporter is directly

or indirectly modulated by CAMK II activity and suggesting a mechanism for the calcium-dependent release process (Paes-de-Carvalho et al., 2005).

The modulation of the nucleoside tranporter by CAMK II raises the intriguing question of what could be its function in the synaptic process. It is very well known that CAMK II is an important enzyme localized in synaptic regions and participating in synaptic plasticity, learning and memory (Lisman et al., 2002). Indeed, this enzyme activity is of fundamental importance in long-term modifications of synaptic physiology such as that occurring in long-term portentiation (LTP) or depression (LTD) (Lledo et al., 1995; Hayashi et al., 2000; Otmakhov et al., 2004), since it belongs to a category of enzymes that when activated can remain in the active state for a long period. CAMK II is stimulated by calcium/calmodulin and when activated by this complex remains in an active and calcium-independent conformation for long periods of time (Lisman et al., 2002). A model for the participation of adenosine transporters and CAMK II in a long-term synaptic modification is shown in figure XXX. Activation of NMDA receptors could promote calcium influx and formation of the calcium/calmodulin complex which could then activate CAMK II. This enzyme could then promote a direct or indirect phosphorylation of the transporter increasing the release of adenosine, and this release could remain high during long periods while the enzyme is active even in the absence of calcium. Adenosine released in the synaptic cleft is able to activate A1 receptors that can by its turn inhibit L-Glu release from the presynaptic neuron (Manita et al., 2004). The inhibition of L-Glu release for long periods of time could then promote the phenomenon of long-term depression of synaptic activity (LTD).

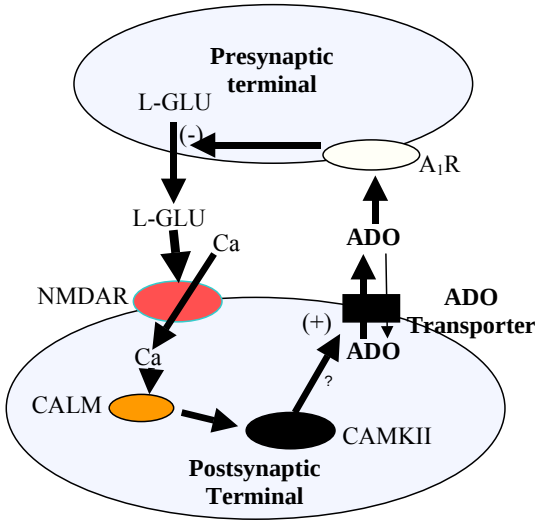

Figure 2. Schematic diagram of a synaptic terminal showing different components of the purinergic and glutamatergic system in presynaptic and postsynaptic cells. NMDAR, NMDA receptor; CALM, calmodulin; ADO, adenosine; CAMK II, calcium/calmodulim-dependent kinase type II; A1R, A1 adenosine receptor.

2.6. Ascorbate Release

Vitamin C is an important antioxidant present in many tissues including the retina. There are two forms of vitamin C depending on its state of oxidation. The reduced form is ascorbate which is taken up by a special family of transporters called sodium vitamin C co-transporters (SVCT). The isoforms SVCT 1 and 2 are glicoproteins with 12 transmembrane domains (Daruwala et al., 1999; Tsukaguchi et al., 1999; Wilson, 2005), which transport L-Ascorbate stereospecifically in a concentrative way. The oxidized form is Dehydroascorbate (DHA) that is taken up by glucose transporters, especially GLUT 1 and 3 (Vera et al., 1993; Rumsey et al., 1997). Both uptake systems are found in the retina (Gerhart et al., 1999; Tsukaguchi et al., 1999). Glucose transporters 1 and 3 are present in different cell types. GLUT 1 is abundant in the basal and apical plasma membrane of retinal pigment epithelium and in many cell types including rod cells and Müller glial cells, while GLUT 3 is restricted to plexiform and ganglion cell layers. SVCT-2 isoform predominates in the retina where it appears to be restricted to the inner nuclear layer (Gerhart et al., 1999).

There are mainly two barriers for the regulation of homeostasis in the retina, the blood-retinal barrier (BRB) and the blood-aqueous barrier (BAB). The first one is subdivided into inner blood-retinal barrier (IBRB) and outer blood-retinal barrier (OBRB). Tight junctions of retinal capillary endothelial cells compose the IBRB while tight junctions of retina pigment epithelial cells compose the OBRB. Epithelial barriers of the cilliary body form the BAB (Hosoya and Tomi 2005). The BRB is responsible for the retinal tissue nourishment; OBRB nourishes the outer retina (pigment epithelium, photoreceptor outer segment layer and outer nuclear layer) while IBRB nourishes more inner layers (a piece of the outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiforme layer, ganglion cell layer, nerve fiber layer and inner limiting membrane) (Hosoya and Tomi, 2005). The IBRB is structurally similar to the brain blood barrier (BBB), being covered by pericytes and Muller glial cells. This barrier presents, as BBB, a dynamic and regulatory interface and exerts a similar transporter function (Hosoya and Tomi, 2005).

Although there are high levels of ascorbate in the retina, many evidences show that SVCT is not the molecule responsible for the entrance of vitamin C within the retina. It appears that vitamin C is taken up on its oxidized form, Dehydroascorbate, and consequently the most important molecule for this transport is the glucose transporter (GLUT) (Hosoya et al., 2004).

The GLUT family of transporters, specially GLUT 1, have already been described on IBRB (Hosoya and Tomi, 2005) and is responsible for vitamin C transport to the retina. Dehydroascorbate is taken up by the IBRB and is rapidly converted to ascorbate, promoting the accumulation of high levels of ascorbate in this tissue (Hosoya et al., 2004).

It was previously described that ascorbate could enter the retina through the lens. It has been described a high concentration of ascorbate in the aqueous humor and its antioxidant role in the lens. In agreement with this, It was observed the expression of SVCT-2 in lens epithelial cells mediating ascorbate uptake (Kannan et al., 2001). In the retina, it has been described that SVCT-2 is present in the inner nuclear layer, where are localized amacrine, bipolar and horizontal cells (Tsukaguchi et al., 1999).

Ascorbate is present at early stages of eye development. It has been described that chick embryos present (at the second day of development) high concentrations of ascorbate in the eye rudiment (Lam et al., 1993). Ascorbate is also a protector against light-induced damage,

and is important for preserving extracelullar dopamine from oxidation mediated by NO in the mammalian retina (Neal et al., 1999). Moreover, ascorbate decreases the voltage-dependent potassium currents in goldfish retinal bipolar cells through a mechanism depending on GTP hydrolysis, Gs protein and PKA (Fan and Yazulla, 1999a). This effect of ascorbate on goldfish bipolar cells is mediated by activation of dopamine D1 receptors, an effect blocked by the antagonist SCH23390 and mimicked by dopamine at the same concentrations (Fan and Yazulla, 1999b). Ascorbate could also block the dark induced efflux of GABA in the goldfish retina (Yazulla, 1985).

It was previously observed interactions between ascorbate and the glutamatergic system. For example, ascorbate is able to regulate the NMDA receptor through interaction with the redox-sensitive regulatory center(s) on NMDA receptor and inhibiting receptor activity. This effect was observed using electrophysiological techniques that demonstrate an inhibition on NMDA-evoked currents in cultured cortical neurons promoted by ascorbate. Moreover, ascorbate was able to inhibit specific L-Glu binding, probably by inducing internalization of NMDA receptors or decrease of L-Glu affinity (Majewska et al., 1990).

Recently, SVCT-2 expression was shown to be required for normal neuronal maturation of the glutamatergic system in hippocampal neurons (Qiu et al., 2007). By recording mini end-plate potentials (mEPSPs) in cultured hippocampal neurons derived from either wild type or embryos lacking SVCT-2, *Slc23a2*$^{-/-}$, it was observed that *Slc23a2*$^{-/-}$ neurons present smaller amplitudes and frequency of mEPSPs when compared with wild type neurons. Moreover, comparing *Slc23a2*$^{-/-}$ and wild-type neuronal morphology, *Slc23a2*$^{-/-}$ neurons exhibit less primary neurite branches and smaller dendrite lengths. Accordingly, *Slc23a2*$^{-/-}$ neurons alsopresent decreased expression of GluR1 AMPA receptor subunits than wild type, indicating the existence of less potential places of excitatory synaptic transmission in these mutants. In the same work, Qiu et al. (2007) observed that SVCT-2 activity protects neurons from oxidative injury induced by H_2O_2 and excitotoxicity induced by NMDA.

L-Glu is able to release Vitamin C through at least two different described mechanisms: the first is through heteroexhange (Grünewald and Fillenz, 1984) and the second is by inducing swelling (Wilson et al., 2000). Both mechanisms involve L-Glu transporters. In the heteroexchange mechanism, L-Glu transporters mediate an exchange of L-Glu by ascorbate. In the second mechanism, L-Glu uptake induces cell swelling and Vitamin C is released through volume-sensitive organic osmolyte/anion channels (VSOAC).

L-Glu-induced Vitamin C release from cultured retinal cells occurs in a calcium-independent/sodium-dependent fashion and is inhibited by tha AMPA/kainate receptor antagonist DNQX, but not by the NMDA antagonist MK801. Interestingly, both NMDA and kainate are able to induce vitamin C release, but the effect of NMDA is completely blocked by DNQX (Portugal C.C. and Paes-de-Carvalho R., submitted). Therefore, different to what is observed in the L-Glu-induced release of GABA or purines (see figure 1), a model for vitamin C release induced by L-Glu in cultures of chick retinal cells is presented in figure 3). Two neurons in series are presented in the model. The first is glutamatergic and expresses NMDA receptors and the second contains AMPA/kainate receptors and is able to release vitamin C. According to this model, addition of L-Glu directly activates the second neuron containing AMPA/kainate receptors and induces vitamin C release, and the effect is logically inhibited by DNQX but not by MK801. However, addition of NMDA activates only the first cell to release L-Glu that by its turn stimulates AMPA/kainate receptors in the second cell and

induces vitamin C release. This explains why the effect of NMDA is blocked by MK801 and also by DNQX.

The release of vitamin C induced by L-Glu is blocked by sulfinpyrazone as well as in the absence of sodium, in a similar way as what happens with the uptake of ascorbate, indicating the participation of a transport system. This is corroborated by the finding that the SVCT2 type of ascorbate transporter is expressed in the chick retina as well as in chick retinal cultures (Portugal and Paes-de-Carvalho, submitted).

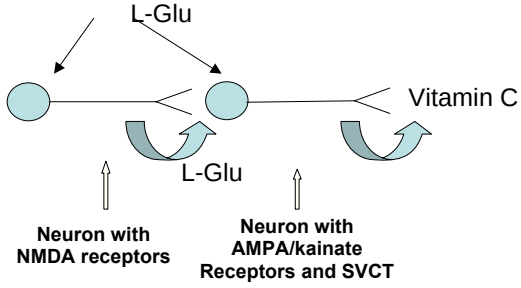

Figure 3. Diagram showing L-Glu and vitamin C-releasing neurons arranged in series and containing different classes of ionotropic receptors in chick retinal cultures.

3. Glutamate Excitotoxicity in the Retina

The exposure of retinal tissue to high concentrations of L-Glu or kainate promotes an extensive damage with a decrease of thickness of the plexiform layers and a decrease of cell numbers, specially in the inner nuclear and ganglion cell layers (Ientile et al., 2001). Photoreceptors are less sensitive and in fact this property was used to make cultures enriched in photoreceptors after long-term exposure to kainate (Hyndman and Adler, 1981; Adler et al., 1982). As Muller glial cells are able to take up L-Glu with high affinity and capacity (Wang et al., 2003), many studies of L-Glu excitotoxicity were performed using purified retinal neuronal cultures (Heidinger et al., 1999).

3.1. Neuroprotection by Adenosine

Previous studies have shown that refeeding purified cultures of chick retinal neurons and photoreceptors with fresh medium promotes an extensive cell death (Paes-de-Carvalho et al., 2003). This phenomenon could be blocked when the cultures are preincubated with adenosine for 24 hours or longer periods, but not when adenosine is given together with fresh medium or preincubated for only two hours before refeeding. This indicates that adenosine is promoting long-term metabolic modifications which are leading to neuroprotection. Interestingly, the protection is mimicked by NBMPR, an adenosine uptake blocker, A2a agonists such as CGS 21680, or 8-bromo cyclic AMP, strongly suggesting that the effect is mediated by activation of A2a receptors and increase of cyclic AMP levels. Furthermore, the effect of NBMPR indicates that an increase of extracellular adenosine released from cultures is also able to protect the cells. This is further corroborated by results showing that the

degradation of endogenous adenosine by incubation of cultures with adenosine deaminase promotes cell death in the same amount as refeeding the cultures with fresh medium. Another interesting finding is that refeeding cultures with conditioned medium obtained from a sister culture does not promote cell death as does refeeding with fresh medium (Paes-de-Carvalho et al., 2003). Since the cell death induced by refeeding is by apoptosis and blocked by general caspase inhibitors (Mejía-García and Paes-de-Carvalho, 2007), it is reasonable to assume that the long-term tratment of cultures with adenosine is leading to permanent metabolic modifications that prevent apoptosis induced by refeeding the cultures. One possibility is that adenosine is promoting the release of trophic factors or transactivating TRKb receptors (Wiese et al., 2007; Lee and Chao, 2001; Rajagopal et al., 2004).

3.2. Blockade of Glutamate Excitotoxicity by Adenosine

The incubation of purified chick retinal neuronal cultures with L-Glu or kainate promotes death of 80-90% of the neurons, including the photoreceptors (Ferreira and Paes-de-Carvalho, 2001). This cell death is greatly attenuated by the NMDA antagonist MK801 or the AMPA/kainate antagonist DNQX, indicating that both types of L-Glu receptors are involved in the phenomenon. In the same way as observed with the cell death induced by refeeding with fresh medium reported in the last section, this effect of L-Glu or kainate is greatly attenuated when cultures are preincubated for 24 or 48 hours with adenosine, A2a receptor agonists, NBMPR or permeable cyclic AMP analogs (Ferreira and Paes-de-Carvalho, 2001).

3.3. Regulation of NMDA Receptors by Adenosine

As stated above, glutamate excitotoxicity in retinal neuronal cultures is mediated by both NMDA and AMPA/kainate receptors. The effect of kainate is attenuated by MK 801, a NMDA receptor channel blocker, indicating that it is primarily mediated by NMDA receptors (Ferreira and Paes-de-Carvalho, 2001). Interactions between adenosine and NMDA receptors were previously described (Koles et al., 2001; Tebano et al., 2005). As a long-term incubation with adenosine or A2a receptor agonists are able to block glutamate excitoxicity in these cultures, one hypothesis was that the nucleoside was modulating the expression of NMDA receptors. In fact, incubation of cultures with adenosine A2a agonists for 24 hours promotes a decrease of (^{3}H)MK801 binding, a decrease of ^{45}Ca influx stimulated by NMDA, and a decrease of NR1 NMDA subunits, strongly reinforcing the hypothesis that adenosine modulates L-Glu excitotoxicity by regulating the expression of NMDA receptors (Ferreira et al., 2002; Ferreira J.M. et al., in preparation).

One interesting finding is that L-Glu excitotoxicity does not occur when cells are cultured in Medium 199 (M199) instead of Basal medium of Eagle (BME) (Ferreira J.M. and Paes-de-Carvalho R., unpublished observations). Instead, these cultures appear healthy and M199 is a medium of choice for growing purified retinal cultures (Adler et al., 1984). As the concentration of L-Glu in M199 is 1 mM while BME does not contain L-Glu, cultures grown in M199 develops in presence of L-Glu since the cell dissociation and seeding of cells in culture dishes. The lack of cell death when L-Glu is added to cultures grown in M199 as opposed to what happens in cultures grown in BME suggests that L-Glu works as a trophic factor and neuroprotective agent, as shown in different cell preparations including the

mammalian retina (Rocha et al., 1999; Martins et al., 2005). However, another possibility is that a long-term incubation with L-Glu leads to modifications in NMDA receptor number and/or composition of subunits.

3.4. Neuroprotection by Nitric Oxide

As in the case of adenosine, preincubation of retinal neuronal cultures with the nitric oxide (NO) donor SNAP is also able to produce neuroprotection against cell death induced by refeeding cultures with fresh medium. The effect is mimicked by Nitroso-glutathione (GSNO) and the substrate of nitric oxide synthase L-arginine (Mejía-García and Paes-de-Carvalho, 2007). Moreover, maximal protection is attained with 24 hours of pretreatment and with the concentration of 100 μM SNAP, and is blocked by the NO scavenger PTIO. The effect of NO is mimicked by YC1, a guanylyl cyclase activator, 8-bromo cyclic GMP, a permeable cyclic GMP analog, or zaprinast, a cyclic GMP phosphodiesterase inhibitor. Accordingly, ODQ, a guanylyl cyclase inhibitor, or KT5823, a PKG inhibitor, completely block the protective effect by NO, showing that this effect is mediated by activation of the classical guanylyl cyclase/cyclic GMP/PKG pathway (Snyder et al., 1991; Bredt and Syder, 1992; Bredt and Snyder, 1994). Interestingly, the effect of SNAP or YC1 is also blocked by inhibitors of many transduction pathways such as PI3 kinase/AKT, MAP kinase, PKA and CAM kinases (Mejía-García and Paes-de-Carvalho, 2007). Recently, we have shown that NO activates these different pathways and is also able to activate CREB phosphorylation (Socodato et al., in preparation, see bellow), suggesting the possibility that NO produces its protective effects through activation of CREB-dependent genes such as Bcl-2 or others (Freeland et al., 2001; Xiang et al., 2006). On the other hand, SNAP regulates the proliferation of retinal glial cells in culture but this effect is not mediated by cyclic GMP and appears to be mediated by a nitrosylation mechanism (Magalhães et al., 2006).

SNAP is also able to inhibit L-Glu excitotoxicity in cultures of retinal neurons, and this effect is dependent of the classical guanylyl cyclase/cyclic GMP/PKG pathway (Ferreira J.M. and Paes-de-Carvalho R., unpublished observations). Long-term incubation with SNAP also regulates the expression of NMDA receptors in the cultures, as measured by (^{3}H)MK 801 binding, ^{45}Ca influx and western blot of NR1 subunits.

4. The Arginine / Nitric Oxide System in the Retina

4.1. L-Arginine Uptake and Metabolism

L-Arginine (L-Arg) has been classified as a “semi-essential” or “conditionally essential” aminoacid. This characterization comes from the fact that L-Arg can be obtained from the diet as a supplementary element besides being synthesized in adult animals during growth or periods of trauma or diseases. In healthy adults, L-Arg is synthesized in sufficient amounts. However, all enzymes responsible for a *de novo* synthesis and catabolism of this aminoacid are not expressed in all tissues. Then, a complex transit of L-Arg and L-citrulline (L-Cit) between organs assures a balance between production and consume of these aminoacids. In this way, the intracellular metabolism as well as the membrane transport are important for the

physiological and pathophysiological role of L-Arg in the organism (for review see Wiesinger, 2001).

L-Arg, besides working as a substrate for the synthesis of NO, is a key molecule for a series of biochemical events and generates several metabolites. In the beginning of the last century it was shown a high arginase activity in mammals (see bellow), hydrolyzing L-Arg in L-ornithine and urea, leading to the discovery of the urea cycle by Krebs and Henseleit in 1932 (Wu e Morris Jr, 1998). This was the first described biochemical cycle involved in the detoxification of excess nitrogen outside the CNS (Wiesinger, 2001).

The endothelium-derived relaxing factor (EDRF), known today to be NO, was discovered in 1980 by Furchgott e Zawadski (Wu e Morris Jr, 1998), and plays a key role in the relaxation of arterial smooth muscle. An important discovery was made in 1988 when it was shown that L-Arg is the precursor for the synthesis of endogenous nitrites and nitrates (Palmer et al., 1988). The discovery of the L-Arg/NO pathway has been considered as very important in animal biochemistry and physiology.

4.2. Diferential Localization of the Molecular Machinery of the L-Arg/NO/L-Cit System in the Retina

The components of the L-Arg/NO/L-Cit system are present in distinct cell populations in the brain (Nakamura et al., 1990; Nakamura et al., 1991). These systems are probably expressed in different cell types in order to keep the cycle of L-Arg synthesis in CNS (Collard, 1995; Wiesinger, 2001).

The regulation of NO synthesis is of fundamental importance for CNS physiology. NO not only works as a chemical messenger, but is also involved in the regulation of neurotransmitter release (Bredt and Snyder, 1992) and developmental functions such as control of cell proliferation and cell death (Peunova and Enikolopov, 1995). One of the limiting key points in the control of NO synthesis is the regulation of availability of the substrate L-Arg. Classically, the cell producing NO is also able to keep the substrate available in the cytoplasm by two distinct mechanisms: 1) Through the cycle of L-Arg synthesis, which is coupled to the cytric acid cycle; or 2) through the uptake of this substrate by some transport systems (y^+, y^+L, $b^{0,+}$, $B^{0,+}$; see bellow).

The transport of L-Arg has been studied in the retina because of its role in the formation of NO. NO is produced in many retinal cell types depending on its physiological state, but in most species amacrine cells and photoreceptors are labeled by the NADPH diaphorase histochemical technique, indicating the expression of NOS in these cells. When stimulated with agents suchs as lipopolysacharide (LPS), NOS is also found in Müller cells. However, endogenous L-Arg was not demonstrated in neurons but was specifically found in Muller glial cells. This differential localization is not a great surprise since L-Arg can be formed *de novo* in Müller cells from reactions involving aspartate/glutamate. This implies that an uptake system should be requested to transport L-Arg from Muller cells to the nitrergic neurons in the retina (Pow, 2001).

The presence of an L-Arg uptake system was demonstrated in cultures of chick embryo retina cells and related to the presence of the NOS enzyme (Cossenza and Paes-de-Carvalho, 2000). In these cultures, NOS immunoreactivity is evident in some neurons (including photoreceptors) and completely absent in glial cells. The uptake system is apparently present

in all cell types including neurons, photoreceptors and glial cells when analysed by autoradiography. When all cell types coexist in the cultures, the autoradiographic grains predominate in glial cells, while neurons present a smaller labeling. However, in purified cultures containing neurons and photoreceptors, the presence of autoradiographic grains is observed since the initial 5 minute period of uptake and an intense labeling, including in the dendritic tree, after 15 minutes of incubation with L-(^{3}H) Arg. These results indicate that neurons and photoreceptors are able to take up L-Arg, but in the presence of neighbor glial cells, the uptake is preferentially performed by these glial cells. Studies of ionic dependence and relative affinity of different substrates indicate the existence of at least two types of transport systems for L-Arg in these cultures, one sodium-dependent and another sodium-independent (Cossenza and Paes-de-Carvalho, 2000).

4.3. Transport of L-Arg and L-Cit in Retina and Other Tissues

The coupling of L-Arg metabolism between various tissues and organs suggests the presence of transport systems for L-Cit and L-Arg in a variety of cell types. The physiological characterization of transport systems for amino acids raised the concept that different groups of amino acids are recognized by carrier proteins according to biochemical and physicochemical properties of the substrates. Neutral amino acids share the transport systems of different charged amino acids (Christensen, 1989). Different amino acid transport systems has been identified and characterized using kinetic properties as substrate recognition, pH influence, dependence on membrane voltage and requirement of sodium ions as a substrate for co-transport (reviewed by Christensen, 1989; Kilberg et al., 1993). Van Winkle (1993) proposed the terminology "transport system" to functionally designate distinct transport processes and used the term "transporter" to denote the protein usually expressed from a cloned gene which catalyses the transport of amino acids across the membrane. Most of the transport of cationic amino acids in different cell types is mediated by the y^+ system, which facilitates the transport of L-lysine, L-ornithine and L-Arg in a sodium-independent and high-affinity manner, and the transport of glutamine and homoserine in a sodium-dependent way (White, 1985; MacLeod et al., 1994; Kakuda & MacLeod, 1994; MacLeod & Kakuda, 1996). Other transport systems such as y^+L, $b^{0,+}$, which are of high affinity and sodium-independent, and $B^{0,+}$, of high affinity but sodium-dependent, also participate in the transport of cationic amino acids in some tissues (MacLeod & Kakuda, 1996).

Recently, several transport proteins denominated CATs (*"Cationic Amino acid Transporter"*), CAT1, CAT2 e CAT3, were cloned and identified as functionally similar to the y^+ system, when expressed in *Xenopus oocytes.* The gene *Cat1* codes for the protein CAT1 which is constitutively expressed. The gene *Cat2* codes for two different isoforms, CAT2a and CAT2b, which are the result of alternative splicing and differ in segments of 41-42 amino acids (Kakuda et al 1998). Recent studies have shown that inflammation mediators such as interleucine-1β, tumor necrosis factor-ɑ(TNF-α) and LPS stimulate the expression of the gene *Cat2,* but not *Cat1,* in rat vascular and astroglial cells (Gill et al., 1996; Stevens et al., 1996). The gene recently identified as *Cat3*, which codes for the CAT3 transporter, is exclusively expressed in the adult rat brain (Hosokawa et al., 1997). Recently, it was described a gene for *Cat-4* in brain, testis and human placenta (Wiesinger, 2001).

The second class of transporters is represented by the formation of heterodimers (with light and heavy chains) in the membrane. The glicoproteins rBAT and 4F2hc constitute the heavy chains and other seven proteins which represent the light chain are able to form the transporter molecule (Bröer et al, 2001). For the L-Arg transport, the proteins y+LAT1 and y+LAT2 represent two isoforms of the y+L system when associated to the glicoprotein 4F2hc (4F2hc/y+LAT), and the $b^{0,+}$AT when associated to rBAT (rBAT/$b^{0,+}$AT) represents the $b^{0,+}$system. The expression of the heterodimer 4F2hc/y^+LAT has been detected in the brain and in cultures of neurons and astrocytes (Bröer et al, 2000; 2001).

In relation to the $B^{0,+}$ system, it was not yet reported any protein, and it is only known that it is a sodium-dependent transport system (Kakuda e MacLeod., 1994). It was identified in rabbit conjuntives a sodium-dependent system in which sodium removal reduces the transport capacity to 6% of control. The pattern of inhibition by other amino acids appears to aggree with the pattern of the $B^{0,+}$ system (Hosoya et al., 1997). In chick retinal cultures, it was also demonstrated the participation of at least two L-Arg transport components, one sodium-dependent and another sodium-independent (Cossenza and Paes-de-Carvalho, 2000).

Very little is known about the transport of L-Cit. All investigated cell types, including astrocytes, microglia and neurons, are able to transport L-Cit with K_m values between 0.4 and 3.4 mM, and the L system for large and neutral amino acids has been proposed to mediate the neuronal transport of L-Cit (Schmidlin et al, 2000). This system is described as sodium-independent and the main system for aromatic and side-chain amino acids (Babu et al., 2003). Their carrier proteins were isolated and designated as LAT (*"L-type amino acid transporter"*). At least three LAT isoforms were already identified (LAT1, LAT2 and LAT3) and as in the case of y+LATs, need to form heterodimers with the proteins 4F2hc or rBAT (Babu et al., 2003). Any regulatory processes were described for this system and, due to the lack of specificity, L-Cit has to compete with many other substrates. Most amino acids which are substrates for the L system are transported with K_m values in the micromolar range (Kanai et al., 1998) and with concentrations many times higher than that of L-Cit. Therefore, the participation of extracellular L-Cit as part of the neural L-Arg/NO/L-Cit cycle appears to depend on the concentrations of all neutral amino acids present (Schmidlin et al., 2000). Other neutral amino acid transport systems have been studied and related to L-Cit transport in other non-neuronal cell types. For instance, in smooth muscle cells from aorta, it was identified a transport pattern which resembles at least two different types, the L system (described above) and the N system which is sodium-dependent and has an identified protein named SN1 (Bode, 2001; Wileman et al., 2003).

5. Regulation of Protein Synthesis in the Retina and the Role of Glutamate Receptors

In the last few years the process of protein synthesis presented many advances in the knowledge of the regulation of mRNA translation, via interaction with specific factors or modulation of activity of components of the translation machinery (Browne e Proud, 2002). These factors consist in specific coupling proteins and kinases which play roles of rigid control of this process (for a review see Proud, 2002). The factors are classified in large groups such as: 1) Initiation factors (involved in the assembly of ribosomes in mRNA strand

to be translated), represented in eucariotes as "eIF", with several subtypes; 2) elongation factors (responsible to carry aminoacyl-tRNA to the ribosome and for translocation of this component from the A to the P site), represented in eucariotes as "eEF", also with many subtypes; and 3) termination factors (responsible to end the translation process), designated as "eTF", also with many subtypes. There is a great number of sophisticated mechanisms that directly regulate the initiation of mRNA translation (for review see Proud, 2002). A number of components of the translation machinery are regulated by signaling events involving the protein mTOR ("*mammalian target of rapamycin"*), a kinase activated by several stimuli such as insulin, growth factors and concentrations of ramified amino acids (reviewed in Proud, 2002; Browne & Proud, 2002; Proud, 2004). For instance, the factor eIF4E has affinity to a coupling protein known as 4E-BP (4E-*"binding protein"*), which blocks its interaction with the target in mRNA, the 5´-cap region. This coupling is reversed by multiple phosphorylations in 4E-BP by mTOR or MAP Kinase (for review see Proud, 2002; Browne & Proud, 2002).

The elongation phase wastes a large amount of energy. At least four high energy bounds are consumed by each amino acid added to the nascent polypeptide chain (two ATP molecules to form the aminoacyl-t-RNA and two GTP molecules for events in the ribosome which involve elongation factors) (Browne & Proud, 2002). The elongation factors known to participate in the elongation phase are eEF1, with the subtypes eEF1A and eEF1B, and eEF2. The first two are involved in the transport of aminoacyl-tRNA to the A site of the ribosome. eEF1A is a GTP binding protein and then its activity depends on GTP hydrolysis. eEF1B is described as a guanylyl nucleotide exchange factor responsible for the exchange of GDP by GTP recovering the activity of eEF1A (Browne & Proud, 2002). eEF2 also uses GTP metabolism and is a factor responsible for translocation of the ribosome from the A to the P site, and consequently the growth of the polypeptide chain. This factor contains a threonine residue in its position 56 of the N-terminal which can be phosphorylated inhibiting its activity (Browne & Proud, 2002).

Ryazanov (1987) showed that eEF2 can be phosphorylated in a calcium/calmodulin-dependent manner and Palfrey and Nairn (1995) have identified eEF2 as a substrate for Ca^{2+}/calmodulin-dependent kinase III (CAMKIII). eEF2 is the only known substrate for this enzyme and then it is now known as eEF2 Kinase (eEF2K) (Browne & Proud, 2002). Nairn e Palfrey (1987) then showed that agents that increase cytoplasmatic Ca^{2+} levels are able to increase eEF2 phosphorylation (Hinke e Nairn, 1992; Mackie et al., 1989). Marin et al. (1997) and Scheetz et al. (1997; 2000) showed a decrease in protein synthesis after eEF2 phosphorylation induced by a short stimulation of NMDA receptors. Nowadays, the structure of the enzyme eEF2K is known and several phosphorylation sites were identified in the enzyme, including a serine at position 500 which represents a region of activation by PKA. Accordingly, the phosphorylation of eEF2 by α-adrenergic agonists in cardiomiocytes was demonstrated (McLeod et al., 2001). Other important sites have been identified as serine 366, which is phosphorylated by a kinase known as p70S6K, which is activated by mTOR, resulting in eEF2K inactivation. This is the mechanism by which insulin activates protein synthesis (Browne & Proud, 2002; Proud, 2002). This same residue is also phosphorylated by $p90^{RSKI}$, a kinase directly activated by Erk of the classical MAP Kinase pathway (Browne & Proud, 2002; Proud, 2002). Knebel et al. (2002) identified Serine 359 as a substrate for SAPK4/p38α (*"stress-activated protein kinase 4"*), a member of the family of kinases activated by stress, and its phosphorylation also inactivates eEF2K. As discussed above, the

elongation phase requires a great amount of energy, and some authors indicate the inhibition of protein synthesis as a fundamental step for cell economy during particular events. In fact, it was described an inhibition of protein synthesis by activation of AMPK ("*AMP activated protein kinase"*), considered as an "ATP sensor", which could phosphorylate and inhibit mTOR (Horman et al., 2002).

5.1. Relation between Protein Synthesis and the Biochemistry of L-Arg

Since the discovery of the NO system, several authors have pointed out a close relationship between L-Arg transport and NO production, as for example fine adjustments of the constitutive transporters in relation to the enzymatic production of NO, increase in the expression of inducible isoforms under special conditions and the regulation by photons in the retina (Kakuda et al., 1998; Nicholson et al., 2001; Sáenz et al., 2002). They have been looking for a good explanation for the phenomenon known as "L-Arg paradox", which states that the production of NO can be increased by the addition of extracellular L-Arg, although the intracellular concentration is much higher than the Km for NOS. Kurz and Harrison (1997) suggested that intracellular L-Arg is sequestered to different "pools" with little access to NOS, while extracellular L-Arg would be preferentially used for NO biosynthesis. Indeed, exogenous L-Arg released from cells that usually do not synthesize NO is recruited by stimulation with L-Glu, depolarization with high potassium concentrations or by peroxynitrite, and then gives support to NO production (Grima et al. 1997;1998; Cossenza & Paes-de-Carvalho, 2000; Vega-Agapito, 1999; 2002). However, the question of how L-Arg accesses the enzyme or the L-Arg "pools" are regulated remains open. McDonald et al. (1997) showed in an elegant study that CAT-1 and NOS-III are compartmentalized in the same complex inside the caveola, then giving a direct evidence of a physical coupling between L-Arg influx and NO synthesis. Lee et al. (2003) showed that a decrease of L-Arg availability blocks the induction of NO production by cytokines in astrocytes. Interestingly, the blockade seems to be due to inhibition of NOS-II expression through a blockade of mRNA translation. The authors suggest that after stimulation by cytokines, L-Arg uptake negatively regulates the phosphorylation "status" of the initiation eucariotic factor type 2α (eIF2α) and then NOS-II translation. eIF2α phosphorylation is attributed to the mammalian kinase homologous to yeast GCN2. In this work, the enzyme is described as regulated by L-Arg availability and then related to the "L-Arg paradox".

In the CNS, NOS-I is not directly associated to membranes or caveola, and its expression is constitutive. In the work by Cossenza et al. (2006), the authors demonstrated that protein synthesis is an important driving force in L-Arg uptake. More than 70% of intracellular L-Arg is incorporated in proteins in developing retinal cell cultures. When incorporation is blocked by cycloheximide, there is an increase of free intracellular L-Arg. The availability of these "pools" was confirmed when the cultures presented an increase of L-Cit production detected both in intracellular and extracellular medium. According to these results, protein synthesis can be playing an active role in the compartmentalization of L-Arg "pools", working as a stock of this amino acid. Therefore, protein synthesis can be working as an intermediate step between L-Arg influx and the regulation of intracellular "pools" available for the synthesis of signaling molecules in their own environments.

5.2. Regulation of Protein Synthesis and L-Arg Availability by NMDA Receptors

During the process of protein synthesis, many steps occur up to the appearance of the polypeptide chain. Several translation factors participate in initiation, elongation and termination steps. eEF2, an elongation factor, is one of the most proeminent phosphorylated proteins in extracts of mammalian tissues. eEF2 phosphorylation results in its inactivation and then is related to inhibition of protein synthesis in the elongation phase (reviewed in Ryazanov, 2002). As discussed above, eEF2 phosphorylation is catalysed by the highly selective calcium/calmodulin-dependent kinase eEF2-kinase. Therefore, it was suggested that intracellular calcium can regulate the rate of protein synthesis through inactivation of eEF2. Recently, it was suggested that activation of L-Glu ionotropic receptors promotes inhibition of protein synthesis trough calcium influx and stimulation of eEF2-K (Marin et al, 1997; Scheetz et al., 1997) in superior colliculus synaptosomes (Scheetz et al., 2000). Cossenza et al. (2006) showed a potential inhibition of amino acid incorporation to proteins by stimulation of NMDA receptors. Using [^{35}S]Methionine ([^{35}S]Met) or L-[^{3}H]Arg incorporation assays, it was found significant reductions in the incorporation of these amino acids during stimulation with NMDA since 2-5 minutes of incubation up to 60 minutes. This effect is probably mediated by calcium influx and eEF2K activation, since the authors showed that the same stimulation is able to increase phosfo-eEF2 levels and this effect is blocked by MK-801, an NMDA receptor antagonist. Curiously, besides the effect of inhibition of amino acid incorporation in proteins, the addition of NMDA also increases intracellular L-Arg concentrations in a time scale very similar to that obtained in [^{35}S]Met or L-[^{3}H]Arg incorporation inhibition assays and the increase of phosfo-eEF2.

A large increase in L-Arg intracellular concentrations is observed when protein synthesis is inhibited by cycloheximide, and no additional increase by NMDA is observed in this case. This result strongly suggests that the increase of intracellular L-Arg concentrations mediated by the NMDA receptor depends on the inhibition of protein synthesis. In this way, one possibility is that a specific source of L-Arg, involving transport or synthesis, could be directing to proteins located in different intracellular compartments and operating as compartmentalized stocks. These synthetic machineries could be rapidly mobilized by activation of membrane receptors through signaling molecules leading to the interruption of protein synthesis and consequently increasing the “pool” of free amino acids.

5.3. L-Arg Availability Mediated by NMDA Receptors and the Production of L-Cit and NO

As discussed above, protein synthesis seems to be closely related with the “pool” of free L-Arg in the cytoplasm. Therefore, it is possible that these intracellular “pools” are “feeding” NOS and sustaining NO production. This hypothesis is confirmed when cycloheximide is added in a 15 minute period of stimulation promoting the increase of L-Cit concentrations, a process blocked by L-Nitroarginine, a NOS inhibitor. L-Glu promotes an increase of intracellular L-Arg concentrations by signaling through NMDA receptors, probably by inhibiting protein synthesis. Cossenza et al (2006) also propose that the activation of NMDA receptors, that classically promotes stimulation of NOS through the calcium-calmodulin

complex, also promotes NOS stimulation by increasing enzyme activity after increasing the concentrations of the substrate L-Arg. This hypothesis is strenghtened by experiments using retinal cell cultures treated or not with cycloheximide. In the situation where protein synthesis is inhibited during the whole experimental period, it is possible to determine an increase of approximately 20% in (^{3}H]Cit concentration when cultures are stimulated for 15 minutes with NMDA, while in the absence of cycloheximide the difference between control and stimulated cultures is approximately 47%. This difference in the results can be explained by two different mechanisms: 1) Mobilization of substrate for NOS through inhibition of protein synthesis mediated by NMDA receptors and 2) Activation of NOS by NMDA receptors through calcium influx and formation of the calcium-calmodulin complex. In cultures treated with cycloheximide the effect of increase in L-Arg intracellular concentrations due to activation of NMDA receptors is not observed, since no protein synthesis is occurring and the levels of free L-Arg are maximal. These results indicate that the increase of intracellular L-Arg concentrations is able to promote an increase in NO production and consequently of L-Cit. In this way, the increase promoted by cycloheximide or NMDA would be displacing the values of V_{max} to NOS to levels related to these higher levels of L-Arg concentrations. This is a plausible phenomenon since L-Arg concentrations are still much lower than the K_m for NOS and then in the linear phase of Michaelis-Menten kinetics. Ientile et al. (1999) showed that the stimulation of NMDA receptors produces an increase of cyclic GMP levels in cultures of chick embryo retinal cells. Therefore, the low levels of L-Cit observed by Cossenza et al. (2006) could reflect concentrations of NO able to stimulate soluble guanilyl cyclase to produce amplified amounts of cyclic GMP. Indeed, unpublished results by Mejía-García T.A, Cossenza M. and Paes-de-Carvalho R. shows that the NO donor S-nitroso-N-acetyl penicilamine (SNAP), as well as the addition of exogenous L-Arg or 3(5'-hidroximetil-2'-furil)-1-benzilindazol (YC-1), a soluble guanylyl cyclase stimulator, increase AKT phosphorylation, a phenomenon mimicked by inhibition of protein synthesis by cycloheximide. These results reinforce the idea that inhibition of protein synthesis and the consequent increase of intracellular L-Arg concentrations are able to produce an increase of NO levels that are relevant for important biological processes such as cyclic GMP accumulation and AKT activation.

5.4. Local Protein Synthesis and the Production of NO

Several works in the literature indicate that many important proteins are synthesized in specific locations in synapse regions. Some of these proteins such as CAMK II are involved in phenomena such as LTP, LTD and synapse formation (Scheetz et al., 2000; Richter & Lorenz, 2002; Mendez & Wells, 2002; Murray et al., 2003; Steward, 2002; Wells et al., 2000). Wu et al. (1998) showed that the activation of NMDA receptors is involved in triggering poly-adenylation of mRNA for CAMKII via CPE (*"cytoplasmatic polyadenylation protein"*). Events of poly-adenylation increase the mRNA poly(A) tail promoting translation initiation. Sheetz et al. (2000) also showed an increase of CAMK II synthesis in synaptosomes of the superior colliculus of young rats after stimulation of NMDA receptors, although the same stimulation considerably reduces total protein synthesis in the synapse. Actually, the local regulation of protein synthesis seems to have important consequences during embryonic development. The extension and retraction of growth cones appears to have

an intimate correlation between protein synthesis and its blockade (Campbell e Holt, 2001). As discussed above, the elongation phase requires a large amount of energy, and some authors suggest that inhibition of protein synthesis is a fundamental economic step in some events. NO also has important effects during CNS development in events such as cell proliferation and survival (Peunova e Enikolopov, 1995), as well as in the synaptic physiology of mature neurons, as for example in the regulation of neurotransmitter release (Barcellos et al., 2000; Blackshaw et al., 2003; Stanton et al., 2003). The discoveries reported by Cossenza et al. (2006) should have important consequences in these phenomena stablishing a new relationship between local regulation of protein synthesis and NO production.

5.5. Substrate Support for NO Production

Work in the literature describes a probable intercellular communication for the enzymatic cycle of L-Arg recovery in CNS (Pow, 1994a; 1994b; Pow e Crook, 1997; Grima et al., 1997; 1998; Cossenza & Paes-de-Carvalho, 2000; Wiesinger, 2001). Indeed, some authors propose that glial cells are fundamental actors in this biochemical scenario, directly participating in the apport of L-Arg for neuronal NO synthesis (Pow, 1994a; 1994b; Pow & Crook, 1997; Grima et al., 1997; 1998; Cossenza & Paes de Carvalho, 2000; Wiesinger, 2001). Its function involves the accumulation of L-Arg via transport systems (Schmidlin & Wiesinger, 1994; 1995; Cossenza & Paes-de-Carvalho, 2000), and a probable release of this amino acid after different stimuli (Grima et al., 1997; 1998; Veja-Agapito et al., 1999; 2001; Cossenza and Paes-de-Carvalho 2000). Studies using cortical astrocytes shows that stimulation of non-NMDA glutamate receptors is able to stimulate L-Arg release from these cells (Grima et al., 1997) and to determine the rate of neuronal production of NO (Grima et al., 1998). Pow (1994) showed in neurohipophysis an immunolabeling for L-Cit in neurons and an immunolabeling for L-Arg mainly in glial cells. The amino acid L-aspartate, involved in the recycling of L-Cit to L-Arg, is exclusively found in glial cells, suggesting the participation of neurons and glial cells in L-Arg recycling. The same pattern is found in retinal Müller glial cells (Pow, 2001). Release assays from glial cells in a model of co-culture of retinal cells (glial cells and neurons separately cultivated and held together during the experiment, but with no direct physical contact) shows that glial cells are able to release L-Arg after depolarization and this amino acid can be taken up by neurons growing in coverslips above the glial layer (Cossenza and Paes-de-Carvalho, 2000). These results were repeated using peroxynitrite to stimulate glial release and the subsequent neuronal uptake of L-Arg using a very similar experimental protocol (Vega-Agapito et al., 1999; 2002). Recent work indicates that the heterodimeric complex 4F2hc/y^+LAT2 is present in astrocytes and working in L-Arg release by exchange with L-glutamine (Bröer et al., 2000).

The present findings indicate the existence of some more pieces in this complicated jigsaw puzzle. According to the reported data, the evident participation of L-Glu in the nitrergic signaling is even more important. The high rates of protein synthesis during the embryonic development points out to a recruitment of extracellular amino acids that could be guided to several intracellular regions in which protein synthesis is occurring, and L-Glu could directly interfere with the availability of free L-Arg. Then, when L-Arg is taken up, its preferential driving force would be to be incorporated in proteins, which is demanding a great

proportion of amino acids. When necessary, this demand would be supported by L-Arg coming from glial cells, at the same time that intracellular regions close to NOS would decrease the local rate of protein synthesis increasing the local substrate concentrations for NO production. It is obvious that we cannot rule out the possibility that this phenomenon is also working in glial cells. According to some data, it was found the expression of NMDA subunits NR1, NR2A and NR2B in glial cells from the avian retina (Lopez et al., 1997; Lopez-Colome et al., 1993; 1995, 1997; Luque e Richards, 1995). If the possible NMDA receptor assembly in glial cells is able to couple to eEF2-K activation, it would be feasible that the mechanism of protein synthesis regulation could be guided to turn free L-Arg available for release.

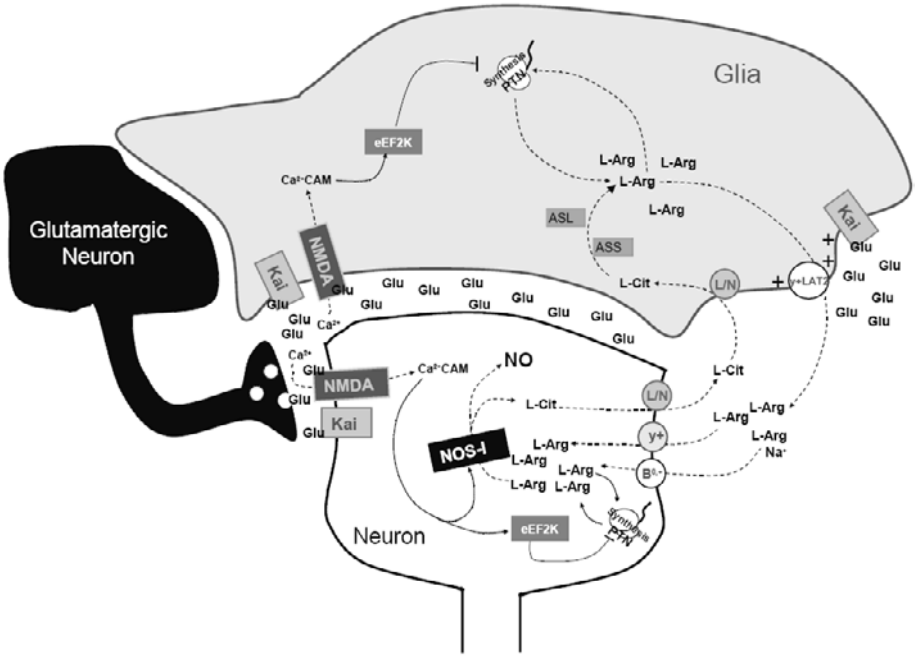

Figure 4. Hypothetic model of the regulation of L-Arg/NO/L-Cit cycle by glutamatergic signaling, as developed from several authors:

In summary, the sequence of events and the general scheme would be as that shown in figure 4: In a glutamatergic synapse, the presence of L-Glu would stimulate AMPA/Kainate receptors leading to a depolarization of the post-synaptic cell. With the change of membrane potential and the binding of agonists, the NMDA receptor would be functionally operant promoting calcium influx. Therefore, the formation of the calcium/calmodulin complex could activate two enzymatic pathways: 1) The enzyme NOS which would catalyse the production of NO from its substrate L-Arg and 2) The enzyme eEF2-K, which would phosphorylate the translation factor eEF2 inhibiting protein synthesis. With this interruption of protein synthesis, the amino acids guided to this process would increase their local concentrations and could then be used in other metabolic pathways like the stimulation of NOS and production of NO. In parallel, the neurons could promote the release of L-Cit which could then be taken up by glial cells. According to some authors, L-Cit could work as a precursor for the recovery of

L-Arg used in this process in a system involving the participation of glial cells and/or other neurons (Aoki et al., 1991; Pow, 1994a; 1994b; Pow & Croock, 1997; Wiesinger, 2001). Then, resynthesized L-Arg could be guided for protein synthesis in glial cell or neurons, or also fill its available "*pools"* for release. The question if glial cells express NMDA receptors coupled to protein synthesis is unknown at present and deserves future investigations. L-Arg would be released from glial cells by depolarizing stimuli involving AMPA/Kainate receptors (Grima et al., 1997; 1998; Bröer et al., 2000; Cossenza and Paes-de-Carvalho, 2000), and their activation would regulate the system y^+LAT2, which would be operating in the outward direction. This L-Arg would then be taken up by neurons through at least two transport systems (y^+ and $B^{0,+}$) and guided to protein synthesis. The protein synthesis would be an essential factor for the recruitment of extracellular amino acids, and the utilization of this "*pool"* could be regulated by L-Glu. The description of these new events contributes in a significative way to the process of neurotransmission involving L-Glu and NO. The question that a neurotransmitter can directly couple different pathways regulating protein synthesis and that this phenomenon is directly related with the synthesis of a signaling molecule is still not discussed in the literature.

L-Arg release from glia (Grima et al., 1997; 1998; Vega-Agapito et al., 1999; 2002; Bröer et al., 2000; Cossenza & Paes-de-Carvalho, 2000); Neuronal uptake of L-Arg released from glia (Cossenza & Paes-de-Carvalho, 2000; Vega-Agapito et al., 2002); L-Arg glial recycling (Pow, 1994a; 1994b; Pow & Croock, 1997; Cossenza & Paes-de-Carvalho, 2000); Neuronal localization of NOS and L-Cit and glial localizationof L-Arg (Aoki et al., 1991, Pow, 1994a; 1994b; Pow e Croock, 1997; Cossenza & Paes-de-Carvalho, 2000); Glial L-Arg release by L-Glu and neuronal production of NO (Grima et al., 1997; 1998); eEF2K activation and inhibition of protein synthesis by calcium influx mediated by activation of NMDA receptors (Marin et al, 1997; Scheetz et al., 1997; 2000; Cossenza et al., 2006); Increase of intracellular L-Arg mediated by activation of NMDA receptors, leading to a greater production of NO (Cossenza et al., 2006). Abreviations used: Glu, glutamate, L-Arg, L-Arginine, L-Cit, L-citrulline, CAM, calmodulin, NOS, nitric oxide synthase, Kai, kainate receptors, NMDA, N-methyl D-aspartate receptors, eEF2K, eucariotic elongation factor 2 kinase, ASS, argininosuccinate synthetase, ASL, argininosuccinate lyase, PTN, protein.

6. Glutamate and Signal Transduction Pathways in the Retina

L-Glu contribution to retinal processing and signal transduction is of prime importance to generate accurate patterns of electrical activity that leaves the retina and reaches higher order integration centers in the cerebral cortex to achieve visual perception. Although many aspects of L-Glu neurochemistry in the retina are well documented, intracellular signaling pathways modulated by activation of L-Glu receptors is still an elusive theme regarding the retina. During this section, we will portray few experimental data on the topic of signaling pathways and calcium homeostasis modulated by glutamate receptors in the vertebrate retina. Furthermore, we will present evidence that L-Glu activates several intracellular signaling pathways in the embryonic chick retina.

The activation of NMDA receptors in mature rat retina induces an increase of matrix metalloproteinase-9 (MMP-9) activity restricted to the ganglion cell layer, an effect mediated by NO production and protein S-nitrosylation (Manabe et al., 2005).

NMDA receptor activation in cultured chick retinal cells also promotes an increase in nitric oxide synthase (NOS) activity and cyclic GMP production (Ientile et al., 1999). Just to correlate, NMDA and kainate administration to turtle retina also promotes a rise in intracellular levels of cyclic GMP, mainly in bipolar, amacrine and some ganglion cells (Blute et al., 1999), which was dependent on NO production (Blute et al., 1999).

Moreover, NMDA receptors are very efficient in inhibiting BDNF-induced AKT phosphorylation in rat retinal ganglion cells (Nakazawa et al., 2005), and this effect is associated indirectly with NMDA-induced phosphatase 1/2A activation (Nakazawa et al., 2005).

NMDA protects rat retinal explants against L-Glu-induced cell death and interestingly, this effect is mediated by BDNF (Martins et al., 2005). Furthermore, in this paradigm, NMDA is capable of increasing the expression levels of mRNA for BDNF and its high affinity receptor TrkB, as well as BDNF protein (Martins et al., 2005). Additionally, chronic L-Glu administration also promotes ERK 1/2 phosphorylation in rat retina Müller cells (Zhou et al., 2007).

L-Glu acting primarily through NMDA and AMPA/kainate receptors, but not metabotropic receptors, modulates CaMKII membrane expression and activity in the adult rat retina (Laabich and Cooper, 1999). However, ionotropic and metabotropic receptors seem to account for L-Glu-induced accumulation of intracellular radiolabeled inositol in cultured embryonic chick retinal cells (Milani et al., 1990). On the other hand, metabotropic L-Glu receptor type 5 (mGluR5) seems to play an important role in generating calcium oscillations in chicken GABAergic amacrine cells (Sosa et al., 2002). mGluR5 is capable of coupling and activating phospholipase C in these cells, increasing calcium from intracellular stores (Sosa et al., 2002), and also controlling calcium influx through TRP channels (Sosa et al., 2002). mGluR8 works as an auto-inhibitory receptor in presynaptic terminals of isolated mammalian photoreceptors (Koulen et al., 1999). This metabotropic L-Glu receptor achieves this characteristic by decreasing the intracellular calcium concentration in the photoreceptor cell (Koulen et al., 1999), and this may provide an inhibitory feedback loop for L-Glu release by these cells.

Both mGluR1and 5 contribute to the decrease in calcium current through L-type calcium channels in cultured neurons of the Xenopus retina (Akopian and Witkovsky, 1996). What is interesting in this case is that these metabotropic receptors modulate calcium currents by a mechanism dependent on IP3 accumulation and calcium release from internal stores (Akopian and Witkovsky, 1996).

Metabotropic and ionotropic L-Glu receptors are efficient in decreasing calcium currents in ganglion cells from slices of the tiger salamander retina (Shen and Slaughter, 1998). Pharmacological data indicate that the metabotropic receptor effect relies on intracellular IP3 generation, activation of ryanodine receptors and release of intracellular calcium stores to modulate L-type calcium channel (Shen and Slaughter, 1998). Ionotropic receptor effect is mediated by calcium influx and activation of calmodulin, with subsequent activation of the calcium/calmodulin-dependent protein phosphatase calcineurin and decrease of calcium current due to phosphatase inhibition of N-type calcium channel (Shen and Slaughter, 1998).

One interesting point is the extracellular-mediated activation of classical kinase systems, such as the PI3K/AKT and ERKs MAP kinases. Recent data demonstrate that L-Glu induces a rapid and transient ERK2 and CREB transcription factor phosphorylation in cultured chick retinal cells, a process reliant of AMPA/Kainate receptors and NO generation (Socodato, R.E.S., Magalhães, C.R. and Paes-de-Carvalho R., in preparation) but, however, independent of NMDA receptors. Moreover, the effect of NO on ERK2 and CREB involves the classical NO/soluble guanylyc cyclase/PKG system. L-Glu and consequently NO-induced CREB phosphorylation is dependent on prior activation of multiple kinases signaling, downstream to the NO pathway, namely the PKA, PKC, CaM kinases, PI3K and Src kinase pathways (figure 1). Moreover, immunocytochemistry data strongly suggest that the increase in CREB phosphorylation at serine 133 in response to L-Glu takes place in Müller cells nuclei. Figure 1. Signaling pathways that are activated by glutamate stimulation of AMPA/Kainate receptors in cultured chicken embryonic retinal cells.

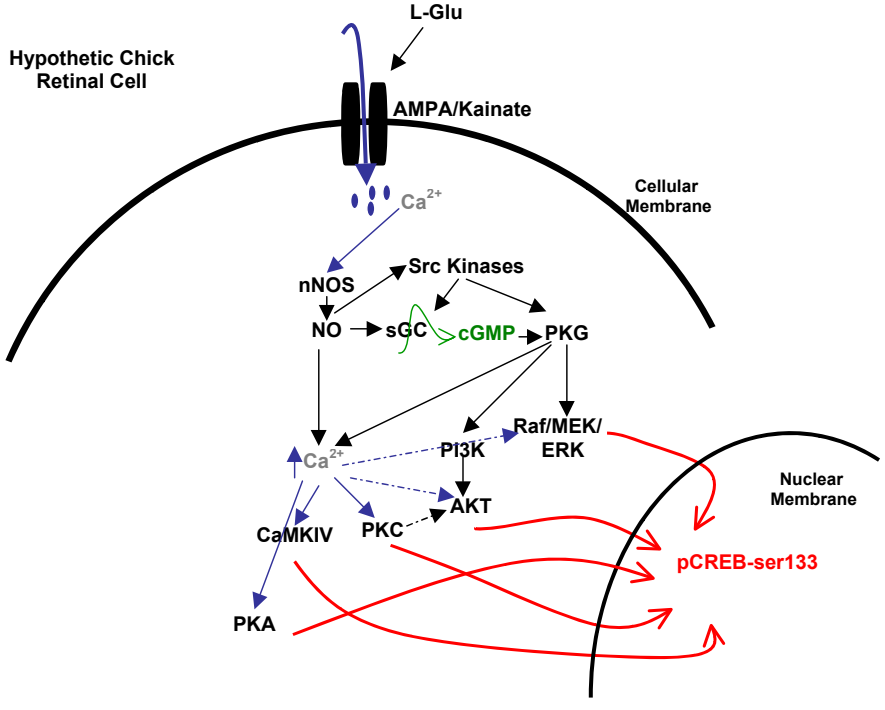

Figure 5. Schematic representation of the signaling pathways involved in L-Glu stimulation of CREB phosphorylation.

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Chapter 2

Peripheral Glutamate Signalling, Immunity and Pain: Novel Aspects in Psychoneuroimmunology

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Abstract

The concept of an intricate network of immuno-neuro-endocrine interactions has evolved in the last 30 years. The scientific field investigating these interactions has been termed "psychoneuroimmunology", and it was proposed that the immune system plays a sensory role, as a "sixth sense", that detects invaders such as pathogens, antigens, allergens and tumors with great sensitivity and specificity. In addition, the fact that each of the systems (i) can produce neurotransmitters, -peptides, hormones, and cytokines, (ii) expresses a common receptor repertoire, and (iii) shares signal transduction pathways, leads to the conclusion that the systems use a common chemical language for intra- and inter-systemic communications. In the last years it became apparent that there are also intimate interactions between the immune system and glutamate receptor-mediated signalling mechanisms. Immune cells have been shown to express different types of glutamate receptors as well as subtypes of the high-affinity glutamate transporter family, similar to observations in other peripheral tissues. Glutamate and its receptors were found to modulate specific and non-specific immune functions, as well as neural activity associated with pain conditions. Furthermore, it was suggested that glutamate may serve as a fast-acting signalling agent between nerval and immune tissues, providing the basis for a multidirectional flow of information among the different organ systems within the body. Taken together, these results indicate that glutamate, besides acting as the major neurotransmitter in the central nervous system, is also involved in peripheral neuroimmunomodulation, working in a receptor/transporter-mediated manner. In summary, new insights into these communication circuits should provide a better understanding of mind-body medicine – a priority for medicine and human life.

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Introduction

Based on the psychoneuroimmunology literature, it is generally accepted that the immune, the endocrine, and the nervous systems are anatomically as well as functionally crosslinked. On the one hand, neuroendocrine and neural signals are involved in the control of humoral and cellular immune responses. On the other, immune mediators, such as cytokines, influence brain functions (Haas and Schauenstein, 1997; Ader, 2000; Haas and Schauenstein, 2001; 2005; Eskandari and Sternberg, 2002; Steinman, 2004; Blalock, 2005; Wrona, 2006; Blalock and Smith, 2007; Besedovsky and del Rey, 2007; Heijnen, 2007; Ziemssen and Kern, 2007). The proposition was put forth that the immune system also serves as a sensory organ that acts as a "sixth sense" to detect foreign invaders (Blalock 2005; Blalock and Smith, 2007). Immune cells were found to produce not only neurotransmitters as well as peptides; they also express receptors for every neurotransmitter or hormone known so far (Haas and Schauenstein, 1997; Steinman, 2004; Heijnen, 2007). In the eighties, the term "synapse" was introduced into the field of immunology (Norcross, 1984). In comparison to the neuronal synapse, the immunological synapse represents the close contact site between T-cells and antigen-presenting cells (APC), and has been shown to play an important role in T-cell antigen recognition, T-cell activation and function (Huppa and Davis, 2003; Steinman 2004; Čemerski and Shaw, 2006; Dustin, 2006; González et al., 2007). In addition, it was found that nerve terminals directly contact immune cells, whereby the sympathetic innervation of immune organs attracted the greatest attention (Felten et al., 1998; Stevenson et al., 2001; Sanders, 2006; Wrona, 2006). In recent years, however, the complexity of neural – immune interactions has become more and more apparent. Moreover, it was even proclaimed that "without nerves, immunology remains incomplete" (Shepherd et al., 2005).

As to the central nervous system (CNS), several data indicate that the hypothalamic-pituitary-adrenal axis (HPA-axis) - the key player in stress responses - regulates immune functions (Turnbull and Rivier, 1999; Haddad et al., 2002; Harbuz et al., 2003). Limbic structures, however, were also implicated in immunoneuroendocrine interactions and found to represent the "real" neuroanatomical substrates for psychoimmunomodulation as they serve as superior regulators of neuroendocrine and autonomic centers (Haas and Schauenstein, 1997). This allows not only an individual modulation of emotional behaviour, but also a selective regulation of stress and immune responses. An increasing number of reports show that cytokines and their receptors are ubiquitously distributed in the brain and, apart from infiltrating leukocytes, glial cells, brain endothelial cells, as well as neurons have been described as major cellular sources (Sawada et al., 1995a, 1995b; Park and Shin, 1996; Botchkina et al., 1997; Haas and Schauenstein, 1997; Acarin et al., 2000; Rothwell and Luheshi, 2000; Joshi et al., 2001; Maślińska, 2001; Strle et al., 2001; Jüttler et al., 2002; Kurowska et al., 2002; Parnet et al., 2002; Utsuyama and Hirokawa, 2002; Andre et al., 2003; Lehtimäki et al., 2003; Wheeler et al., 2003; Fee et al., 2004; Galanakis et al., 2006; Figiel and Dzwonek, 2007). In summary, these results provide the basis for the use of a common language between the different systems. A better understanding of this language will help to decipher the mechanisms underlying health and disease, which ultimately should ensure global acceptance of mind-body medicine.

Distribution of Glutamate Receptors/Transporters in Immune Tissues

In the last years it has become apparent that the major excitatory neurotransmitter glutamate and its receptors (ligand-gated ion channels as well as G-protein coupled metabotropic receptors) are ubiquitously distributed also in peripheral non-neuronal tissues (Skerry and Genever, 2001; Nedergaard et al., 2002; Hinoi et al., 2004; Kalariti et al., 2005; Gill and Pulido, 2005). As to the immune system, it was concluded that glutamate is involved in signalling in immune cells and that the expression of both ionotropic (iGluR) and metabotropic glutamate receptors (mGluR) may have important regulatory functions in immunocompetent cells (Boldyrev et al., 2005). Initial evidence that mononuclear phagocytes may express receptors for dicarboxylic neuroexcitatory amino acids came from a study by Malone et al. (1986). Human peripheral mononuclear cells responded chemotactically to 4-carboxy-L-glutamic acid, an analogue of glutamic acid, and kainate, an ionotropic glutamate receptor agonist. The chemotactic response was stereospecific, cell-type-specific and occurred at nanomolar concentrations, underpinning the notion that the response involves a specific, high-affinity receptor. Interestingly, glutamic acid itself acted as a competitive antagonist in this experimental system without any agonist activity (Malone et al., 1986). In our own studies we used flow cytometric analysis to measure changes in the phagocytic activity of human monocytes after incubation of whole blood from healthy volunteers with various concentrations of glutamatergic agonists and antagonists (Haas et al., 2003; 2005). The results so far indicate that kainate (KA) exerts up to 90% stimulation (Figure1), whereas CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), an antagonist at AMPA/kainate glutamate receptors, significantly suppresses the percentage of phagocytotic monocytes (Figure 2). No significant effects were observed on the mean fluorescence intensity per cell. While glutamate receptor reactive agents influence monocyte functions; monocytic cells, in turn, can affect glutamatergic synaptic transmission in the brain (Hegg and Thayer, 1999). This reveals a reciprocal network of interactions between the immune system and glutamate receptor-mediated signalling. In addition, macrophages in other tissues (e.g. alveolar macrophages, peritoneal macrophages, osteoclasts) have also been shown to express different types of glutamate receptors as well as transporters (Nurieva et al., 1998; Chenu, 2002; Dickman et al., 2004; Kalariti and Koutsilieris, 2004; Mason, 2004; Spencer et al., 2007). Furthermore, Rezzani et al. (2003) found that mGluR subtypes 2/3, 4 and 5 were differentially expressed in dendritic cells (DC) of rats, whereas Pacheco et al. (2006) did not observe mGluR1 and 5 expression on human monocyte-derived DC surface. These results indicate a different pattern of expression of glutamate receptors in DCs from different species. In addition, after the observation that the natural polyamine spermine triggers histamine secretion, and the fact that the NMDA (N-methyl-D-aspartate) receptor antagonist MK-801 (dizocilpine/(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) was able to inhibit this action, a functional polyamine site associated with the NMDA receptor complex was suggested on rat peritoneal mast cells (Purcell et al., 1996). However, others did not confirm these results and suggested rather the involvement of pertussis toxin-sensitive G proteins in the secretory effects of polyamines on rat peritoneal mast cells (Daeffler et al., 1999). Nevertheless, nonselective effects of NMDA receptor ligands on mast cells could not be excluded.

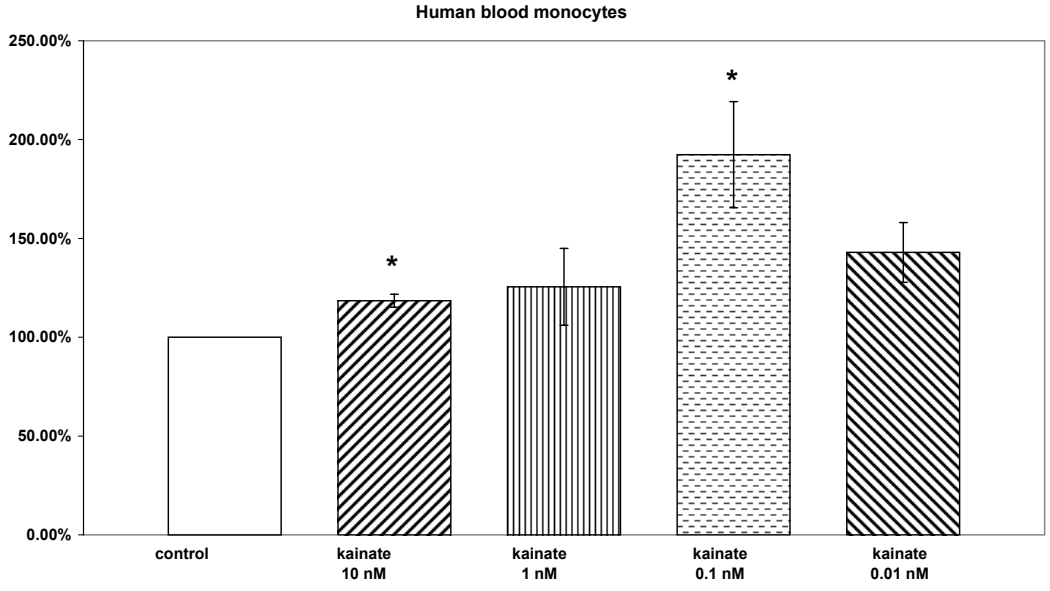

Figure 1. Modulatory effects of the ionotropic glutamate receptor agonist kainate on phagocytic activity of human blood monocytes. Kainate (0.1 nM and 10 nM) significantly increased phagocytic activity of human blood monocytes (* $p \leq 0.05$). Error bars represent s.e.m. Experiments with 10 nM kainate were performed in triplicate. Experiments with 0.1 nM kainate were performed in duplicate.

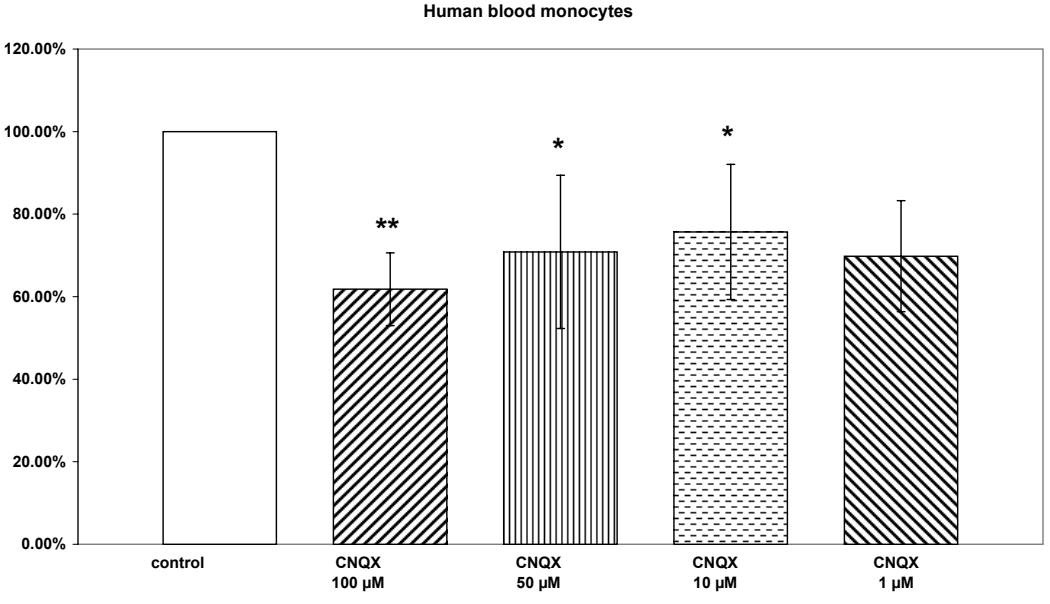

Figure 2. Modulatory effects of the ionotropic glutamate receptor antagonist CNQX on phagocytic activity of human blood monocytes. CNQX (10, 50 and 100 µM) significantly decreased phagocytic activity of human blood monocytes (* $p \leq 0.05$; ** $p \leq 0.01$). Error bars represent s.e.m. Experiments were performed in quintuplicate.

A specific interaction of L-glutamate with human T-lymphocytes as well as HL-60 human promyelocytic leukaemia cells was first described by Kostanyan and colleagues (1997,

1998a). More recently, it was found that human T-lymphocytes express different subtypes of NMDA receptors, whereby the molecular composition of the subtypes appeared to be dynamically regulated by cell activation (Miglio et al., 2005a). In resting peripheral blood lymphocytes and Jurkat T-cells the receptors are heterotetrameres composed of the NMDAR1 (NR1) and NR2B subunits, whereas in activated T-cells they consist of NR2A and NR2D subunits. Furthermore, this study shows that the receptors are functionally active in modulating T-cell activation (Miglio et al., 2005a). In addition, it should be underscored that there are species differences in glutamate receptor expression. In lymphocytes from rodents, for example, only two different subtypes of glutamate receptors (NR1 and mGluR group III) were functionally expressed (Boldyrev et al., 2004). The AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor subunit GluR3 was discovered in normal human T-cells, human T leukaemia cells (Jurkat), mouse anti-myelin basic protein (MBP) T-cells as well as T-cells from multiple sclerosis (MS) patients (Ganor et al., 2003; Sarchielli et al., 2007). The group of Ganor et al. (2007) also showed that interactions between the immune system and glutamate receptor mediated signalling (particularly GluR3) differed with T-cell activation states. The authors found that glutamate, via GluR3 activation, could activate only resting, but not T-cell receptor (TCR)-activated T cells. After TCR activation of T-lymphocytes there was a dramatic down-regulation of GluR3 from the cell surface, which was a result of an autocrine/paracrine proteolytic cleavage mediated by granzyme B (GB), a potent serine protease released by activated T-cells themselves and NK (natural killer) cells (Ganor et al., 2007). A consequence of this may be that the cleavage and release of GluR3 into the extracellular milieu may increase its antigenicity, and thus the production of anti-self GluR3 autoantibodies, which have been found to play a role in the pathophysiology of epilepsy (Ganor et al., 2004; 2005, 2007, Cohen-Kashi Malina et al., 2006). As to metabotropic glutamate receptors, it is accepted that individual members of mGluR group I and II are expressed and functionally active in both murine thymocytes as well as cells derived from the thymic stroma (Storto et al., 2000). Furthermore, this study provides evidence that peripheral mGluRs in thymocytes are developmentally regulated, suggesting a role for mGluR signalling in T-cell development. In addition, there are differences in the distribution pattern of mGluRs in thymic cells. The mGluR5 subtype is expressed most strongly in medullary cells, whereas mGluR2/3 and mGluR4 are moderately expressed in lymphocytes of the medulla and weakly in cortical lymphocytes (Rezzani et al., 2003). Similar to observations with ionotropic glutamate receptors, the expression of mGluRs also differs depending on the state of activation of the cells. In resting human blood lymphocytes mGluR5 is expressed constitutively, whereas mGluR1 is expressed only upon T-cell activation (Pacheco et al., 2004). With respect to the clinical significance of glutamate receptor expression in immune cells, recently, the group of Poulopoulou et al. (2005a) observed a significant reduction in the expression of mGluR2 mRNA in peripheral blood T-lymphocytes of patients with sporadic amyotrophic lateral sclerosis (ALS). These findings suggest that glutamatergic dysfunctions in ALS patients may not be restricted to the CNS and that T-lymphocytes may represent a novel peripheral marker for the early diagnosis of ALS (Poulopoulou et al., 2005a).

It is generally accepted that macrophages and dendritic cells express the cystine/glutamate antiporter (x^-_c system) (Watanabe and Bannai, 1987; Pacheco et al., 2006). This Na^+-independent glutamate transport system, originally described in the plasma

membrane of human fibroblasts (Bannai, 1986), transports extracellular cystine in exchange for intracellular glutamate. Less is known about the expression of the high-affinity Na^+/K^+-dependent glutamate uptake system ($X_{\bar{A}G}$ system = EAATs) on immune cells. Rimaniol and colleagues (2000), however, reported that macrophages derived from human blood monocytes express not only the cystine/glutamate antiporter but also EAATs with kinetic parameters similar to those determined for astrocytes and neurons. Freshly isolated tissue macrophages did not possess EAATs, whereas macrophages activated by adhesion to plastic, cultured splenic macrophages as well as blood monocytes stimulated with cytokines (tumor necrosis factor-α, TNF- α) did. Furthermore, this study shows that these transporters are functionally active and suggests that EAATs on peripheral macrophages may be involved in the regulation of glutathione (GSH) synthesis (Rimaniol et al., 2000; see also below). In the thymus, GLAST transporter mRNA as well as protein expression was found in cells scattered throughout cortex and medulla. Based on the dendritic shape, these GLAST-positive cells were likely antigen-presenting cells (Berger and Hediger, 2006). In the spleen, GLAST mRNA labeling and protein immunostaining was widespread in red pulp and more restricted in white pulp. GLT-1 immunostaining was primarily restricted to cells in the germinal center. Similarly, in salivary gland lymph nodes, widespread GLAST expression was found in cortex and medulla, whereas relatively restricted expression of GLT-1 was detected in germinal centers (Berger and Hediger, 2006).

These data indicate that glutamate receptors as well as transporters are ubiquitously distributed in the immune system. This provides the basis for a dialogue between the major excitatory neurotransmitter glutamate and immune cells, and likely contributes to an expeditious multidirectional flow of information between the immune and the nervous systems.

The Role of Glutamate Signalling in Immune Functions and Pain

Most attention has been focused on the role of glutamate as key immunomodulator in T-cell mediated immunity (Pacheco et al., 2007). However, it should be mentioned that other immune cells are also involved in glutamate-receptor mediated signalling pathways. For example, stimulated polymorphonuclear leukocytes (PMN) release glutamate, which in turn regulates human vascular endothelial barrier function via activation of endothelial mGluRs (Collard et al., 2002). In neutrophil respiratory burst activation, known to be Ca^{2+}-dependent, glutamate and NMDA exert a potentiating effect (Kim-Park et al., 1997). Our own studies indicate changes in the phagocytic activity of human monocytes after treatment with iGluR agonists and antagonists (Haas et al., 2003, 2005, see also above). Furthermore, keeping in mind that an immune response can be conditioned (Ader, 2003), it is important to note that glutamate has been found to be involved in recall of a conditioned NK cell response (Kuo et al., 2001). This effect was believed to be mediated through NMDA receptors, since the conditioned response was interrupted by the NMDA receptor antagonist dizocilpine maleate (Kuo et al., 2001). Last but not least, it has been shown that the “immunological synapse” uses glutamate as immunotransmitter (Pacheco et al., 2006). Similar to neurological synapses, dendritic cells (DC) (the antigen presenting cells) release glutamate during T-cell – DC

contact formation. This DC-derived glutamate acts early during T-cell – DC interaction via activation of mGluR5 on T-cells, thereby impairing interleukin-6 (IL-6) production and, consequently, T-cell activation. However, after productive antigen presentation, mGluR1 became expressed on T-cells. In this way, DC-derived glutamate works via mGluR1 to enhance T-cell activation as well as proinflammatory (IL-2, IL-6, IL-10, TNF-α, interferon (IFN)-γ) cytokine secretion (Pacheco et al., 2006). This study demonstrated that peripheral glutamate could influence differential or even opposing immune functions by activating different receptor subtypes.

The glutamine/glutamate metabolism per se has long been described in resting as well as activated lymphocytes and macrophages (Brand et al., 1987; Ardawi, 1988; Klegeris et al., 1997; Gras et al., 2006; Porcheray et al., 2006). Abnormally high venous plasma glutamate levels have been reported in several diseases, such as cancer, human immunodeficiency virus infection, stroke, amyotrophic lateral sclerosis (ALS) and other neurodegenerative disorders (Dröge et al., 1988; Eck et al., 1989a, 1989b; Plaitakis and Constantakakis, 1993; Hack et al., 1996; Tremolizzo et al., 2004; Aliprandi et al., 2005). These data led to the conclusion that elevated glutamate concentrations may be an etiologic factor for impaired immune functions (Dröge et al., 1988; Eck et al., 1989a, b), and may result, at least in part, from a decreased glutamate transport activity (Hack et al., 1996). However, elevated glutamate levels can also be found more locally, e.g. in the synovial fluids of patients with active arthropathies (McNearney et al., 2004). In these cases, it was suggested that glutamate contributes rather to peripheral inflammatory processes. Moreover, normal elderly subjects have been reported to have high venous glutamate levels as well (Hack et al., 1996), and high amounts of glutamate, but without signs of chemical inflammation, have been found in the tendons of patients with tennis elbow as well as in Jumper's knee/tendinosis (Alfredson et al., 2000, 2001). In addition, immunohistochemical analyses demonstrated the occurrence of NMDAR1 in tendon tissue, and acetylcholinesterase histochemistry showed that these receptors were localized in association with nerve structures in human patellar tendons (Alfredson et al., 2001). Keeping in mind the diversity of glutamate receptors/transporters as well as their signal transduction pathways, it is reasonable to assume that glutamate can exert diverse effects on immune functions. Vice versa, the immune system will also affect glutamate-receptor signalling pathways in different ways, e.g. depending on the stage of development, state of activation or local immune cell interactions. Increasing numbers of researchers lately have been trying to unravel some aspects of these complex interactions.

In 2001, Lombardi et al. investigated whether glutamate might modulate human lymphocyte functions by a receptor-mediated mechanism. In these studies, glutamate did not modify either the intracellular calcium concentration $[Ca^{2+}]_i$ or the cell proliferation in resting lymphocytes. However, when cells were activated, both responses were significantly modified. Glutamate significantly potentiated the effects of anti-CD3 mAb or phytohaemagglutinin (PHA)-induced $[Ca^{2+}]_i$ rises, and significantly inhibited the mitogen-stimulated lymphocyte proliferation. Furthermore, these effects appeared to be mediated by NMDA as well as AMPA/KA receptors, whereby AMPA/KA receptor compounds showed a greater efficacy (Lombardi et al., 2001). A continuing study showed that the inhibitory effect of glutamate on lymphocyte proliferation was not mediated by activation of iGluRs, but rather linked to depletion of intracellular GSH stores (Lombardi et al., 2004). Moreover, in another study NMDA receptor antagonists inhibited PHA-induced T-cell proliferation, but they did

not affect IL-2-induced proliferation of PHA blasts (Miglio et al., 2005a). In addition, exposure of leukaemic Jurkat T-cells to NMDA receptor antagonists inhibited cell growth, whereas glutamate as well as NMDA increased Jurkat T-cell adhesion to fibronectin (Miglio et al., 2007). A possible explanation of these confusing results may be that the expression of iGluRs on lymphocytes has been shown to be dynamically regulated by cell activation (Miglio et al., 2005a). Consequently, as described by Pacheco et al. (2006, see also above), different subtypes of receptors are expressed on immune cells in different states of activation, and, accordingly, mediate different responses. Low and high concentrations of glutamate may also affect lymphocyte functions in different ways. In this context, Sarchielli et al. (2007) observed that glutamate as well as AMPA in the range of 10 nM to 10 μM enhanced the proliferation of myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) specific lymphocytes, whereas excess concentrations of glutamate (values from 20 μM to 1 mM) exerted an opposing and inhibitory effect on lymphocyte proliferation in healthy subjects as well as MS patients. In addition, ion channels other than glutamate channels may be involved in the modulation of glutamate mediated neuroimmune interactions as shown by Poulopoulou and colleagues (2005b). Their data indicate that glutamate has a dual effect on voltage-gated potassium channels in human T-lymphocytes. Glutamate, at concentrations within normal plasma levels, facilitates channel activation, an effect that was mimicked by the mGluR agonist ACPD (trans-(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid). On the other hand, glutamate, at concentrations known to induce suppression of mitogen-stimulated lymphocyte proliferation, significantly decreases potassium currents. The authors accordingly suggest that the antiproliferative effect of high glutamate levels may, at least in part, result from its inhibitory effect on potassium currents (Poulopoulou et al., 2005b). However, glutamate receptor signalling is also involved in other cellular events. NMDA and to a lesser extent L-AP4 (L-2-amino-4-phosphonobutyric acid), an mGluR group III agonist, increased reactive oxygen species (ROS) levels in rat and human lymphocytes (Tuneva et al., 2003; Boldyrev et al., 2004; Mashkina et al., 2007), whereby combined administration of homocysteic acid (HCA) and NMDA produced still higher levels of intracellular ROS in murine lymphocytes than in the presence of NMDA alone (Boldyrev et al., 2005). Interestingly, ROS levels also increased in NK cells following incubation with NMDA, whereas B-cells were not affected (Mashkina et al., 2007). Ganor et al. (2003) found that glutamate triggered the integrin-mediated adhesion to laminin and fibronectin as well as the chemotactic migration of T-cells towards the chemokine CXCL12/stromal cell-derived-factor-1α. More recently, it was shown that glutamate as well as AMPA enhanced the chemotactic migration of T-cells towards the chemokines CXCL12/stromal cell-derived-factor-1, RANTES (Regulated on Activation Normal T-cell Expressed and Secreted) and MIP-1α (macrophage inflammatory protein) in normal subjects as well as MS patients, whereby higher values were found in MS patients during relapse and patients with neuroradiological evidence of disease activity (Sarchielli et al., 2007). In 1998, it was determined that glutamate could also modulate the interaction of cytokines with their receptors (Kostanyan et al., 1998b). It was shown that glutamate converted the binding of [^{125}I]rHuIL-lβ and [^{125}I]rHuIL-6 to HL-60 leukaemic cells from high affinity into low affinity binding. In contrast, preincubation of HL-60 cells with 0.1 μM glutamate resulted in an increase in affinity and number of [^{125}I]rHuTNF-α binding sites. Thus, glutamate decreased the sensitivity of HL-60 cells to IL-1β and IL-6, but increased TNF-α binding (Kostanyan et al., 1998b). In addition, glutamate enhanced the TNF-α-induced differentiation of HL-60 cells

(Astapova et al., 1999), and modulated the cytotoxic effect of TNF-α in this cell line (Gibanova et al., 2005). Glutamate can also modulate cytokine secretion. At a concentration of 1 mM, glutamate increases the secretion of IFN-γ and IL-10, whereas 5 mM glutamate significantly inhibited the secretion of IFN-γ, IL-10 as well as IL-5, and had no effect on TNF-α, IL-4, and IL-2 (Lombardi et al., 2004).

As to metabotropic glutamate receptors, group I mGluRs differentially modulate T-cell activation depending on the specific activation of either mGluR1 or 5 (Pacheco et al., 2006; see also above). However, data about the signalling pathways involved in these effects are partly conflicting. In contrast to the results of Storto et al. (2000) obtained from murine thymocytes, Pacheco et al. (2004) reported that neither mGluR1 nor mGluR5 in human peripheral blood lymphocytes was coupled to the phosphoinositide-signalling machinery operating in the CNS. Instead, mGluR1s were coupled to the mitogen-activated protein (MAP) kinase cascade, and mGluR5 increased cAMP levels. These different signalling pathways along with the differential expression pattern in resting versus activated T-cells may explain why glutamate exerts an inhibitory effect via constitutively expressed mGluR5, whereas it has a stimulatory role when mGluR1 becomes expressed upon T-cell activation (Pacheco et al., 2004; 2006). In addition, there is some disagreement about changes in intracellular calcium levels following group I mGluRs stimulation in human T-cells. Pacheco et al. (2004) reported that agonist-induced group I mGluR stimulation did not evoke Ca^{2+} responses in human T-cells, whereas Miglio et al. (2005b) observed increases in intracellular calcium levels and subsequent immediate early gene (*c-fos* and *c-jun*) expression following group I mGluR activation. However, aside from methodological differences, changes in the activation state of the immune cells may again explain such contrary results. Last but not least, it should be mentioned that group I mGluR stimulation inhibited also activation-induced cell death of human T-lymphocytes (Chiocchetti et al., 2006), which is in line with data obtained in the brain, where mGluRs have been shown to reduce neuronal apoptosis (Copani et al., 1995; Nicoletti et al., 1999; Allen et al., 2000; Vincent and Maiese, 2000; Rong et al., 2003).

In the last years an accumulating body of evidence has emerged in support of the notion that glutamate receptors play a crucial role in pain pathways, including those involved in neuropathic pain, inflammatory, or joint-related pain (Carlton, 2001a; Bleakman et al., 2006). In the following, interactions of glutamate-receptor mediated signalling mechanisms and pain-related states shall be critically discussed. Recent observations suggest that glutamate has an important impact in sensory transduction in the periphery. Different types of iGluRs (NMDA, AMPA, as well as kainate (KA) receptors) were found in unmyelinated axons of rat glabrous skin, and exogenously applied glutamate resulted in nociceptive behaviours (Carlton et al., 1995; Coggeshall and Carlton, 1998). The same iGluRs were also detected in unmyelinated cutaneous axons at the dermal-epidermal junction in human hairy skin (Kinkelin et al., 2000). Furthermore, iGluRs were found expressed on large-diameter myelinated axons in the sural and medial plantar nerves (Coggeshall and Carlton, 1998). Following injection of complete Freund's adjuvant (CFA) into the hindpaw of rats, a significant increase in the number of peripheral primary afferent axons expressing NMDA, AMPA, and KA receptors was observed, suggesting that this may be a contributing factor to peripheral sensitization during inflammation (Carlton and Coggeshall, 1999). Furthermore, also sympathetic efferents are involved in the signalling pathways of pain. iGluRs were found to be expressed by peripheral postganglionic sympathetic fibers (Carlton et al., 1998), and

CFA-induced inflammation again resulted in an increase in proportions of postganglionic sympathetic axons expressing these receptor subtypes (Coggeshall and Carlton, 1999). Since glutamate is released in the dorsal horns of the spinal cord in response to peripheral nociceptive stimulation (Vetter et al., 2001), it is not surprising that both ionotropic as well as metabotropic glutamate receptors are present in dorsal root ganglion cells, where they are critically involved in spinal sensory transmission (Boyce et al., 1999; Gerber et al., 2000a; 2000b; Huettner et al., 2002; Karlsson et al., 2002; Ruscheweyh and Sandkühler, 2002; Szekely et al., 2002; Rustioni, 2005; Lucifora et al., 2006; Maile et al., 2007). In view of the fact that glutamate receptors are hetero-oligomeric complexes of several receptor subunits, it is reasonable to assume that the receptor subunits are differentially affected by peripheral inflammation or painful hypersensitivity states. In this context, Karlsson et al. (2002) observed that fewer dorsal horn neurons express the NMDAR subunit 2A (NR2A) in peripheral nerve-injured rats as compared to control rats. Similarly, Iwata et al. (2007) reported that in the superficial dorsal horn the ratio of the number of neurons expressing NR2A subunit decreased, whereas the ratio of the number of neurons expressing NR2B subunit increased after nerve ligation. This is in line with the observation that selective NR2B antagonists possess antinociceptive activity, which makes them to promising novel targets for the treatment of chronic pain states (Boyce et al., 1999; Kovács et al., 2004; Brown and Krupp, 2006; Gogas, 2006). However, studies on nociceptive behaviours reveal that besides NMDA receptors also AMPA and KA receptors play a substantial role in mediating the development of hyperalgesia and allodynia (Sluka et al., 1994; Zhou et al., 1996; Sang et al., 1998; Sutton et al., 1999; Leem et al., 2001; Ahn et al., 2004; Blackburn-Munro et al., 2004; Jones et al., 2006). Furthermore, a subtype-specific regulation of synaptic transmission once more became apparent, since the AMPA receptor subunits GluR1 and GluR2 were found to modulate spinal synaptic plasticity and inflammatory pain in a reciprocal manner (Hartmann et al., 2004).

Apart from iGluRs, also multiple mGluR subtypes have been implicated in nociceptive transmission. mGluR1 and 5 were found expressed on unmyelinated as well as myelinated peripheral afferent fibers (Bhave et al., 2001; Zhou et al., 2001). Carlton's et al. study (2001b) suggested group II mGluRs on a proportion of axons in cutaneous digital nerves of rats. As mentioned above, the expression of mGluRs was also described at the level of the spinal cord. Group I mGluRs were defined in human and rat dorsal horn, whereby changes in the expression of the mGluR5 subtype were observed during development (Valerio et al., 1997a; 1997b; Jia et al., 1999; Alvarez et al., 2000). Similar to observations in immune tissues (see above), Western immunoblotting showed that the mGluR5 protein was present at higher levels in the spinal cord of neonatal rats than in adult rats (Valerio et al., 1997a). mGluR2/3 were found in rat dorsal root ganglion cells (Valerio et al., 1997b; Carlton et al., 2001b; Carlton and Hargett, 2007), as well as at extrasynaptic loci in rat spinal dorsal horn synapses (Azkue et al., 2000). Group III mGluRs appear to be highly and very specifically expressed in dorsal root ganglion cells (Valerio et al., 1997b; Azkue et al., 2001; Carlton and Hargett, 2007). mGluR8 was expressed by small, medium, as well as large diameter cells (Carlton and Hargett, 2007), whereas immunostaining for mGluR4a was detected only in small to medium-sized cells (Azkue et al., 2001). Furthermore, a considerable percentage of dorsal root ganglion cells expresses both group II and III mGluRs (Carlton and Hargett, 2007). As to the pharmacological modulation, there is increasing evidence that the different groups of mGluRs affect pain states in different ways. Group I mGluR antagonists may provide a useful

therapeutic strategy for the treatment of various pain conditions. Peripheral application of the selective, non-competitive, mGluR5 antagonist MPEP (2-methyl-6-(phenylethynyl)-pyridine) and the selective, non-competitive, mGluR1 antagonist CPCCOEt (7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester) to mice blocked glutamate-induced thermal hypersensitivity and attenuated nociceptive scores in the formalin model of inflammatory pain (Bhave et al., 2001). Similarly, peripheral injection of another mGluR1 antagonist (AIDA = (RS)-1-aminoindan-1, 5-dicarboxylic acid) into rats blocked the glutamate-induced increase in mechanical sensitivity and attenuated phase 2 formalin nociceptive behaviours (Zhou et al., 2001). Spinal administration of mGluR1 antagonists before injection of kaolin and carrageenan into the knee joint cavity of rats significantly blocked the reduction of paw withdrawal latency, whereas post-treatment reversed thermal hyperalgesia (Zhang et al., 2002). However, knee joint inflammation itself was not affected by either treatment. More recently, it was shown that, compared to AIDA, intra-articular MPEP was most effective on non-evoked pain as well as mechanical hyperalgesia in both the induction and maintenance of knee joint inflammation (Lee et al., 2007). Furthermore, it should be pointed out that selective antagonism of mGluR5 differentially modifies acute, persistent, and/or chronic pain states (e.g. associated with persistent inflammation or nerve injury) (Walker et al., 2001; Varney and Gereau 4th, 2002; Zhu et al., 2004; Su and Urban, 2005). Systemic administration of MPEP, for example, produced only reversal of inflammatory hyperalgesia, i.e. acted solely against the pain that accompanies inflammatory pathology, whereas it did not affect acute nociception or hyperalgesia associated with nerve injury (neuropathic pain) (Walker et al., 2001). However, even though all these data indicate that mGluR5 antagonists have strong analgesic efficacy, they do not affect other aspects of inflammation (e.g. paw oedema) (Walker et al., 2001; Zhu et al., 2004). As to the signalling pathways, it was shown that ERK/mitogen-activated protein kinases were activated in spinal cord dorsal horn neurons after noxious stimulation (Ji, 2004), and that selective stimulation of mGluR1 and/or mGluR5 was necessary for ERK activation after peripheral inflammation (Karim et al., 2001). While a number of studies have focused on group I mGluRs in pain conditions, less is known about the specific role of group II and III mGluRs in pain processing. Several data indicate that group II agonists may have analgesic properties in pain states (Neugebauer et al., 2000; Sharpe et al., 2002; Yang and Gereau 4th, 2002; 2003; Jung et al., 2006). Similar to observations with group I antagonists, also group II agonists affect acute, persistent and chronic pain states in different ways. mGluR2/3 agonists showed no effect on acute thermal nociception but reduced paw-licking behaviour in the formalin model of persistent pain and reduced mechanical allodynia in the neuropathic pain model (Simmons et al., 2002). Jones et al. (2005), however, observed that upon repeated dosing, tolerance may develop to the analgesic effects of mGluR2/3 agonists. Thus, further research is needed to better understand the mode of action of group II mGluR agonists in pain processing. The role of group III mGluRs in nociception appears to be even more variable. Activation of group III mGluRs inhibited the responses of primate spinothalamic tract cells to brief innocuous as well as noxious cutaneous mechanical stimuli (Neugebauer et al., 2000). Contrary to this finding, Chen and Pan (2005) observed that the group III mGluR agonist L-AP4 (L(+)-2-amino-4-phosphonobutyric acid) had no significant effect on dorsal horn neurons in normal rats, but suppressed allodynia and inhibited hypersensitivity of dorsal horn projection neurons associated with neuropathic pain. On the other hand, stimulation of mGluR8 relieved formalin and carrageenan-induced hyperalgesia in inflammatory pain, whereas it was ineffective in

established inflammatory or neuropathic pain states (Marabese et al., 2007a). Moreover, at the level of the periaqueductal gray, mGluR7 and 8 mediated opposite effects on rostral ventromedial medulla cell activities as well as thermal nociception, i.e. they could either relieve or worsen pain perception (Marabese et al., 2007b).

Last but not least, the role of high affinity glutamate transporters in peripheral neuroimmunomodulation and sensory transmission shall be summarized. As mentioned above, macrophages derived from human monocytes express both the cystine/glutamate antiporter (x^-_c system) as well as the high affinity glutamate transporters (EAATs) (Rimaniol et al., 2000). Furthermore, this study provided evidence that macrophages may be as efficient as neural cells in clearing extracellular glutamate. In addition, EAATs on macrophages along with the cystine/glutamate antiporter were shown to be critically involved in the regulation of glutathione (GSH) synthesis (Rimaniol et al., 2000; 2001). In particular, EAATs on macrophages affect GSH synthesis in two ways. First, EAAT expression on macrophages results in glutamate-dependent enhancement of glutathione synthesis by providing intracellular glutamate for direct insertion in glutathione. Second, extracellular cystine is transported into macrophages in exchange for glutamate via the cystine/glutamate antiporter. Then, extracellular glutamate is taken up by EAATs, thereby fuelling the intracellular pool of glutamate. This permits an increase in x^-_c velocity, which leads to enhanced cystine uptake, albeit under competition conditions (called *trans*-stimulation), and, consequently increases glutathione synthesis. Moreover, macrophages have recently been shown to express both EAATs as well as glutamine synthetase and it was suggested that this may play a role in mediating neuroprotection against glutamate in the HIV-infected brain (Vallat-Decouvelaere et al., 2003; Porcheray et al., 2006). Several data indicate that glutamate transporters are also involved in normal sensory transmission as well as pathological pain states (reviewed by Tao et al., 2005). Both expression levels as well as uptake activity of spinal glutamate transporters were altered following peripheral nociceptive stimulation (Sung et al., 2003), whereby in most cases the expression of spinal glial transporters (GLAST, GLT-1) was downregulated after a transient initial upregulation (Niederberger et al., 2003; Sung et al., 2003; Weng et al., 2005; Niederberger et al., 2006). Also the spinal neuronal transporter EAAC1 was downregulated following nerve injury (Wang et al., 2006). Interestingly, this downregulation appears to be regulated by central glucocorticoid receptors in rats, which may be mediated through the transcription factor NF-κB (Wang et al., 2006). How glutamate uptake inhibitors affect sensory transmission and pain states is not entirely clear. Whereas inhibition of glutamate uptake produced significant pronociceptive effects under physiological conditions (Liaw et al., 2005), and intrathecal application of a non-selective glutamate transport inhibitor, L-trans-pyrrolidine-2,4-dicarboxylic acid (10 µl of 100 µM solution) induced hypersensitivity to peripheral mechanical and thermal stimuli (Weng et al., 2006), others did not confirm these observations. They rather found that inhibition of glutamate uptake activity produced antinociceptive effects (Niederberger et al., 2003; 2006), even though the underlying mechanisms of these responses are not known. Several distinct models for the action of glutamate transporter inhibitors in pathological pain states have been established. Among others, these include the occurrence of dorsal horn neuronal death, increase of spinal GABA contents, activation of inhibitory presynaptic mGluRs, desensitization of postsynaptic glutamate receptors, or blockade of inverse operation of glutamate transporters (Tao et al., 2005). In summary, even though these data indicate that glutamate transporters may be new

targets for the treatment of pain states, further studies are needed to explore the exact role of spinal glutamate transporters in sensory transmission, inflammation and pain.

Conclusion

Glutamate is the most abundant dicarboxylic amino acid in the brain and was long believed to be primarily an excitatory neurotransmitter in the central nervous system. In recent years it has become apparent that glutamate and its receptors are also ubiquitously distributed in peripheral, non-neuronal tissues, and several lines of evidence indicate that glutamate plays an important role in the regulation of a number of other biological responses as well. Recent advances in molecular biological techniques as well as immunocytochemical and *in situ* hybridization methodologies have lead to a detailed knowledge of the distribution of GluRs in peripheral non-neuronal tissues, including the immune system. Both glutamate receptors and high affinity transporters have been detected in immune tissues and were shown to be dynamically regulated depending on the stage of development and state of activation of the immune system or local immune cell interactions. High and low glutamate levels have been shown to affect immune responses in different ways, and peripheral glutamate receptors were found to play a crucial role in nociceptive transmission. Novel glutamate receptor reactive compounds have been shown to affect immune cells in individual ways. In particular, the emerging field of peripherally acting, subtype-specific, positive and negative allosteric modulators of mGluRs offers an exciting potential for novel therapeutics in immunological disorders as well as inflammatory or pain states (Neugebauer and Carlton, 2002; Chiechio et al., 2004; Kew, 2004; Ritzén et al., 2005; Schkeryantz et al., 2007). Taken together, these data underscore complex autocrine and paracrine signalling roles for glutamate in the regulation of immune functions as well as pain conditions. Since the experience of pain affects our mood and our mood affects immune functions, the results summarized here should not only improve our understanding of neuroimmune interactions, but also expand our perspective of mind-body medicine (Figure 3).

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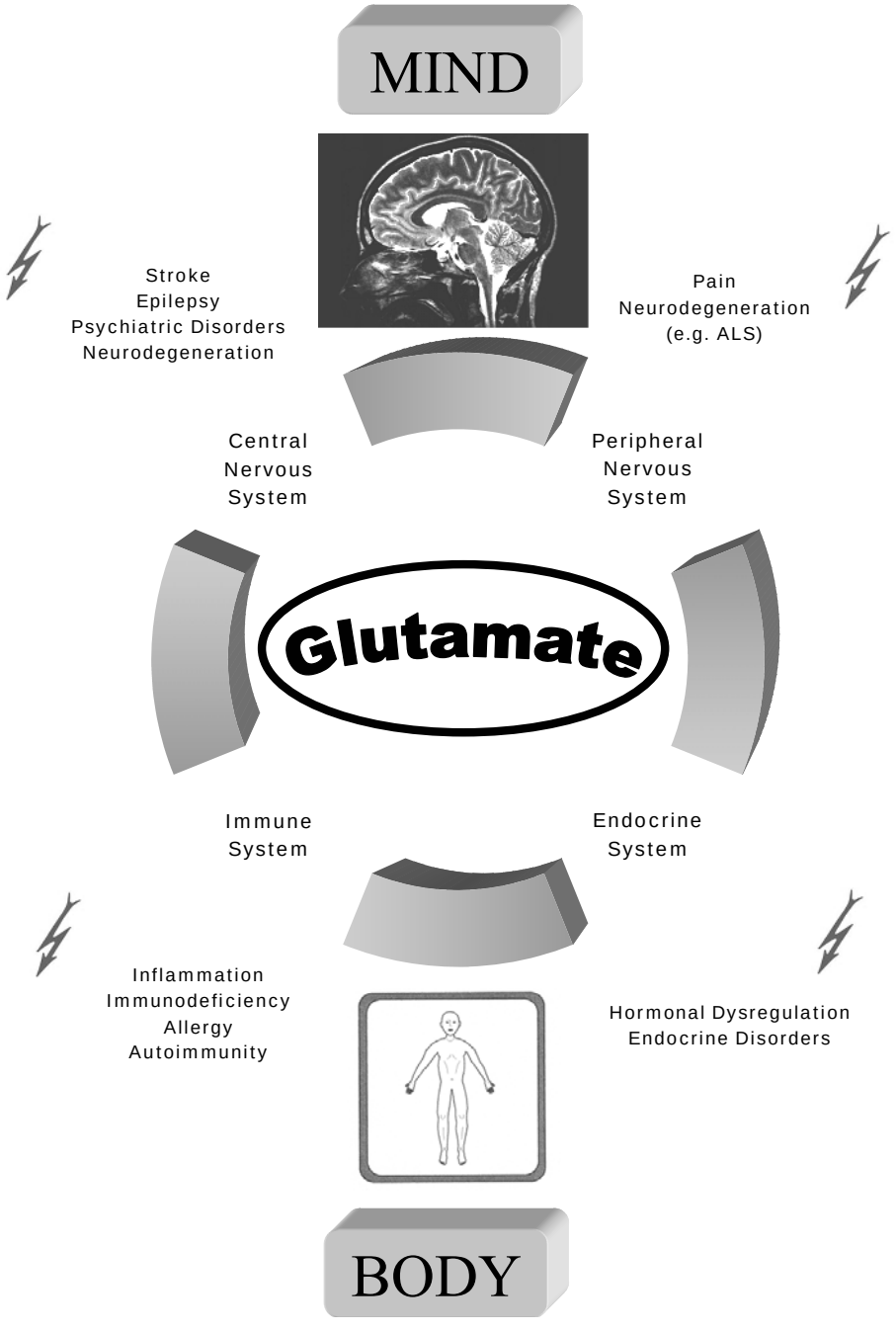

Figure 3. The role of glutamate in mind-body medicine.

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Chapter 3

Insights from Structure-Function Studies of AMPA Receptor Agonists and Competitive Antagonists

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Abstract

The first high resolution structure of an ionotropic **glutamate** receptor ligand-binding core was published a decade ago (Armstrong et al., 1998). This discovery was a breakthrough within the **glutamate** receptor field and has enabled a cascade of structure-function studies on the mechanism of action of this receptor family. Insights from these studies have contributed significantly to the understanding of the series of events coupling ligand-binding to channel motions for the **glutamate** receptors, but may also add valuable input to researchers working with other ligand activated receptors.

This chapter focuses on a line of studies where X-ray analysis has been combined with electrophysiological studies of a range of ligand-receptor combinations. Here the agonist 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (**AMPA**) has been employed as an important pharmacological tool due to the large number of substituents, which have been introduced on the isoxazole ring. This has revealed important information on ligand selectivity and kinetic properties of the receptor complex. Given that more than fifty structures of the **AMPA** receptor ligand-binding domain have been determined, our centre of attention is the **AMPA** receptor family, however where appropriate, we will correlate the results to the closely related **kainate** receptors.

Earlier studies explored the central ligand-binding pocket in order to gain agonist selectivity. In this chapter we describe how recent efforts resulted in ligands that protruded out from the **AMPA** binding site and reached into a novel sub-domain to obtain highly subunit selective agonists.

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Important information has also been obtained on the desensitization mechanism. It was recently revealed that desensitization results from a disruption of the interactions between the upper parts of the ligand-binding core. We speculate that a novel interdomain twist observed in some crystal structures might facilitate these movements. In addition, we will discuss the high-resolution structures of **AMPA**-derived competitive antagonists bound to the ligand-binding site of the **AMPA** receptor GluR2.

In this chapter we first present a basic introduction to the **AMPA** receptors followed by a brief description of the agonist and antagonist induced changes in the receptor complex. Subsequently, we discuss selected structure-function studies grouped after position of the substituent in the **AMPA** isoxazole ring.

Introduction

Basic Structure and Function of the AMPA Receptors

AMPA receptors are essential players in mediating the fast excitatory neurotransmission within the mammalian central nervous system. They belong to the ionotropic **glutamate** receptors which are divided into three classes; **NMDA**, **AMPA** and **kainate** receptors, where the latter two classes exhibit the highest degree of pharmacological and physiological similarity. The **AMPA** receptor class consists of four members; GluR1, GluR2, GluR3 and GluR4 (also referred to as GluRA-D), which assemble into tetramers (Rosenmund et al., 1998; Chen et al., 1999; Greger et al., 2007) believed to be organized as a dimer of dimers (Armstrong and Gouaux, 2000; Tichelaar et al., 2004). Functional **AMPA** receptors can be composed of both homomeric and heteromeric assemblies of the four subunits (Hollmann, 1999).

An **AMPA** receptor subunit can be viewed as composed of three modules, each exhibiting sequence and structural homology to conserved domains found even in bacteria (Mayer, 2006). Two modules show homology to bacterial periplasmatic amino-acid binding proteins (Fig. 1) with the most N-terminal segment not required for assembly of functional homomeric **AMPA** receptors (Pasternack et al., 2002), but being important for restricted assembly of the **AMPA** receptors from members within the family and not with e.g. the **kainate** receptors (Ayalon and Stern-Bach, 2001; Ayalon et al., 2005). The second module forms the ligand-binding domain and is interrupted by the pore-forming helices. The pore-forming segments, containing two transmembrane helices and a reentrant loop, show sequence similarities with the bacterial potassium channels (Panchenko et al., 2001; Kuner et al., 2003). A third transmembrane helix follows the ligand-binding domain enabling several proteins involved in receptor trafficking to bind the intracellular C-terminal tail.

As might be expected based on the modular assembly of the **AMPA** receptor monomer, recent findings show that the three linkers between the ligand-binding domain and the pore region (Fig. 1) influence both the agonist potency and the desensitization kinetics (Schmid et al., 2007) as well as forming the binding site for the non-competitive antagonists **GYKI53655** and **CP-465,022** (Balannik et al., 2005). These findings underline an essential region in the targeting of novel allosteric modulators towards the **AMPA** receptors.

We still await high resolution full length structures of the **glutamate** receptors. These may reveal e.g. the importance of the increased flexibility observed for the bi-lobed binding domain of the **kainate** receptors as compared to the **AMPA** receptors. To date, only low

resolution structures of full length receptors have been reported (Safferling et al., 2001; Tichelaar et al., 2004).

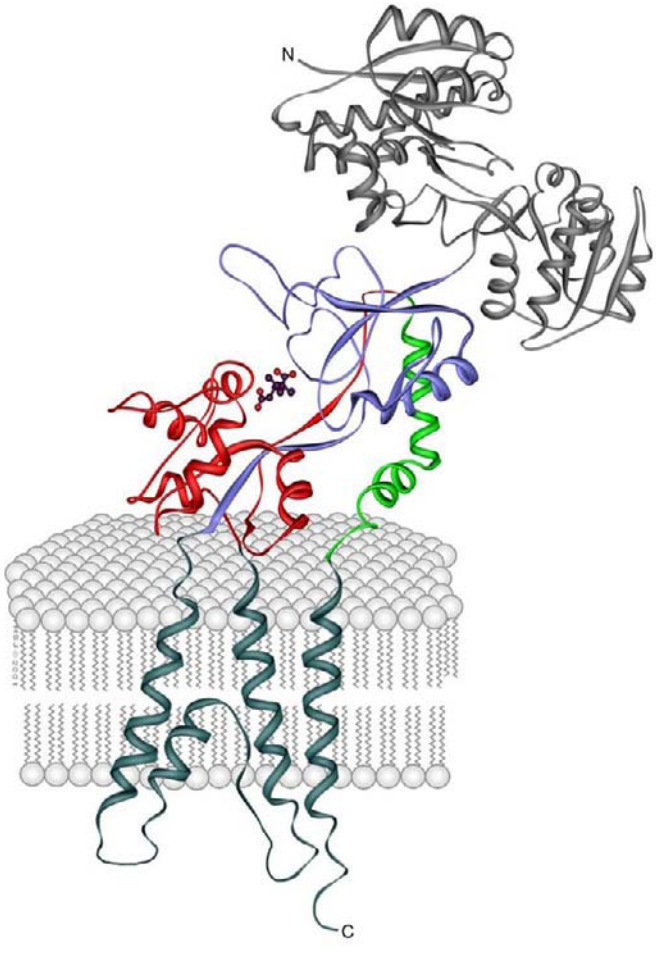

Figure 1. Illustration of a single **AMPA** receptor subunit. The agonist **kainate** binds in the cleft between the two lobes (D1 and D2) of the ligand-binding domain and is displayed in ball and stick. The N-terminal LIVBP-like domain is gray, segment S1 is blue, S2 is red and the alternatively spliced flip/flop region is colored green. This model was generated using the published structures of LIVBP (2LIV), the ligand-binding domain from GluR2 complexed with kainate (1GR2) and the membrane-domain from KscA (1BL8), PDB ID codes in parenthesis. The figure was kindly designed and provided by Henrik Sindal Jensen, H. Lundbeck A/S.

Post-Transcriptional Modifications Add Further Diversity

Post-transcriptional modifications fine tune the kinetic properties and channel permeability. Alternative splicing occurs within the binding domain of all four **AMPA** receptor types resulting in two distinct isoforms termed flip and flop (Fig. 1, shown in green) (Sommer et al., 1990; Monyer et al., 1991). Generally, flip variants predominate in early

developmental stages, whereas the two isoforms are expressed in about equal amounts in adult animals.

The flip/flop region leads to two kinetically distinct isoforms of the **AMPA** receptor; the flip isoform displaying slower desensitization kinetics (decay-time higher than 3.5 ms), whereas the flop variant displaying faster kinetics (decay constant less than 3 ms) upon **glutamate** application (Fig. 9) (Mosbacher et al., 1994; Lomeli et al., 1994; Koike et al., 2000; Partin, 2001; Pei et al., 2007). The flip/flop ratio may have important functional consequences, since it provides a mechanism to regulate the kinetic response. Implications of the flip/flop ratio has been observed under pathological conditions e.g. the flip isoform has been shown to be increased in spinal motor neurons during amyotrophic lateral sclerosis as compared to control group and this is believed to contribute to the selective vulnerability of motor neurons during this disease (Tomiyama et al., 2002 and see also review by Kawahara and Kwak, 2005). Additionally, several different C-terminal splice forms affect the targeting and trafficking of the receptors within the neurons (Gallo et al., 1992; Sommer et al., 1992; Gregor et al., 1993; Kohler et al., 1994; Schiffer et al., 1997).

RNA editing results in an arginine to glycine change just upstream of the flip/flop region altering the recovery kinetics (Lomeli et al., 1994). Furthermore, a glutamine is exchanged with an arginine in the pore region, which strongly influences the calcium permeability (Hume et al., 1991; Sommer et al., 1991).

Transmembrane **AMPA** receptor regulatory proteins (TARPs) control the trafficking of **AMPA** receptors (Tomita et al., 2003; Nicoll et al., 2006). However, recent findings demonstrate that these proteins additionally regulate the kinetic properties of the **AMPA** receptors and that the flip/flop region is involved in this mechanism. Currents activated by **kainate** and **glutamate** on the faster desensitizing flop isoforms were potentiated more than those of the corresponding flip variant (Kott et al., 2007).

Coupling of Agonist Binding to Channel Motions

The breakthrough in our molecular understanding of the ligand-receptor interaction is based on the ability to engineer a soluble form of the ligand-binding domain of GluR2 and the subsequent crystallization (Kuusinen et al., 1995; Chen et al., 1998; Armstrong et al., 1998). Since then more than fifty crystal structures of representatives of all the ionotropic **glutamate** receptors in complexes with agonists and antagonists have been reported.

The ligand-binding domain crystallizes as dimers, with an interface between the D1 domains (Fig. 2). Binding of a ligand to the **glutamate** binding site induces a closure of the D1-D2 domains, where the degree of closure is ligand dependent. Full agonists (such as **glutamate** and **AMPA**) induce a ~21° closure, while partial agonists (such as **kainate**) stabilize lower degrees of closure (~13°). Antagonists (like (*R,S*)-2-amino-3-(5-tert-butyl-3-phosphonomethoxy-4-isoxazolyl)propionic acid (**ATPO**) and 6,7-dinitroquinoxaline-2,3-dione (**DNQX**)) stabilize even lower or a more open conformation.

The agonist induced stabilization of the closed conformation can roughly be ascribed to three contributions; *first* an interaction present for all agonists between the α-carboxy and α-amino groups of the agonist and residues in both D1 (Pro478, Thr480 and Arg485) and D2 (Ser654 and Glu705) (Fig. 3) (Armstrong and Gouaux, 2000). *The second* contribution is the interaction of e.g. the ε-carboxy group in **glutamate** or the isoxazole group including

substituents in **AMPA**, with one or both D1 and D2. *The third* contribution originates from direct interactions between residues in D1 and D2 at the interface of the binding pocket, brought into proximity by the agonist induced closure. Distortion of these interactions by the ligands can greatly reduce the potency. More specifically, by eliminating the "interdomain bridge" ($Glu402_{D1}$-$Thr686_{D2}$, Fig. 4) by mutagenesis destabilize the closed state of the ligand-binding domain resulting in a reduced agonist affinity and efficacy (Robert et al., 2005). In support of these findings, mutations forming additional interactions can increase the apparent agonist affinity and slow down the deactivation kinetics and the recovery from desensitization (Weston et al., 2006a).

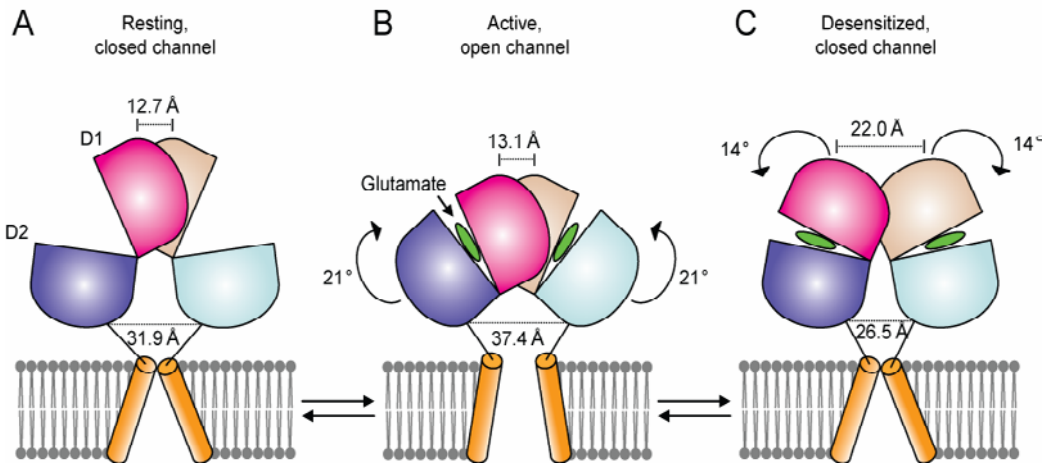

Figure 2. Agonist induced conformational states of the **AMPA** receptor. The tetrameric **AMPA** receptor exists in equilibrium between basically three functional states: a resting (A), an active (B) and a desensitized state (C). D1 and D2 denotes domain 1 and 2 of the ligand-binding pocket, respectively. A) In the absence of agonist, the bi-lobed ligand-binding domain is in a relaxed open conformation and the ion channel is closed. B) Binding of **glutamate** stabilizes a closed ligand-binding domain and this is believed to "pull" the transmembrane helices apart to open the pore. However, this state may rapidly be left, leading to either desensitization in the continued presence of agonist or deactivation as a result of removal of the agonist. The model in Panel C is based on a recent X-ray structure of a **glutamate**-bound desensitized state revealing a disrupted D1-D1 (purple and pink) interface, compensating for the **glutamate** induced binding cleft closure (Armstrong et al., 2006). Disrupting the D1-D1 interface relieves the strain on the chains to the channel domain and the ion-channel closes. Indeed, X-ray structures reveal that the linker segments, which represent the first three membrane helices from the full length receptor, are more separated in the active/**glutamate** bound conformation (B) as compared to the resting state (A) and desensitized state (C) (Armstrong et al., 2006). Distanced between D1 from the two subunits have been estimated by measuring distances between residue 739 in each protomer (distances denoted in Ångstroms above the dimers). The relative movements in the pore region have been estimated based on linker separation in the three structures (written below the dimers). Model of the resting, closed state is based on X-ray structures of the apo state (PDB: 1FTO) and the active receptor is based on the crystal structure of the agonist binding domain in complex with the full agonist **glutamate** (1FTJ) (Armstrong and Gouaux, 2000). The model of the desensitized receptor is based on the crystal structure of the mutant GluR2-S1S2J(S729C), which binds **glutamate** and is trapped in the desensitized conformation by a spontaneously formed disulfide bridge between the introduced cysteine residues (PDB: 213V) (Armstrong et al., 2006). Only two subunits of the tetrameric receptor have been illustrated for clarity.

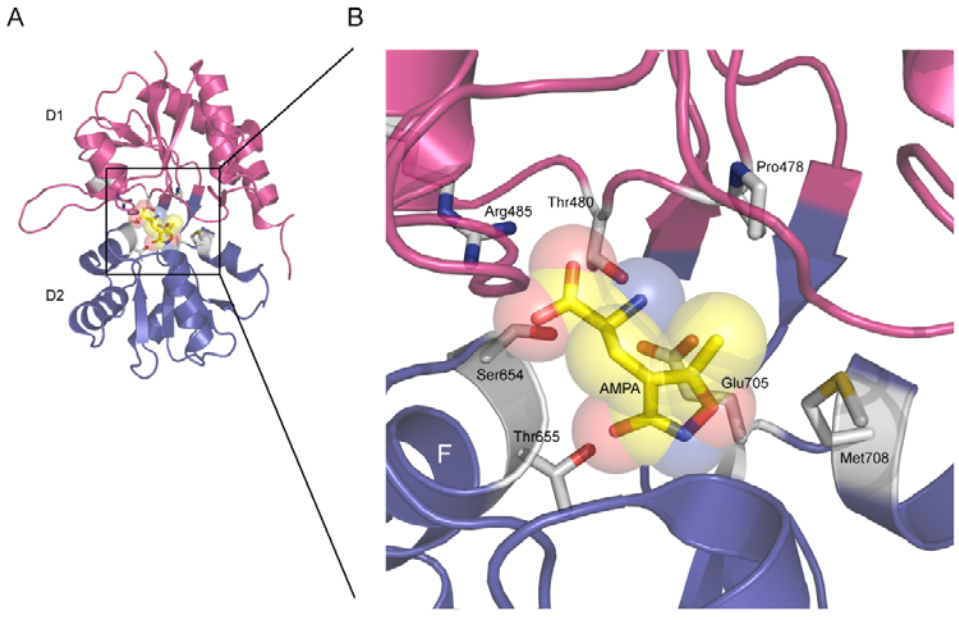

Figure 3. **AMPA** in the agonist binding cleft of GluR2. A) The overall structure of the ligand-binding domain of GluR2. D1 is displayed in red and D2 is blue. Selected interacting residues are highlighted in sticks and colored gray. **AMPA** is colored yellow and displayed with transparent spheres. B) Zoom on the central ligand-binding pocket, in the same orientation as in A. Numbers on residues correspond to the full length GluR2 receptor. The figure is based on the X-ray structure with PDB ID-code: 1FTM, (Armstrong and Gouaux, 2000).

It has been proposed that ligands initially bind the D1 followed by movement of D2 towards D1 inducing an increase in the distance between the protrusions into the transmembrane region, leading to channel opening (Fig. 2B). The agonist-bound complexes crystallize in a dimeric configuration reflecting the open or activated state. However, in the whole receptor, the concurrent changes in the membrane spanning domains impose an apparent constraint on the binding domain dimer resulting in a disruption of D1-D1 interaction and a subsequent reduction in the distance between the protrusions to the transmembrane region and transition into the desensitized state (Fig. 2C) (Armstrong et al., 2006). The propensity to induce the desensitized state increases in general for agonist inducing a higher degree of domain closure. In support of this model, it was recently possible to trap the **kainate** receptors, GluR5, GluR6 and GluR7, and also the **AMPA** receptor GluR2, in the active state by introducing intermolecular disulfide cross-links between the two D1 regions at the dimer interface, thereby hindering the conformational rearrangements leading to desensitization (Weston et al., 2006b).

Mutations that destabilize the D1–D1 interface are shown to markedly accelerate desensitization (Horning and Mayer, 2004). In contrast, mutations or compounds stabilizing the D1-D1 interface interactions reduce desensitization. Several of these compounds exhibit differential effects on desensitization and deactivation (for reviews see Black, 2005; Arai and Kessler, 2007). The enhancing effects of the ampakines (Lynch, 2006) on fast synaptic transmission suggest a potential in treatment of several neurological disorders (like

schizophrenia, depression and Parkinson's disease) as well as in the development of cognitive enhancing drugs (Black, 2005; Lynch, 2006).

Elements Influencing the Degree of Closure of the Ligand-Binding Domain

Two residues $Tyr450_{D1}$ and $Leu650_{D2}$ in GluR2 (Fig. 4) are for a large number of ligands not involved directly in hydrogen bonds, but act as "wedges" between the agonist and their respective domains, thereby having a strong impact on the degree of domain closure. **Kainate** is a naturally occurring non-desensitizing (Koike et al., 2000) partial agonist at GluR2. The crystal structure of GluR2 and **kainate** (Armstrong et al., 1998) shows that Tyr450 is located as a wedge between the isopropenyl moiety of **kainate** and D1 (Fig. 4). Mutating Tyr450 to the smaller alanine results in a increased desensitization rate and a 40-fold higher potency of **kainate** (Fig. 5) (Holm et al., 2005b), supporting the model that the tyrosine hindered domain closure. Similar findings are reported on the binding-site leucine. Mutating Leu650 (Fig. 4) to a threonine changed the domain closure upon **kainate** binding to 15° compared to 13° in the wild type (Armstrong and Gouaux, 2000; Armstrong et al., 2003). This increase in closure resulted in a 2.7-fold increase in potency and a 12-fold higher efficacy of **kainate** at the mutant.

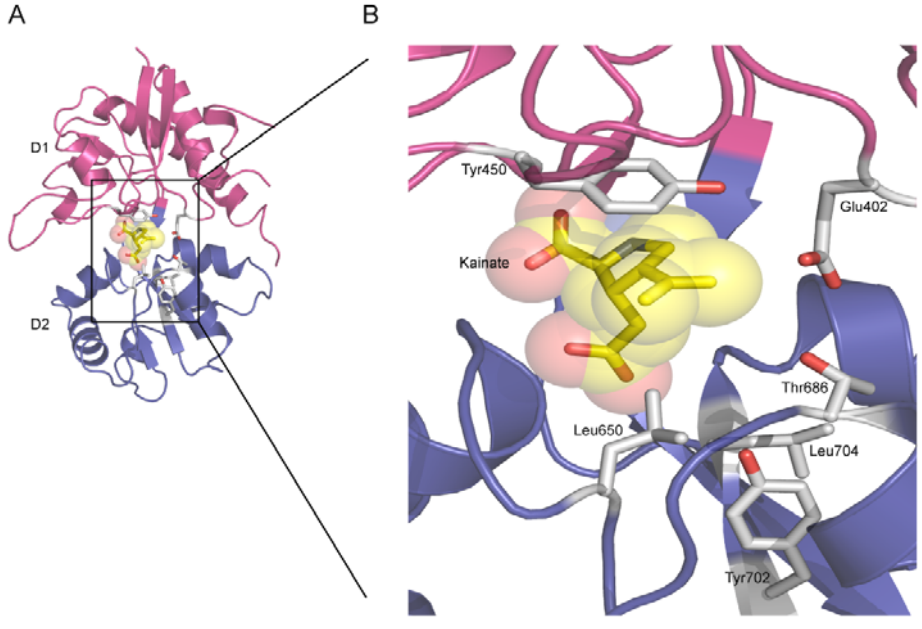

Figure 4. Amino acids influencing domain closure. Selected residues influencing closure of the ligand-binding domain (Tyr450, Leu650, Tyr702 and Leu704). $Glu402_{D1}$ and $Thr686_{D2}$ form an "interdomain bridge" stabilizing the active receptor conformation. Panel B is a magnification of the central binding pocket, in the same orientation as in A. D1 is red and D2 is colored blue. **Kainate** is colored yellow and displayed with transparent spheres.

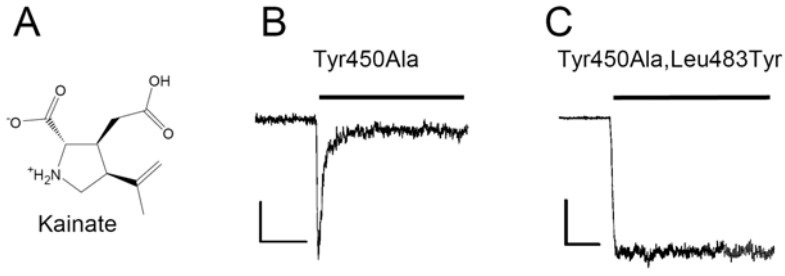

Figure 5. GluR2 mutants strongly affect desensitization kinetics. A) The molecular structure of **kainate**, which has been employed to activate the currents displayed in panel B and C. B) In contrast to the non-desensitizing currents elicited on wild type GluR2 receptors, **kainate** revealed desensitizing currents when tyrosine 450 was exchanged to an alanine. C) "Adding" the other mutation (Leu483Tyr) blocked the conformational changes necessary to desensitize the receptor. Leu483Tyr is in the interface between the subunits and is believed to "dominate" over the binding site mutant, Tyr450Ala. Currents are activated by a 100 ms ultra fast application (within 300 μs) of 1 mM **kainate** to outside-out patches from *Xenopus laevis* oocytes expressing the respective receptors. Scale bars in panel B is 100 pA and 20 ms, whereas in panel C the bars represent 400 pA and 20 ms.

Superimposition of GluR2 in complex with the **AMPA** analogue, 2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[*d*]-4-isoxazolyl)propionic acid (**4-AHCP**) on the **glutamate** complex shows that Leu704 (Fig. 4) hinders a tight D1-D2 closure leaving the domain in a partially closed state (17°) also here correlating with the compound displaying a lower efficacy of 0.38 as compared to **glutamate** (Nielsen et al., 2005). Even the nearby residue, Tyr702 (Fig. 4), allows 2.2° tighter domain closure by **kainate** when mutated to phenylalanine, correlating with an increased efficacy (Tyr702Phe: 0.47 ± 0.023 and wt GluR2: 0.21 ± 0.032) (Frandsen et al., 2005).

As discussed above, the degree of domain closure stabilized by the agonist is the most important feature for determining the agonist efficacy and studies using **willardiine** derivatives elegantly demonstrated the underlying mechanism. **Willardiine** (Fig. 10) is a naturally occurring compound, isolated from the plant *Acacia willardiana* (Gmelin, 1961), and by only substituting the 5-position with a hydrogen or halogens of increasing size, five complexes exhibiting different domain closure were investigated and the single channel conductances determined (Jin et al., 2003). The larger **I-willardiine** stabilized the ligand-binding domain in a fairly open conformation (9.2° more open than the **glutamate** structure) while **H-willardiine** stabilized the ligand-binding domain in a closed conformation (only 3.4° more open than the **glutamate** structure). The study suggested that each of the subunits could be activated independently, but that it required activation of at least two subunits to open the channel. The degree of domain closure defines the probability of the pore region to be in the open state e.g. for a bound **glutamate** the probability for the subunit to be in the open conformation is approximately 0.7. Thus efficacy is defined by the distribution between the different conductance states, with partial agonist inducing a relative larger fraction of the receptors in low conductance states as compared to full agonists stabilizing higher conductance levels (Jin et al., 2003).

Antagonists Stabilize the Binding Domain in an Open and Inactive Conformation

Competitive antagonists bind specifically in the ligand-binding pocket (like agonists), but traps the two lobes in an open conformation (Armstrong and Gouaux, 2000; Furukawa and Gouaux, 2003; Hogner et al., 2003; Mayer et al., 2006) insufficient to induce the required changes in the pore helices to open the ion channel (Fig. 6). Recent findings even report a domain opening upon association with a competitive antagonist (Kasper et al., 2006).

The **quinoxalinediones** were among the first group of selective antagonists at the **AMPA** receptors (Honoré et al., 1988). 6-Nitro-7-sulfamoylbenzo[*f*]quinoxaline-2,3-dione (**NBQX**) (Fig. 6C) was more selective than the parent compound 6-cyano-7-nitroquinoxaline-2,3-dione (**CNQX**) (Fig. 6C), however in clinical trials this group of compounds failed as drug candidates due to very limited solubility and consequent precipitation in the kidneys (Herrling, 1997). Armstrong and Gouaux published in 2000 the GluR2 complex with **DNQX** (Fig. 6C), another member of the **quinoxalinedione** family. This structure revealed that **DNQX** traps the ligand-binding domain in an open structure (Fig. 6B) by preventing the $Glu402_{D1}$ and $Thr686_{D2}$ interactions (Fig. 4) that stabilizes the lobes in a closed conformation upon binding of agonists (Armstrong and Gouaux, 2000; Hogner et al., 2003). Interestingly, **DNQX** recruits a water molecule and a sulfate ion to mediate the interactions with the D2 residues Glu705, Ser654 and Thr655 (Fig. 3), thereby participating in the interactions performed by substituents in the 3-position of **AMPA** derived agonists. Notably, the binding of the phosphate moiety, in the **AMPA** derived antagonist **ATPO** coincide with the binding side of the sulfate ion in the **DNQX** complex (Hogner et al., 2003). The α-carboxylate and the α-ammonium group of **ATPO** interacts with the highly conserved amino acids; Pro478, Thr480, Arg485 and Glu705 (Fig. 3) as do the corresponding groups of **AMPA** and all known agonists, however the isoxazole ring of the two molecules interacts differently (Hogner et al., 2003). **ATPO** interacts with the D2 residues Ser654, Thr655 (Fig. 3) directly with the 3-phosphonate group, whereas **AMPA** recruits a water molecule to form comparable hydrogen bonds. This water molecule places **AMPA** closer to D1 while **ATPO** hinders closure of D1-D2 thereby acting as an antagonist.

The structure of **ATPO** in complex with the **kainate** receptor GluR5 (Hald et al., 2007) indicates why **ATPO** only interacts with the **AMPA** receptors and GluR5 (Wahl et al., 1998a) and not with GluR6. Analysis of the binding site interactions explained this profile by the presence of an alanine at position 689 in GluR6 which is replaced by a serine in GluR5 and all the **AMPA** receptors (Hald et al., 2007). In the presence of **ATPO**, this serine may coordinate the network of water molecules better as compared to the alanine in GluR6. To date no apo structure of GluR5 has been published, however comparing the degree of domain closure induced by **glutamate** and **ATPO**, reveals a 28.2° more open conformation stabilized by **ATPO**. In comparison, at GluR2 the antagonist only induced a 19.2° more open conformation. Apparently, GluR5 allows a markedly higher degree of closure of the ligand-binding domain since the structural variability of the GluR5 ligand-binding domain upon association with different ligands is reported to range 30°, whereas at GluR2 the values range no more than 19° (Hald et al., 2007).

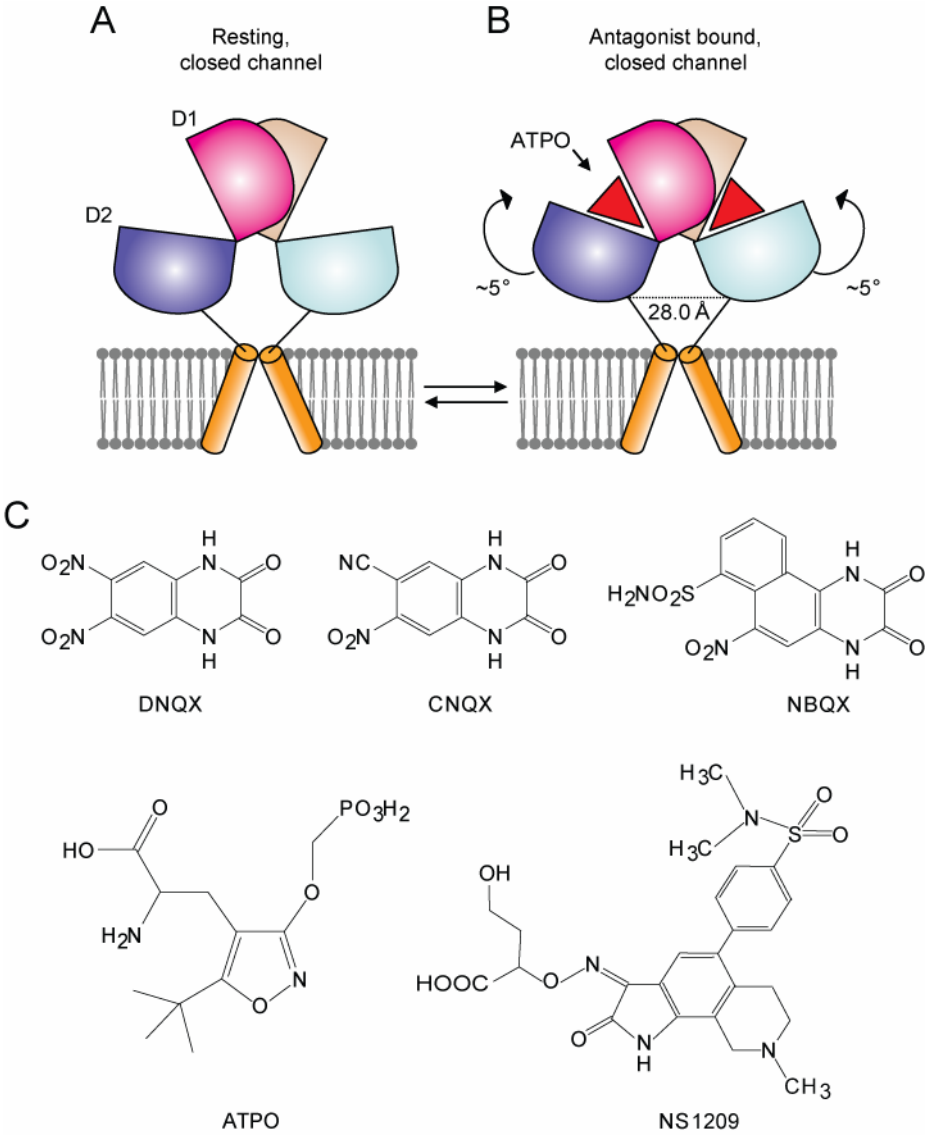

Figure 6. Binding of competitive antagonists (**ATPO**) stabilizes D1-D2 in a slightly closed conformation, which does not open the ion channel. A) The resting state of the **AMPA** receptor, with a ligand-binding domain in a relaxed open conformation and a closed ion channel. B) Binding of an antagonist, **ATPO**, results in slight closure of D1-D2, but the channel remains closed. The 5° closure is based on the reported values for domain closures reported as in the range 2.5-5.1° for **ATPO** (Hogner et al., 2003) and 3.0-6.0° for **DNQX** (Armstrong and Gouaux, 2000) as compared to the structure with no agonist/antagonist bound (Armstrong and Gouaux, 2000). Model of the resting, closed state is based on X-ray structures of the apo state (PDB: 1FTO) (Armstrong and Gouaux, 2000). Model of the antagonist bound, closed channel state is based on the GluR2-S1S2J:**ATPO** (PDB id code 1N0T, Hogner et al., 2003, linker distances from Hald et al., 2007 and GluR2-S1S2J:**DNQX** (PDB id code 1FTL, Armstrong and Gouaux, 2000). Only two subunits of the tetrameric receptor have been illustrated for clarity. C) Selected competitive antagonists discussed in the text.

In contrast to the subtle domain closure observed in the GluR2 co-crystal structures with **ATPO** or **DNQX** (Fig. 6B), (*S*)-8-methyl-5-(4-(*N*,*N*-dimethyl-sulfamoyl)phenyl)6,7,8,9,-tetrahydro-1*H*-pyrrolo[3,2-*h*]-isoquinoline-2,3-dione-3-*O*-(4-hydroxybutyrate-2-yl)oxime [**(*S*)-NS1209**] (Fig. 6C) induces a domain opening of the binding domain (Kasper et al., 2006). **(*S*)-NS1209** forms a hydrogen bond to Thr686 and thereby prevents the "interdomain lock" formed between $Glu402_{D1}$ and $Thr686_{D2}$ (Fig. 4) (Kasper et al., 2006). Interestingly, **(*S*)-NS1209** is crystallized in a mixed structure with **glutamate** bound to the other monomer in the dimer. This is the first structure of a mixed dimer and the 33 Å linker separation corresponds to the distance induced by the partial agonist **kainate** (Kasper et al., 2006) suggesting that the structure may not represent the conformations in native receptors with two apo forms and two **glutamate**-bound subunits. Rather this novel state, with domain opening and 33 Å linker separation (with no channel opening) probably represents a new distorted form of the channel that does not allow any current (Kasper et al., 2006). Future studies of mixed receptor complexes might provide insights to the conformational states corresponding to the distinct conduction states observed for the **AMPA** receptor family and add further details to the coupling of ligand-binding to movements of the transmembrane helices.

AMPA as a Lead Structure: Structure-Function Studies with the AMPA Molecule in Focus

Since **AMPA** was synthesized in 1980 (Krogsgaard-Larsen et al., 1980) and developed as a radioligand in 1982 (Honoré et al., 1982) extensive efforts have been used to explore derivatives of this compound (reviewed in Bräuner-Osborne et al., 2000; Madsen et al., 2001b; Stensbøl et al., 2002). Series of distinct functional groups have been introduced at different positions of the isoxazole ring (Fig. 7). In the following sections we will discuss the **AMPA** derivatives focusing on reports where structural and electrophysiological data are available.

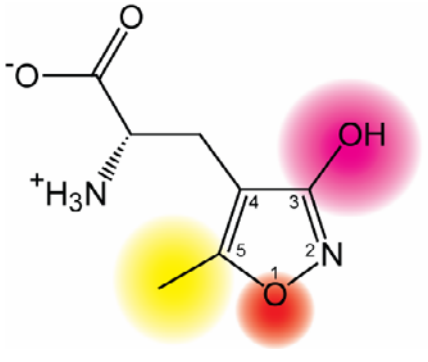

Figure 7. The **AMPA** molecule. "Red" highlights the 1-position, "pink" highlights the 3-position while "yellow" highlights the 5-position of the isoxazole ring structure.

Changing the 1-Position of the AMPA Isoxazole Ring -Effects on Receptor Kinetics

Substitution of the oxygen in the 1-position of the isoxazole ring in **AMPA** for a sulphur was introduced to improve the brain penetration of the compound (Matzen et al., 1997). When analyzed in the rat cortical wedge preparation (*R,S*)-2-amino-3-(3-hydroxy-5-methyl-4-isothiazolyl)propionic acid (**thio-AMPA**) (Fig. 8) was less potent than **AMPA**. However, when evaluating the convulsive potencies after subcutaneous administration of **AMPA** and the sulphur-substituted analogue, the latter proved the most potent *in vivo*, indicating better penetration of the blood brain barrier (Matzen et al., 1997).

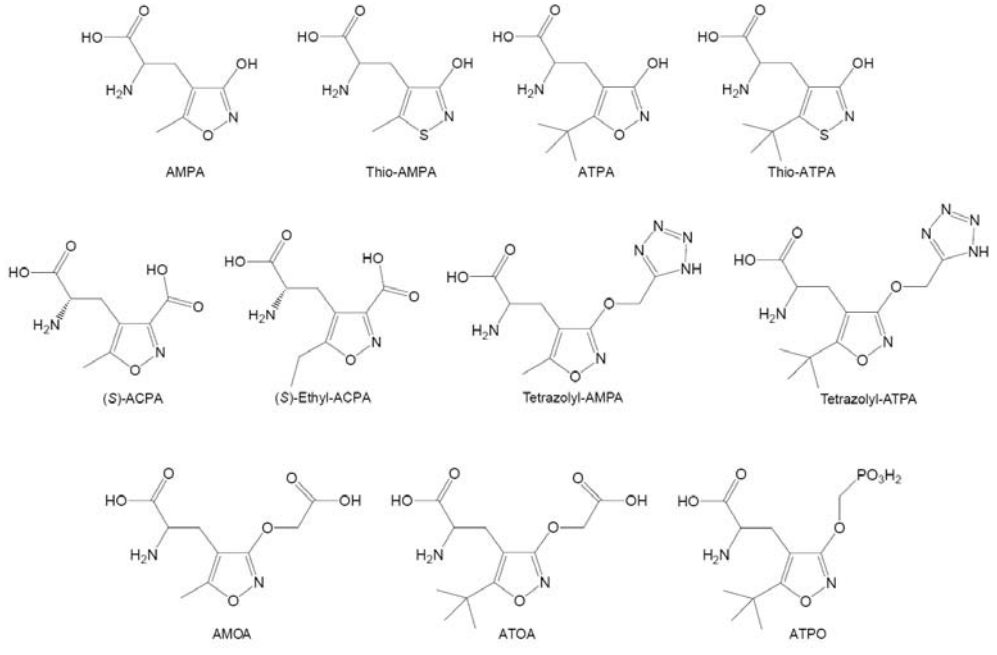

Figure 8. Selected substitutions in the 1- and 3-position of the **AMPA** isoxazole ring. Structures of some **AMPA**-derived agonists with changes in the 1-position of the isoxazole ring (first row). **AMPA**-derived agonists (**ACPA** and **ethyl-ACPA**) and competitive antagonists (**tetrazolyl-AMPA, tetrazolyl-ATPA, AMOA, ATOA** and **ATPO**) with different substituents in the 3-position are displayed in the second and third row.

Introduction of the sulphur atom additionally changed the kinetic properties. In particular (*R,S*)-2-Amino-3-(5-tert-butyl-3-hydroxy-4-isothiazolyl)propionic acid (**thio-ATPA**) (Fig. 8), where also the methyl group at position five has been replaced by a tertbutyl group, has been studied in greater detail. **Thio-ATPA** revealed curiously high maximal currents when compared to **ATPA** under two-electrode voltage clamp (Stensbøl et al., 2001). A detailed kinetic analysis showed that **thio-ATPA** desensitized GluR2flop more than three-fold slower than **glutamate**, when analyzed in outside-out patches from *Xenopus leavis* oocytes (Fig. 9) (Holm et al., 2005a), indicating that **thio-ATPA** delays the conformational changes leading to desensitization. The GluR2 co-crystal showed that **thio-ATPA** induces an 18° domain closure

as compared to the ~21° closure induced by **glutamate**. Notably, **Br-HIBO**, induced the same degree of domain closure as **thio-ATPA**, while revealing distinct kinetics (Fig. 9), indicating that factors beyond domain closure influences the kinetic response (Holm et al., 2005a).

Analyzing the structural differences of **thio-ATPA** and **Br-HIBO** bound to the ligand-binding domain of GluR2 pointed at two elements that might define the agonist specific desensitization kinetics (Holm et al., 2005a). Firstly, Leu650 (Fig. 4) adapts two distinct agonist-dependent conformations with the side-chain χ1 angle in a *gauche* arrangement in complex with **Br-HIBO, glutamate**, **AMPA** or **ATPA** compared to an *anti* arrangement upon binding of **thio-ATPA** or **kainate**. Secondly, a twist of D2 relative to D1 of the ligand-binding domain was observed in the **Br-HIBO** complex but not in the **thio-ATPA** complex. Superimposition of the two structures reveals a relative twist at 6° of D1 and D2 stabilized by **Br-HIBO** as compared to the **thio-ATPA** co-complex (Fig. 9D) (Holm et al., 2005a), proposing that receptors stabilized in a "twisted" state more easily destabilize the D1 interface and undergo the conformational changes leading to desensitization as observed for **Br-HIBO**.

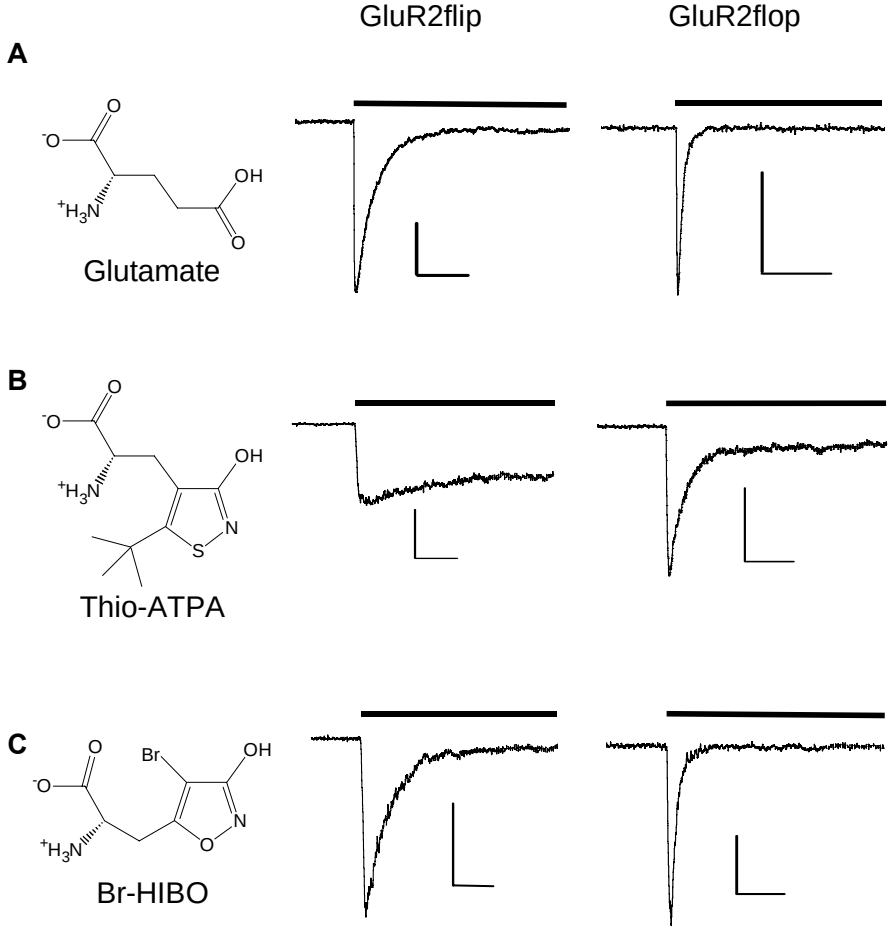

Figure 9. Continued on next page.

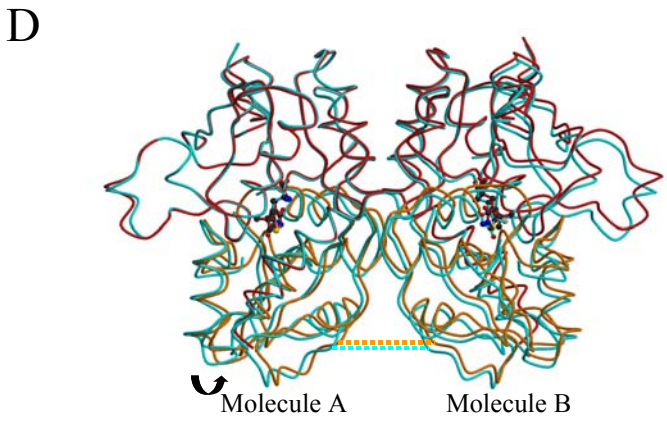

Figure 9. Agonist structure strongly affects the desensitization kinetics on GluR2 splice variants. A) The molecular structure of **glutamate** and representative currents elicited on GluR2 flip and flop, respectively. Responses desensitized with the time constants 9.8 ± 0.8 ms and 2.0 ± 0.2 ms on GluR2flip and flop, respectively. Panel B shows **(*S*)-thio-ATPA** and currents evoked on GluR2 flip (τ >100 ms) and flop (τ = 6.6 ± 0.7 ms). Panel C illustrates **(*S*)-Br-HIBO** and currents evoked on GluR2 flip (τ = 13 ± 1 ms) and flop (τ = 3.0 ± 0.1 ms). Currents are activated by a 100 ms ultra fast application (within 300 μs) of the respective agonists to outside-out patches from *Xenopus laevis* oocytes expressing the respective receptors. Vertical and horizontal scale bars represent 500 pA and 20 ms, respectively, for all traces, except for **Br-HIBO** on GluR2flop where the vertical bar is 200 pA. D) Twist of D2 relative to D1. Residues from D1 of molecule A were used for the superimposition. D1 residues of the GluR2-S1S2J**:(*S*)-thio-ATPA** (pdb code: 2AIX) dimer are coloured red and D2 residues are coloured orange. The GluR2-S1S2J**:(*S*)-Br-HIBO** (pdb code: 1M5C) dimer is coloured cyan. **(*S*)-Thio-ATPA** and **(*S*)-Br-HIBO** are shown in ball-and-stick representation. Dotted lines indicate the distance between Ile633 of the two protomers of the GluR2-S1S2J**:(*S*)-thio-ATPA** dimer (orange, 34.8 Å) and GluR2-S1S2J**:(*S*)-Br-HIBO** dimer (cyan, 34.5 Å). The D1-D2 domain closure is 18° in both structures, and the arrow indicates the different twist of D2 between the two structures.

Substitutions at the 3-Position of the AMPA Isoxazole Ring -from Agonists to Competitive Antagonists

The pharmacophore models used for the **AMPA** derivatives has largely been based on the assumption that the hydroxyl group at the 3-position would be bioisosteric with the **glutamate** γ-carboxyl group. Hence, it came as a surprise when the GluR2-**AMPA** co-crystal (Armstrong and Gouaux, 2000) showed that instead the 3-hydroxyl group and a water molecule was localized as the γ-carboxyl group to mediate the interaction between the 3-hydroxyl and helix F from D2 (Fig. 3). This explained why a substitution of the 3-hydroxyl group with the larger carboxyl group in (*S*)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl)propionic acid (**ACPA**) (Fig. 8) could be accommodated and still act as an agonist. The 3-carboxyl group of **ACPA** replaces the 3-hydroxyl group and the water molecule and hydrogen bonds directly to Thr655 (Fig. 3). Additionally, **ACPA** is positioned slightly closer

to D1, probably explaining that the carboxyl-substituted agonist is more potent at the **AMPA** receptors as compared to **AMPA** itself (Hogner et al., 2002; Brehm et al., 2004).

Combining a 5-ethyl group and a 3-carboxyl group, (*R,S*)-2-amino-3-(3-carboxy-5-ethyl-4-isoxazolyl)propionic acid (**ethyl-ACPA**) (Fig. 8), increases the potency at GluR3 (EC_{50} = 0.89 [0.72-1.1] μM) and GluR4 (EC_{50} = 0.28 [0.22-0.37] μM) (Brehm et al., 2004). The X-ray structure of **(*S*)-et-ACPA** alone was subsequently docked into the binding-site of GluR2 and this model revealed that due to the larger substituents in the 3-position, major rearrangements in the pocket would be induced if larger substituents were to be accommodated in the 5-position (Brehm et al., 2004).

Bulky modifications in the 3-position of the isoxazole ring result in competitive antagonists as described for **ATPO**. (*R,S*)-2-Amino-3-(3-tetrazolylmethoxy-5-methyl-4-isoxazolyl)propionic acid (**tetrazolyl-AMPA**) and (*R,S*)-2-amino-3-(3-tetrazolylmethoxy-5-tert-butyl-4-isoxazolyl)propionic acid (**tetrazolyl-ATPA**) (Fig. 8) (Frølund et al., 2005) display comparable antagonism at GluR2. **Tetrazolyl-ATPA** has almost no affinity for the **kainate** receptor GluR5, still **tetrazolyl-AMPA** show moderate activity at this receptor family (IC_{50} = 160 ± 4.2 μM) (Frølund et al., 2005). It might seems surprisingly that the **kainate** receptor selective agonist derived **tetrazolyl-ATPA** has lost binding to the **kainate** receptor GluR5 (IC_{50} > 2500 μM), apparently, the combination of the 3-tetrazol group and the 5-tertbutyl group abolishes interactions within the GluR5 binding cleft, whereas the **AMPA** receptor binding site is still able to accommodate **tetrazolyl-ATPA** as competitive antagonist.

Another line of studies examine the effect of inserting a carboxymethoxyl group in the 3-position of **AMPA** and **ATPA** to derive the competitive antagonists (*R,S*)-2-amino-3-(3-carboxymethoxy-5-methyl-4-isoxazolyl)propionic acid (**AMOA**) (Krogsgaard-Larsen et al., 1991) and (*R,S*)-2-amino-3-(3-carboxymethoxy-5-tert-butyl-4-isoxazolyl)propionic acid (**ATOA**) (Wahl et al., 1998b), respectively (Fig. 8). Both **AMOA** and **ATOA** display antagonistic activity at the **AMPA** receptors (Madsen et al., 1996; Frølund et al., 2005), where **AMOA** is **AMPA** receptor selective and exhibits moderate potency at **AMPA** receptors expressed in *Xenopus laevis* oocytes (IC_{50} ~ 40-100 μM) (Wahl et al., 1998a; Frølund et al., 2005). However, the phosphonomethoxy–derivative, **ATPO**, is 10-fold more potent at the **AMPA** receptors as compared to **AMOA** (Wahl et al., 1998a) and consequently **ATPO** was analyzed in details in X-ray structures as discussed above (Hogner et al., 2003).

The simplified mechanism of converting these agonists (**AMPA** and **ATPA**) into competitive antagonists (**AMOA**, **ATOA, ATPO**, **tetrazolyl-AMPA**, **tetrazolyl-ATPA**) is through conserving specific ligand-receptor interactions within the agonist binding pocket while adding bulky groups in the 3-position of the isoxazole ring and thereby minimizing the antagonist induced closure of the bi-lobed ligand-binding domain.

Substitutions at the 5-Position of the AMPA Isoxazole Ring Confer a Selective Profile

Changes in the 5-position modifies the selectivity both within the **AMPA** receptor family as well as between the **AMPA** and **kainate** receptors. The 5-methyl group of **AMPA** points into a pocket occupied by Met708 (Fig. 3), which is forced away in the GluR2-**AMPA** structure as compared to the extended conformations observed in the **kainate** and **glutamate**

bound structures (Armstrong and Gouaux, 2000). 2-Amino-3-(5-tert-butyl-3-hydroxy-4-isoxazolyl)propionic acid (**ATPA**) is one of the most selective **AMPA** derived agonists (Fig. 10) (Clarke et al., 1997) and exhibits more than 90-fold preference for the **kainate** receptor GluR5 as compared to the **AMPA** receptor GluR1 (Stensbøl et al., 1999). Mutagenesis studies revealed that the main determinant for the selectivity could be ascribed to a serine in GluR5 located at a position equivalent to a methionine (Met708) in GluR2 and the other **AMPA** receptors (Nielsen et al., 2003). A structural explanation for this is found in the crystal structures of GluR5, showing more room to accommodate the bulky tertbutyl group in **ATPA** with the serine coordinating a unique water network while additionally participating in a D1-D2 contact ($Tyr444_{D1}$-$Ser741_{D2}$) stabilizing the active conformation (Naur et al., 2005; Hald et al., 2007).

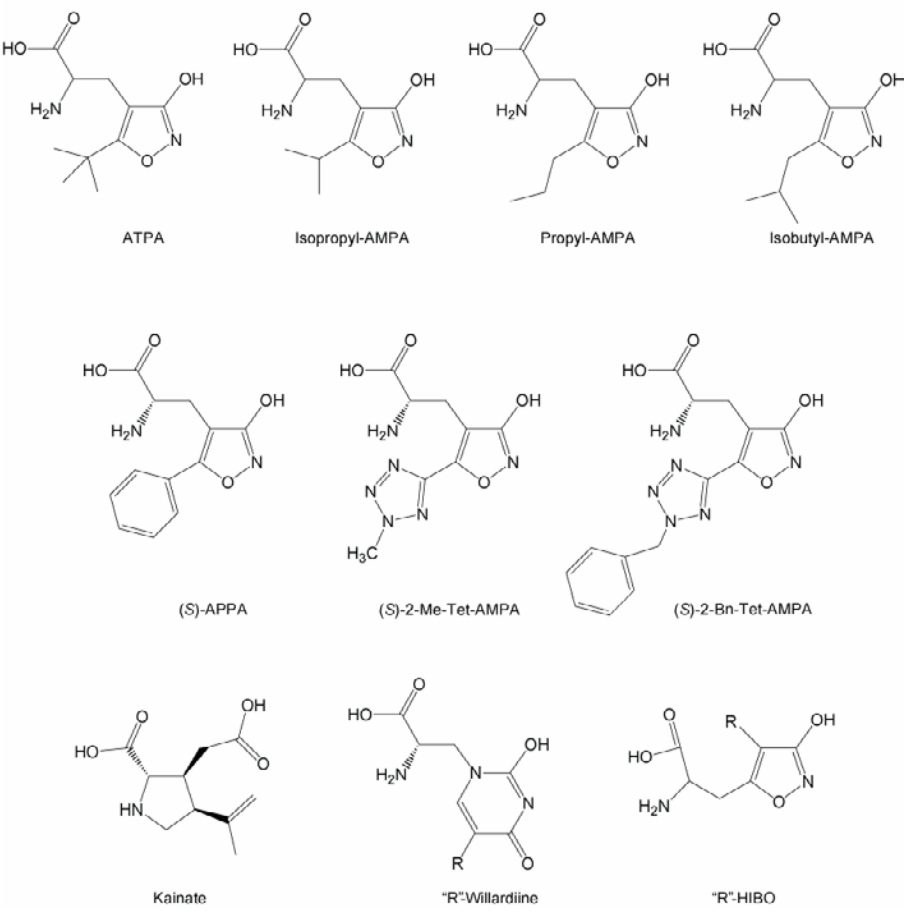

Figure 10. Selected substitutions in the 5-position of the **AMPA** isoxazole ring and various non-**AMPA** like agonists. Structures of some **AMPA**-derived agonists with changes in the 5-position (**ATPA**, **isopropyl-AMPA**, **propyl-AMPA**, **isobutyl-AMPA**, **(*S*)-APPA**, **2-Me-Tet-AMPA** and **2-Bn-Tet-AMPA**). Last row displays three agonists of non-**AMPA** like structure, all representing pharmacological tools, which have contributed significantly to the understanding of the mechanism of action of the **AMPA** receptors. "R" denotes the position in **willardiine** and **HIBO** where different functional groups have been inserted.

Another line of compounds was developed in which the tertbutyl group of **ATPA** was replaced by different heteroaromatic ring substituents. 2-Amino-3-(3-hydroxy-5-phenyl-4-isoxazolyl)propionic acid (**APPA**) (Fig. 10), with a 5-phenyl group, displayed a week agonistic profile in the *S*-form, however the *R*-form was a weak competitive antagonist (Ebert et al., 1994a; Ebert et al., 1994b). Continuing these approaches, it was also possible to design selectivity *within* the **AMPA** receptor family. By further increasing the size of the 5-substituent, Vogensen and co-workers obtained 2-amino-3-[3-hydroxy-5-(2-methyl-2*H*-5-tetrazolyl)-4-isoxazolyl]propionic acid (**2-Met-Tet-AMPA**) (Fig. 10). This is the most potent **AMPA** derivative showing an 18-fold preference for GluR4flop (EC_{50} = 0.009 ± 0.002 μM) as compared to GluR1 when analyzed on homomeric recombinant receptors expressed in *Xenopus laevis* oocytes under two-electrode voltage clamp (Vogensen et al., 2000). Interestingly, the 2-Met-Tet moiety is located at the interface between D1 and D2 but repositions the isoxazole ring and locates the 3-hydroxyl group closer to D2, thereby inducing a direct interaction between the 3-hydroxyl group and Thr655 (Fig. 3).

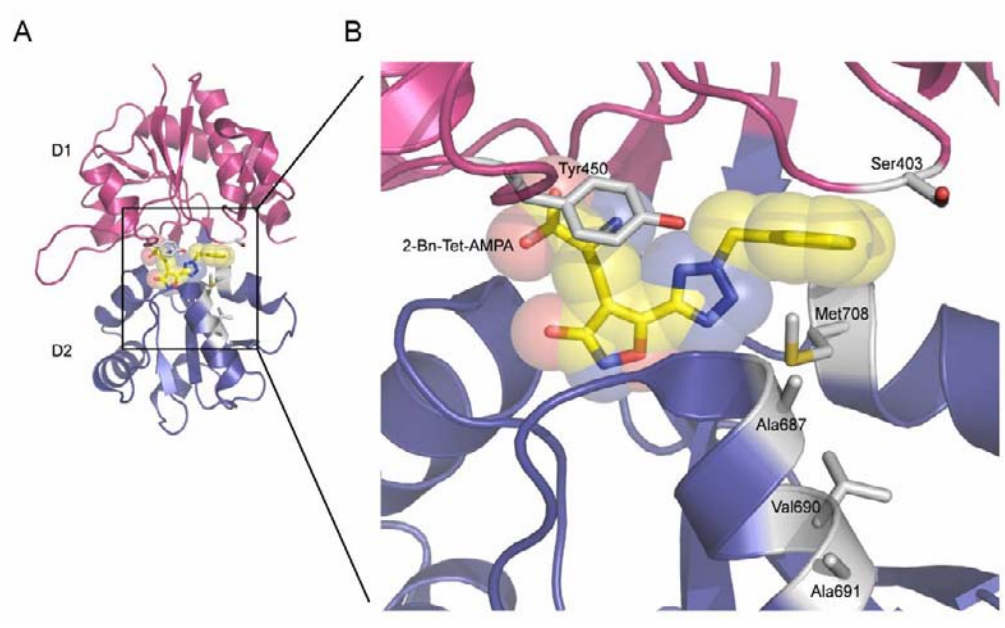

Figure 11. **2-Bn-Tet-AMPA** protrudes out of the central ligand-binding pocket and gains a selective profile. Ser403, Ala687, Val690 and Ala691 (GluR2 numbering) differ between GluR1 compared to GluR2-4. These residues are close to the benzyl-moiety and are believed to contribute to the highly selective profile of **2-Bn-Tet-AMPA** towards GluR2-4 as compared to GluR1 (Vogensen et al., 2007; Jensen et al., 2007). A) Overall structure of the ligand-binding domain of GluR2 complexed with **2-Bn-Tet-AMPA** (pdb ID code: 2P2A , Vogensen et al., 2007). D1 colored red and D2 colored blue. B) Zoom on the binding pocket with selected residues displayed in sticks and colored gray. **2-Bn-Tet-AMPA** is colored yellow and displayed with transparent spheres. Same orientation as in A. Met708 is highlighted to underline the importance of its reorientation which "opens up" to this new sub-domain of the binding cleft while retaining the agonistic profile of the compound (Vogensen et al., 2007). Tyr450 is included to illustrate the location of the central binding pocket.

The binding mode of the 2-Met-Tetrazolyl moiety in **2-Met-Tet-AMPA** at the interface between D1 and D2 suggests the possibility for further extension of this 5-substituent. Substituting the methyl group for a benzyl moiety, to obtain (*R,S*)-2-amino-3-[3-hydroxy-5-(2-benzyl-2*H*-5-tetrazolyl)-4-isoxazolyl]-propionic acid (**2-Bn-Tet-AMPA**) (Fig.10) revealed a highly selective compound displaying a 38-fold preference for GluR4 (K_i = 0.20 ± 0.09 μM) as compared to GluR1 in receptor binding assays (Vogensen et al., 2007). In the GluR2 crystal structure the benzyl moiety protrudes into a new cavity (Fig. 11) outside the ligand-binding domain, where agonist substituents have not previously been observed. Here, the aromatic ring of the benzyl moiety exhibits hydrophobic interactions with residues from both D1 and D2 ($Thr707_{D2}$, $Tyr711_{D2}$ and $Trp767_{D1}$). This novel binding mode may contribute to the highly selective profile as also revealed by homology modeling.

Homology models of **2-Bn-Tet-AMPA** bound to GluR1, GluR3 and GluR4 were constructed based on the crystal structure of GluR2 complexed with **2-Bn-Tet-AMPA** (Jensen et al., 2007; Vogensen et al., 2007). This pin-pointed four residues close to the benzyl group which differed between GluR1 (Asp399, Glu683, Met686 and Ile687) and GluR2-4 (GluR2 numbers: Ser403, Ala687, Val690 and Ala691) (Fig. 11), respectively. Obviously this diversity is speculated to contribute to the pronounced selectivity recorded within the **AMPA** receptor family (Jensen et al., 2007) and although the residues do not directly interact with the benzyl-group, they strongly influence the network of interactions in the binding-pocket, thereby shaping the selectivity profile. Additionally, these four residues are close to the methionine residue (Met708, Fig. 11), which has shown a marked degree of flexibility adapting agonist of varying structure in this region (Armstrong and Gouaux, 2000; Kasper et al., 2002; Lunn et al., 2003). This newly identified domain of the **AMPA** receptor ligand-binding pocket is likely to be further explored in the search of new subtype selective compounds directed towards each of the four members of the **AMPA** receptor family.

As mentioned previously the water mediated interactions between the **ACPA** and **Br-HIBO** and particular Tyr702 (Fig. 4) also form a basis for obtaining intersubunit selectivity (Banke et al., 2001; Hogner et al., 2002). This has led to exploration of **HIBO** analogous (Fig. 10) where **Cl-HIBO** exhibits a 275-fold preference for GluR1 over GluR4 and almost 1600-fold for GluR2 compared to GluR3 (Bjerrum et al., 2003). **Isopropyl-HIBO** displayed 50-100-fold selectivity for GluR1 and GluR2 relative to GluR3 and GluR4 in binding studies (with K_i-values at 0.41 ± 0.02 μM and 0.95 ± 0.06 μM at GluR1 and GluR2, compared to 35 ± 4 μM and 43 ± 12 μM at GluR3 and GluR4, respectively) (Madsen et al., 2005). Extension into larger substituents, as **hexyl-HIBO,** looses affinity (Madsen et al., 2001a), leaving **Cl-HIBO** as one of the most selective agonists reported on the **AMPA** receptors so far (Bjerrum et al., 2003).

In this chapter we have highlighted some important findings from current studies of the structure and function of **AMPA** receptors. Future development of agonists targeted at the GluR1 and GluR2 receptor families will obviously take advantage of the highly selective **Cl-HIBO** as a lead structure, while **2-Bn-Tet-AMPA** would be the agonist of choice when one wants to target GluR3 and GluR4. To date, no human disease are definitely proved to be linked to mutations in the gene for a **glutamate** receptor (Mayer, 2006), however **AMPA** receptor agonists and competitive antagonists already serve as indispensable pharmacological tools that may someday be developed into clinically applicable compounds.

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Chapter 4

Glutamate Signalling in the Skin

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Abstract

Many cellular elements of the skin appear to involve glutamate for signalling. This has been most extensively studied in nociceptive nerve endings (supplied by C and Aδ fibres). Both ionotropic and metabotropic glutamate receptors are involved in inflammation, and modulate hyperalgesia and allodynia. Less work has been on low threshold mechanoreceptors in the skin (supplied by Aβ fibres), although there is clear evidence for a modulatory role here too. There is evidence that other structural cellular elements, such as keratinocytes use glutamate for signaling, and may be important for skin growth and wound healing. Finally, excessive release of glutamate and/or aberrant expression of metabotropic glutamate receptors may be contributory factors in the development of skin melanomas. There appears to be significant clinical potential in the development of topically applied glutamate receptor active compounds to treat a range of different skin disorders.

Introduction

The skin is the largest organ of the body. In the human body it accounts for 18% by volume, weighs 4 kg, measures 1.7 m^2 and is 3 mm thick. In its multiple roles of protection, containment, thermoregulation and sensing, it is comprised of numerous types of cells forming a complex layered structure. The skin also plays an important role in our interpersonal communication through touch, and its visual condition provides information about our health to others, for example in sexual attraction.

While glutamate is established as the most significant transmitter in the central nervous system, there is accumulating evidence to suggest that it plays important roles in many other tissues including the skin (Hinoi et al., 2004;Skerry and Genever, 2001). The current focus is on glutamate receptor-mediated signalling, although it is possible that amino acids other than

glutamate, e.g. aspartate, may actually act as the signalling molecule. A role for GABA, which is a synthetic product of glutamate, may play some role in normal and pathological processes in the skin, but there is less information (Watanabe et al., 2006). Glutamate may act as a transmitter or modulator between cellular elements associated with sensory nerve endings. Similar roles have been discovered in other peripheral systems, for example the intraganglionic laminar endings in the gastrointestinal tract (Raab and Neuhuber, 2007) and sensory terminals on muscle spindles (Bewick et al., 2005). In the skin glutamate signalling may also regulate cell proliferation and differentiation during development and wound healing, and hence aberrant signalling might be associated with pathological proliferation and neoplasia (Kalariti et al., 2005). Thus, a more precise understanding of glutamate's role in the skin could lead to potential therapies for somatosensory disorders, skin conditions and skin cancers.

Sensory Nerves in the Skin

Glutamate Receptor Labelling

Evidence for a role for glutamate signalling has been found in a range of different afferent and efferent nerve types (Aas et al., 1989;Jensen et al., 2005;Lorenzo et al., 1994;Raab and Neuhuber, 2007). In the skin, afferent myelinated and unmyelinated nerve profiles in the rodent and human dermal-epidermal junction express ionotropic glutamate receptors (Coggeshall and Carlton, 1998;Kinkelin et al., 2000). Labelling for NR1 (NMDA), GluR1 (AMPA) and GluR5,6,7 (kainate) receptor subunits was observed. Labelling was patchy along the length of identified fibres. Up to 20% of unmyelinated axons were labelled for ionotropic receptors. Approximately 50% of myelinated axons were labelled for NR1 but significantly fewer were labelled for non-NMDA receptor subunits. Approximately 28% of unmyelinated axons express kainate receptors (Du et al., 2006). These percentages depend to some extent on the particular nerves examined. The proportion of labelled nerves increased after inflammation of the skin (Carlton and Coggeshall, 1999), although this may represent an enhancement of nerves with existing receptors present. The afferent nerve endings associated with Merkel cells appear to be unusual in that no selective labelling for NMDA or AMPA receptors could be found (Cahusac et al., 2005;Senok et al., 2003). Metabotropic glutamate (mGlu) group I (mGlu1/5) receptors, which are positively coupled by Gq to phospholipase C, were most prevalent on unmyelinated fibres (Bhave et al., 2001;Walker et al., 2001;Zhou et al., 2001). Group II (mGlu2/3) receptors, which are negatively coupled by Gi/Go to cAMP, were found in equal proportions on unmyelinated and myelinated fibres (approximately 30%) (Carlton et al., 2001). A recent study of dorsal root ganglion (DRG) cells found that group III (mGlu8) receptors were expressed by 75% of cells, including all fibre diameter types, and that there was significant colocalization with group II (mGlu2/3) receptors (Carlton and Hargett, 2007). The same study found that mGlu1 and mGlu2/3 are expressed most often by medium and small diameter cells.

Merkel associated nerve terminals show labelling for two G proteins Gαo and Gαi (Tachibana et al., 2001), which may be linked to group II and III mGlu receptors. Only a rather weak and "capricious" labelling was found for mGlu5 receptors at these terminals (Tachibana et al., 2003).

Sources of Glutamate

There are several possible sources of glutamate to bind at the receptors found on nerve endings. Electrical stimulation of A and C fibres produces an increase in glutamate (but not aspartate) in the extracellular space of peripheral tissue (de Groot et al., 2000;Jin et al., 2006). Local perfusion of capsaicin which selectively activates C fibres also increases levels of glutamate. Glutamate levels also increase following inflammation (Jin et al., 2006;Lawand et al., 2000;Omote et al., 1998). Given the effect of antidromic activation, one likely source is from the nerves themselves. Virtually all DRG and trigeminal ganglion cells are labelled by vesicular glutamate transporters, either VGLUT1 or VGLUT2 (Brumovsky et al., 2007;Landry et al., 2004;Li et al., 2003b), but it seems that there is differential expression of these proteins peripherally and centrally according to fibre type. Many nerve terminals in the skin contain clear vesicles (Kruger et al., 2003), and VGLUT1, VGLUT2 and VGLUT3 proteins have been identified in corpuscular (Aβ fibres) and free nerve endings (Aδ and C fibres) (Nunzi et al., 2004). However terminals (neurites) associated with Merkel cells were not clearly labelled for VGLUT proteins, only weak and variable labelling was found for VGLUT2 (Brumovsky et al., 2007;Nunzi et al., 2004)[1]. Other possible sources of glutamate are: keratinocytes (see below), Merkel cells (see below), Schwann cells (Araque et al., 1999) and macrophage-like epithelial cells (Nordlind et al., 1993).

Role of Glutamate Signalling

Depolarization of nerve endings in the skin by stimulation (mechanical, thermal, chemical) could result in the release of glutamate. Another mechanism is 'antidromic' propagation of activity from central terminals by the so-called dorsal root reflex (Willis, 1999). This mechanism causes inflammation at sites near to, but not directly at, the initial source of noxious stimulation. Released glutamate could act on the same or neighbouring nerve endings. The specific effect of glutamate on the endings would depend on the type of glutamate receptors expressed there.

a) Nociceptive fibres.

Subcutaneous injection of glutamate (0.1 ml at 1 – 100 mM) produced dose-dependent pain in humans, described as "moderate" intensity (Gazerani et al., 2006). An area of secondary pinprick hyperalgesia was seen in most participants which lasted up to 10 minutes. Ventral root reflexes in a rat preparation were observed after the tail skin application of various excitatory amino acids, including glutamate, domoate, AMPA, kainate and quisqualate (Ault and Hildebrand, 1993). Mechanical hyperalgesia in animals can be

[1]Although it need not overly concern us here, a contrasting picture emerges for the central terminations of different fibre types in the spinal cord dorsal horn and trigeminal medullary dorsal horn. Central terminals of all large myelinated Aβ and some smaller myelinated Aδ fibres express VGLUT1 and/or VGLUT2 (Alvarez et al., 2004;Morris et al., 2005;Oliveira et al., 2003;Todd et al., 2003). Smaller myelinated Aδ or unmyelinated C fibre terminations either do not contain either VGLUT or only show low levels of VGLUT2 (Brumovsky et al., 2007;Todd et al., 2003), but see (Li et al., 2003a). These data suggest in apparent contravention of Dale's Law that while most low-threshold mechanoreceptor afferents release glutamate at both their central and peripheral terminals, nociceptive afferents release glutamate peripherally and a peptide centrally.

produced by injection into the skin of NMDA, AMPA and kainate, and the effects attenuated by respective receptor antagonists (Zhou et al., 1996). Up to 1 mM glutamate applied to nerve endings in rat skin has surprisingly modest excitatory effects on the firing of Aδ and C fibres, increasing their firing by just 1 Hz (Du et al., 2001). Neural response thresholds to thermal stimuli decreased while thresholds to mechanical stimuli were unaffected. This effect appears to be restricted to smaller diameter fibres, having no detectable effect on Aβ fibres (but data not shown). Peripherally-acting NMDA receptor antagonists block allodynia in a model of neuropathic pain (Christoph et al., 2005). Antagonists for both NMDA and AMPA receptors were also effective at blocking enhanced activity following antidromic stimulation of afferent nerve fibres (Cao et al., 2007). In a Phase II clinical study, the AMPA/kainate antagonist LY293558 given intravenously reduced capsaicin produced inflammatory pain possibly by an action on peripheral nerves, however central effects of the drug limited its clinical usefulness (Sang et al., 1998). Injection of kainate into the skin reduces both thermal and mechanical neural thresholds (Du et al., 2006). Since kainate receptors are presumably activated by glutamate, this latter result seems to be at odds with this group's previous study where no reduction in mechanical thresholds was observed to glutamate (Du et al., 2001). Use of a selective kainate receptor antagonist demonstrated that kainate receptors were involved in nociceptor sensitisation in normal skin and in the early stages of inflammation, and that only AMPA and NMDA receptors were also involved in longer-term inflammation.

Direct application into the skin (1 – 100 nM) of group I mGlu receptor agonists 3,5-Dihydroxyphenylglycine (3,5-DHPG) and 2-Chloro-5-hydroxyphenylglycine (CHPG, mGlu5 selective) were more potent than ionotropic agonists AMPA and NMDA in producing mechanical hyperalgesia (Walker et al., 2001). Group II and III mGlu agonists were ineffective. Pronounced increases (~ 15 Hz) in neural firing from wide dynamic range afferents was observed following local injection into the skin (Walker et al., 2001). These effects, including hyperalgesia, were blocked by a selective mGlu5 receptor antagonist. Inflammatory hyperalgesia produced by paw incision could also be blocked by a mGlu5 receptor antagonist (Zhu et al., 2005), and these findings are supplemented by electrophysiological recordings from nerve fibres responsive to noxious mechanical stimulation (Su and Urban, 2005;Walker et al., 2001). There is some evidence that selective activation of mGlu1 receptors in skin results in reduced mechanical nociceptive thresholds, with no effect on thermal heat thresholds (Zhou et al., 2001). Activation of group I mGlu receptors can cause the release of other inflammatory mediators such as prostaglandins (Hu et al., 2002). Some studies only show antagonist effects if administered before the onset of inflammation, and others show effects after its onset, and the particular results may depend on whether a formalin or carageenan-based inflammation model was used (Karim et al., 2001). Given the disparate results from ionotropic and metabotropic glutamate receptors it appears that these all play some role in peripheral nociception. On current data it is difficult to decide which receptor type predominates functionally. It may be that there is a certain redundancy built into sensory signalling so that failure of one system/receptor is covered by another parallel system/receptor. In addition, there may be interactions between ionotropic and metabotropic glutamate receptor actions, e.g. (Cho et al., 2003).

The effect of agonist activation of group II mGlu receptors contrasts markedly with those for group I mGlu (and ionotropic) receptor activation. Subcutaneous injection of the selective mGlu2/3 receptor agonist (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate reduces mechanical and thermal hyperalgesia and allodynia in inflamed skin (Yang and Gereau, 2002;Yang and

Gereau, 2003). The effect is only seen against inflammation, as neither agonist nor antagonist (LY341495) had any effect on nociceptive thresholds in normal skin. Endogenous activation of these group II mGlu receptors during inflammation is apparent because administration of the antagonist in inflamed skin resulted in a prolongation of allodynia compared with the recovery to normal sensitivity in untreated skin (Yang and Gereau, 2003). This suggests that group II mGlu receptors provide negative feedback control for over-stimulation of nociceptive nerve endings during inflammation.

A preliminary study reported that subcutaneous injection of a group III mGlu receptor agonist had similar effects to group II agonists in reducing hyperalgesia in inflamed skin (Yang et al., 2006).

In summary, these studies indicate that, for nociceptive (small myelinated and unmyelinated) fibres, ionotropic and group I metabotropic glutamate receptor activation leads to inflammation, enhancement of neural firing, and hence hyperalgesia and/or allodynia. In contrast, activation of group II and III mGlu receptors reduces these effects. The colocalization of group I and group II, and of group I and group III, is found among approximately half of smaller diameter DRG cells (Carlton and Hargett, 2007). A small percentage is triple-labelled for group I/II/II receptors. There may be differences in the relative expression of receptors peripherally in the skin compared with DRG, but it seems likely that many terminals in the skin will have glutamate receptors which are both positively and negatively linked to inflammatory nociceptive processes there. This will allow for fine tuning of responses and for negative feedback to control for excessive inflammation.

b) Low threshold mechanoreceptor fibres.

No corresponding studies have been done of the various low threshold slowly and rapidly adapting mechanoreceptor (Aβ) nerve fibres, even though some of these appear to release glutamate and express glutamate receptors. It may be that they play some role in allodynia (see section below on *Corpuscular mechanoreceptors and Schwann cells*), where formerly innocuous tactile stimuli become noxious. It is also possible that glutamate plays a more subtle role in modulation/amplification of tactile sensitivity which cannot easily be measured using current animal behavioural models.

Cells within the Epidermis

A number of different cellular components which have their cell bodies within the epidermis have been implicated in glutamate signalling. Only the most important of these will be reviewed here.

Merkel Cells

Merkel cells are found in the basal layer of the epidermis and have a neural crest origin (Halata et al., 2003). They most often form a close association with nerve terminals of slowly adapting type I large (Aβ) myelinated afferents (Iggo and Muir, 1969). Merkel cells have many functional and structural characteristics only present in sensory cells, such as non-selective ion channels and espins (Meyers et al., 2003;Sekerkova et al., 2004). At least 3

distinct voltage-gated currents have been recorded in isolated Merkel cells (Yamashita et al., 1992) and, more recently, 6 subunits for K^+ and Ca^{2+} ion-channels were found to be preferentially expressed by Merkel cells (Haeberle et al., 2004). Mechanical stimulation of Merkel cells causes increases in intracellular calcium (Chan et al., 1996;Halata et al., 2003;Tazaki and Suzuki, 1998), and triggers calcium induced calcium release (Senok and Baumann, 1997). All these data are consistent with the suggestion that the Merkel cell acts as a mechanotransducer which then signals by chemical transmission to nerve endings of slowly adapting type I afferents. Large dense-core granules (vesicles) in Merkel cells are located adjacent to synapse-like contacts with nerve endings (Hartschuh and Weihe, 1980). It may come as no surprise then that several essential components for a glutamatergic synapse are found here. Merkel cells express presynaptic active-zone molecules and synaptic vesicle proteins (Haeberle et al., 2004;Hitchcock et al., 2004;Nunzi et al., 2004). VGLUT1 and VGLUT2 are generally co-expressed, and expression was densest adjacent to the nerve terminal (Haeberle et al., 2004;Nunzi et al., 2004). A subpopulation of Merkel cells also express VGLUT3, suggesting that they co-release other transmitter besides glutamate, such as serotonin (Nunzi et al., 2004). A functional role for glutamate seemed assured when it was demonstrated that the broad spectrum ionotropic antagonist kynurenate reduced responses of slowly adapting type I mechanoreceptors (Fagan and Cahusac, 2001). Further work implicated a specific NMDA receptor subunit, since only polyamine site blockers (e.g. ifenprodil) and ion channel blockers (e.g. MK 801) were effective (Cahusac et al., 2005). The surprising finding was that the NMDA receptor NR2A/B subunit involved was found not on the nerve terminals as expected, but on the Merkel cells themselves (Cahusac et al., 2005). This added further evidence for a mechanoreceptive role for the Merkel cell, but failed to disclose the chemical transmitter that signals from Merkel cell to nerve terminals. Metabotropic mGlu5 receptors are expressed on Merkel cells (Tachibana et al., 2003) and group I mGlu receptor antagonists have an excitatory effect on slowly adapting type I unit activity (Cahusac and Senok, 2006). Since there is evidence that glutamate may be released by Merkel cells (see above), together these data suggest that there are glutamate autoreceptors (both ionotropic and metabotropic) which modulate the mechanosensitivity or the output of Merkel cells, so providing negative feedback control. Another source of glutamate is from the nerve endings associated with Merkel cells, and suggest a reciprocal synapse (Mihara et al., 1979). Finally, there may be bi-directional glutamate signalling with other neighbouring cells, such as keratinocytes (see below) and Langerhans cells. A particularly close relationship between the latter and Merkel cells has been observed in hair follicles (Narisawa, 2003).

Corpuscular Mechanoreceptors and Schwann Cells

Like Merkel endings, Pacinian corpuscles are innervated by large (Aβ) myelinated afferents. They are responsible for rapidly adapting type II afferent responses. The nerve terminals are encapsulated by concentric layers of flattened lamellae derived from Schwann cells. Cultured Schwann cells have been shown to release glutamate (Jeftinija and Jeftinija, 1998). The inner core lamellae (near the nerve terminal) have voltage-gated Na^+ and K^+ channels (Pawson et al., 2000;Pawson and Bolanowski, 2002). A recent study has found that the both the lamellae and the nerve terminals express VGLUT1 and mGlu1 receptors (among other proteins) (Pawson et al., 2007). Furthermore, the nerve terminals contain clear vesicles.

This means that glutamate released by either or both elements (terminals and lamellae) can bind to receptors on either or both elements. As well as glutamate signalling from lamellae to nerve terminal (which may modulate the mechanotransduction process), such an arrangement allows for a reciprocal 'synapse' from nerve ending to lamellae. It also accommodates autoreceptors on each structure. If a glutamate signalling role for lamellae is established, then the classical view that lamellae merely act as a mechanical high pass filter will need to be revised. Pharmacological experiments with selective receptor agonists and antagonists may unravel which of the various signalling scenarios are functionally important.

The other rapidly adapting mechanoreceptor in the skin, the Meissner corpuscle, is co-innervated by unmyelinated C fibre afferent terminals, in addition to Aβ endings (Paré et al., 2001). It is thus possible that glutamate signalling may occur between these afferent endings and give rise to mechanical allodynia.

Keratinocytes

Keratinocytes are the major type of cell found in the epidermis. In normal skin, basal epidermal keratinocytes express NMDA, AMPA and metabotropic glutamate receptors, and glutamate transporters EAAC1 and GLT-1 (Genever et al., 1999;Morhenn et al., 1994;Nahm et al., 2004). The different receptors are differentially distributed. The NMDA (NR1 and NR2A/B subunits) receptors are expressed uniformly over keratinocytes, while AMPA (GluR1 and GluR2, but not GluR1 subunits) receptors are concentrated on the basal surfaces of these cells. mGlu1 receptors are also abundant, but absent in some basal keratinocytes, while mGlu2/3 receptors are restricted to discrete clumps of cells. During healing following full-thickness wounds in skin, the NR1 receptor subunit and EAAC1 increased throughout suprabasal keratinocytes layers. Other glutamate receptor levels were unchanged. During embryo-genesis (E14) in rats, the NR1 receptor subunit is evenly expressed in keratinocytes throughout basal and suprabasal layers. However, by E18 NR1 expression is restricted to basal layer keratinocytes. The NMDA receptor blocker MK 801 prevented cultured keratinocytes from forming monolayer colonies, suggesting a functional role for these receptors (Genever et al., 1999). MK 801 also decreases keratinocyte growth and yet increases apoptosis, implying a link to psoriasis (Morhenn et al., 2004). Glutamate may be released and actively recycled by keratinocytes and other epidermal cells including dermal fibroblasts (Cooper et al., 1998). Release from afferent nerve fibres (as detailed above) could also provide a source of glutamate, consistent with the observation that denervation inhibits keratinocyte proliferation (Hsieh and Lin, 1999). In summary, studies suggest glutamate signalling occurs among keratinocytes and neighbouring cellular elements. Signalling may involve a number of mechanisms, including second messenger pathways, Ca^{2+} signalling and detection, and ion fluxes through membrane channels. Skin disorders, such as psoriasis (Fischer et al., 2004), keloid formation, harlequin ichthyosis and squamous cell carcinomas (Nahm et al., 2004), may be amenable to topical application of drugs acting at glutamate receptors.

Melanocytes

Melanocytes occupy the basal layer of the epidermis, and also like Merkel cells, are derived from the neural crest. AMPA (GluR2 and GluR4 subunits) receptors, NMDA (NR2A and NR2C subunits) receptors, and possibly mGlu1α receptors are expressed by cultured human melanocytes (Hoogduijn et al., 2006). There is some disagreement with other investigators over the particular receptors expressed, e.g. (Frati et al., 2000;Hoek et al., 2004). Specific glutamate transporters (GT-1 and GT-3) and decarboxylases are also expressed by these cells, although it was not possible to demonstrate glutamate release. In some cells intracellular Ca^{2+} was increased by infusion with AMPA and NMDA. A functional role for the ionotropic receptors is suggested by the rapid (and reversible) morphological changes to melanocytes on exposure to NMDA and AMPA receptor antagonists. Release of glutamate from keratinocytes would allow signalling between these different types of cells. The crucial melanocyte differentiation and proliferation factor MiTF is reduced by blockade of AMPA receptors (Hoogduijn et al., 2006). Reduced activation of MiTF *in vivo* could lead to the depigmentation disorder vitiligo (where melanocytes stop producing melanin) or to the development of melanoma. Aberrant expression of the mGlu1 receptor has been associated with the spontaneous development of melanoma (Pollock et al., 2003). Human melanoma cells display elevated release of glutamate, and treatment with mGlu1 receptor antagonists suppresses cell proliferation and decreases extracellular glutamate (Namkoong et al., 2007). Treatment with a glutamate release inhibitor resulted in significant inhibition of tumour growth.

Conclusion

Many of the principal cells of the skin appear to utilize some form of glutamate signalling. The most compelling evidence is available for the afferent innervation of the skin. Increasing our fundamental knowledge here could lead to more effective treatments for peripheral pain and inflammation. Less research has been done on low threshold mechanoreceptors (Merkel nerve endings, Ruffini endings, Meissner and Pacinian corpuscles). Further work on keratinocytes and melanocytes has the potential to lead to improved topical treatments for a number of skin ailments, ranging from psoriasis through verrucae to melanoma. Topical application of drugs to the skin would have several advantages over systemic application, notably: fewer side-effects, a lower dose requirement, and more targeted action.

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Chapter 5

Regulatory Mechanism of Plasticity at $GABA_A$ Receptor-Mediated Inhibitory Synapses

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Abstract

The neuronal activity modulates the efficacy of synaptic transmission at both excitatory and inhibitory synapses in the central nervous system. Such an activity-dependent synaptic plasticity has been regarded as a cellular basis for learning and memory. Since balanced regulation of excitation and inhibition is critical for the information processing, clarification of detailed mechanism to control inhibitory synaptic plasticity is important. Fast neuronal inhibition is mainly mediated by gamma-amino butyric acid receptor type A ($GABA_AR$). In this article, we summarize recent advances regarding the regulatory molecular mechanism of plasticity at $GABA_AR$-mediated synapses. We shortly review several forms of plasticity at GABAergic synapses in various brain regions, and then focus on synaptic plasticity in the cerebellum. At the GABAergic synapses on a Purkinje neuron, four forms of synaptic plasticity have been reported. They are short-term potentiation or depression of presynaptic release probability of synaptic vesicles, long-term potentiation of postsynaptic $GABA_AR$ responsiveness and long-term depression. We discuss how different patterns of neuronal activity leads to different forms of synaptic plasticity, what kind of intracellular signaling cascades including protein kinases/phosphatases control the induction of plasticity, and how $GABA_AR$ binding proteins are implicated in modification of $GABA_AR$ function.

Introduction

In the central nervous system, the amino acid neurotransmitter GABA (γ-amino butyric acid) mediates inhibitory synaptic transmission predominantly. GABA is synthesized from glutamate by two isoforms of glutamic acid decarboxylase (GAD), GAD65 and GAD67

(Erlander et al., 1991; Esclapez et al., 1994; Soghomonian & Martin, 1998). The action of GABA is mediated by two types of receptors. One is ionotropic $GABA_A$ receptor ($GABA_AR$) consisting of five subunits forming a central pore, which mediates fast (~tens of msec) inhibitory synaptic transmission (Unwin, 1993; Barnard et al., 1998). The other is metabotropic $GABA_B$ receptor ($GABA_BR$) with seven-transmembrane segments which causes slow response (~seconds) through heterotrimeric G protein (Kaupmann et al., 1997 and 1998; White et al., 1998; Jones et al., 1998; Kuner et al., 1999). $GABA_AR$ inhibits neuronal excitation through hyperpolarization and/or shunting the plasma membrane conductance by permeating Cl^-. On the other hand, $GABA_BR$ activates intracellular second messenger cascade and inhibits neuronal excitability relatively slowly through opening a type of K^+ channels (GIRK) (Misgeld et al., 1995). There are at least 19 subunits (α1-6, β1-3, γ1-3, δ, ε, ρ1-3, θ, π) which compose a $GABA_AR$ hetero-pentamers (Rabow et al., 1995; Davies et a., 1997; Hedblom & Kirkness, 1997; Bonnert et al., 1999; Whiting, 1999; Moss & Smart, 2001). Each $GABA_AR$ subunit has 4 transmembrane segments with the large N- and the tiny C-terminal extracellular regions (Moss & Smart, 2001). The most predominant subunit composition of $GABA_AR$ is $2\alpha1, 2\beta2$ and $1\gamma2$, followed by $2\alpha2, 2\beta3$ and $1\gamma2$, and $2\alpha3$, $2\beta3$ and $1\gamma2$ (Fritschy & Brunig, 2003; Mohler, 2006). GABA binds to the interface of α and β subunits (Mohler, 2006). The homomeric pentamer consisting of only ρ subunits, which is sometimes called $GABA_C$ receptor ($GABA_CR$) is expressed almost exclusively in the retina (Cutting et al., 1991; Lukasiewicz, 1996; Moss & Smart, 2001). $GABA_CR$ is insensitive to bicuculline, a selective antagonist for other $GABA_AR$. $GABA_AR$ is a target of clinically relevant drugs including benzodiazepines, barbiturates, neurosteroids, anesthetics and alcohol (Mohler, 2006). The binding sites of benzodiazepines are thought to lie at α and γ subunits. Altered expression and/or function of $GABA_AR$ has been related to neurological or psychiatric disorders such as epilepsy, ischemia, schizophrenia and depression (Duncan, 1999; Olsen et al., 1999; Lewis, 2000; Coulter, 2001; Nutt & Malizia, 2001; Blum & Mann, 2002; Fritschy and Brunig, 2003). Thus, regulation of $GABA_AR$ is critical for the normal function of central nervous system.

The synaptic transmission efficacy is modulated by the neuronal activity in the central nervous system, which has been regarded as a cellular basis for learning and studied extensively at excitatory synapses (Malenka and Nicoll, 1999; Bailey et al., 2000; Ito, 2001; Lisman and Zhabotinsky, 2001; Hansel et al., 2001; Kandel 2001). In the hippocampal CA1 region, repetitive activation of presynaptic neurons induces long-term potentiation (LTP) or depression (LTD) of transmission efficacy through activation of NMDA-type glutamate receptor (Bliss and Lomo, 1973; Bliss and Collingridge, 1993; Malenka, 1994). Whether LTP or LTD is induced is determined by the pattern (such as frequency) of presynaptic neuronal activity. When NMDA receptor is activated, the concentration of intracellular Ca^{2+} increases, activating signaling pathways including protein kinases and phosphatases such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein phosphatase 2B (also called calcineurin) (Malenka, 1994). As a result, the level of protein phosphorylation is altered, and the properties and/or surface number of AMPA-type glutamate receptors change (Hayashi et al., 2000; Lee et al., 2000; Sheng and Kim, 2002). In this way, neuronal activity modulates the excitatory synaptic transmission depending on the history of synaptic usage.

On the other hand, studies on the plasticity at inhibitory synapses have been limited. Usually, inhibitory synaptic input by itself does not excite a neuron. In juvenile the

equilibrium potential for Cl^- is more positive than in mature, and $GABA_AR$ activation leads to depolarization rather than hyperpolarization. However, this excitatory action of $GABA_AR$ is seen only in early developmental stages. During development the intracellular Cl^- concentration decreases due to the increased expression of Cl^- transporter KCC2 (Ganguly et al., 2001). Nevertheless, the use-dependent plasticity has been reported at various inhibitory synapses (Kano, 1995; Marty and Llano, 1995; Komatsu, 1996; Oda et al., 1998; Nusser et al., 1998; Aizenman et al., 1998). In some cases, heterosynaptic excitatory synaptic inputs trigger plasticity of inhibitory synaptic transmission (Kano et al., 1992; Marsicano et al., 2002; Chevaleyre & Castillo, 2003).

This review describes recent advances in the study on regulatory mechanism of GABA-mediated inhibitory synaptic transmissions in the central nervous system.

Activity-Dependent Regulation of GABAergic Synaptic Transmission

LTP and LTD are induced at inhibitory synapses in various regions of the central nervous system such as the hippocampus, cerebral cortex, cerebellum, deep cerebellar nucleus, brain stem, and lateral superior olive (Komatsu, 1994 and 1996; Kano, 1995; Marty and Llano, 1995; Nusser et al., 1998; Aizenman et al., 1998; Gaiarsa et al., 2002). LTP at glycinergic synapses on a Mauthner cell plays a critical role in the escape behavior of a goldfish (Oda et al., 1998). The increase in intracellular Ca^{2+} concentration triggers plasticity not only at excitatory synapses but also at inhibitory synapses (Gaiarsa et al., 2002). LTP or LTD is induced at inhibitory synapses in the hippocampus depending on the level of intracellular Ca^{2+} increase brought about by glutamatergic synaptic inputs (Caillard et al., 1999a,b; Wang and Stelzer, 1996; Lu et al., 2000). The Ca^{2+} influx through voltage-gated Ca^{2+} channels and/or NMDA receptors contribute to the Ca^{2+} increase (Figure 1). Similarly, at the GABAergic synapses on a cerebellar Purkinje neuron, excitatory input from a climbing fiber, which strongly depolarizes the Purkinje neuron and causes large increase in the intracellular Ca^{2+} concentration through opening of voltage-gated Ca^{2+} channels, induces LTP of inhibitory synaptic transmission, which is called rebound potentiation (RP) (Kano et al., 1992). The Ca^{2+} release from the intracellular store (endoplasmic reticulum, ER) caused by activation of metabotropic glutamate receptor type 1 (mGluR1) also contributes to the induction of RP (Figure 1) (Hashimoto et al., 1996). At inhibitory synapses in the visual cortex, $GABA_BR$ activation causes the Ca^{2+} release from ER, resulting in the LTP induction (Figure 1) (Komatsu, 1994 & 1996). Thus, the neuronal activity-dependent intracellular Ca^{2+} increase seems to be the key mechanism for induction of plasticity at both inhibitory and excitatory synapses.

At GABAergic synapses on a deep cerebellar nuclei neuron, another mechanism works to induce plasticity (Morishita & Sastry, 1993 & 1996; Aizenman et al., 1998; Ouardouz & Sastry, 2000). After the hyperpolarization caused by burst spiking of a presynaptic Purkinje neuron, a postsynaptic cerebellar nuclear neuron shows rebound depolarization (Aizenman et al., 1998). LTP or LTD is induced by this rebound depolarization depending on its intensity and duration. The postsynaptic Ca^{2+} increase is required for both LTP and LTD. This inhibition-triggered rebound depolarization is ascribed to synchronous opening of the low-

threshold voltage-gated Ca^{2+} channel. It was postulated that when the rebound depolarization is below the threshold of postsynaptic firing, moderate Ca^{2+} increase occurs and LTD is induced. On the other hand, when the depolarization is large enough to reach the threshold of action potential generation, it backpropagates into dendritic arbors, triggering the Ca^{2+} influx through high-threshold Ca^{2+} channel, resulting in the LTP induction. Here, the effect of inhibitory synaptic input is not simply inhibitory. In this case, the intrinsic properties of excitable neuronal membrane enable inhibitory synaptic transmission-mediated induction of plasticity (Figure 1).

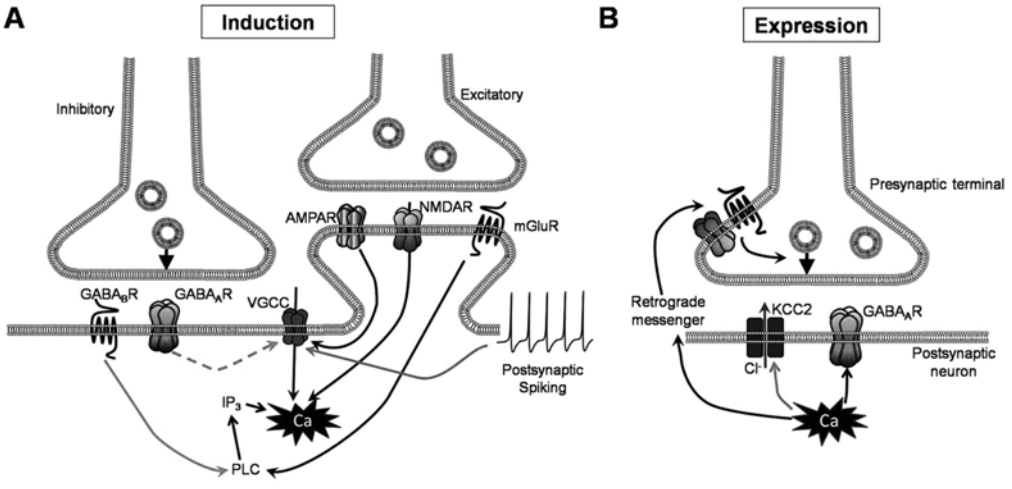

Figure 1. Induction and expression of inhibitory synaptic plasticity. A, Homosynaptic activation, heterosynaptic excitatory inputs and/or neuronal firing, trigger the induction of synaptic plasticity. Increase in the intracellular Ca^{2+} concentration plays an essential role in most forms of synaptic plasticity if not all. Excitatory heterosynaptic inputs leads to the intracellular Ca^{2+} increase through activation of NMDAR and/or voltage-gated Ca^{2+} channel (VGCC) in the hippocampus and the cerebellum. VGCC is activated by AMPA-type of glutamate receptor and/or backpropagating postsynaptic action potential. $GABA_AR$ activation leads to opening of VGCC during rebound depolarization in the deep cerebellar nucleus neurons. Group 1 mGluR activates PLC and then causes Ca^{2+} release from the internal store. In the visual cortex, $GABA_BR$ also activates PLC. B, Expression mechanism of inhibitory synaptic plasticity. Postsynaptic increase in the intracellular Ca^{2+} concentration leads to modulation of function and/or surface number of postsynaptic $GABA_ARs$ in the cerebellum and hippocampus. It also changes driving force of Cl^- ion flux through regulation of Cl^- transporter KCC2 in the hippocampus. The postsynaptic Ca^{2+} increase also affects the probability of GABA release from the presynaptic terminal via retrograde messengers.

High frequency stimulation (typically at 50 - 100 Hz) of presynaptic nerve fibers has been used as the conditioning to induce plasticity at both inhibitory and excitatory synapses (Bekkers and Stevens, 1990; Bliss & Collinglidge, 1993; Nicoll & Malenka, 1995; Linden and Connor, 1995: Deisseroth et al., 1996; Gaiarsa et al., 2002). About a decade ago, more physiological neuronal activity was found sufficient for triggering the induction of plasticity (Debanne et al., 1996; Markram et al., 1997; Bi & Poo, 1998). Correlated spiking of pre- and postsynaptic neurons at several Hz induces LTP or LTD at both excitatory and inhibitory synapses (Holmgren & Zilberter, 2001; Gaiarsa et al., 2002). This form of synaptic plasticity was named as spike-timing dependent plasticity (STDP), because the temporal order of pre-

and postsynaptic neuronal firings determines the polarity of plasticity. When the presynaptic neuronal firing precedes the postsynaptic firing, LTP is induced. On the other hand, LTD is induced when firing of the presynaptic neuron comes after the postsynaptic firing. Thus, it seems that the synaptic input strong enough to evoke the postsynaptic firing is strengthened, and that failed to contribute to the postsynaptic firing is weakened. This form of regulation was first proposed as a fundamental mechanism for learning and memory by Hebb in 1949, and was experimentally demonstrated about 50 years later. At the GABAergic synapses in the entorhinal cortex, STDP similar to that at excitatory synapses takes place after pairing of pre- and postsynaptic action potentials at 2 Hz for 5 minutes (Haas et al., 2006). Presynaptic stimulation earlier than postsynaptic firing induces LTP, whereas that delaying to postsynaptic firing induces LTD. Both LTP and LTD are induced depending on the Ca^{2+} influx through L-type of Ca^{2+} channel. Inhibitory synapses on a CA1 pyramidal neuron in the hippocampus also exhibit STDP with different temporal characteristics from that at excitatory synapses (Holmgren and Zilberter, 2001). Interestingly, neither LTP nor LTD is induced by the postsynaptic action potential firing alone, suggesting the important role of presynaptic activity, although the mechanism has not been clarified. Fast postsynaptic chelating of intracellular Ca^{2+} by BAPTA inhibits both LTP and LTD, while slow Ca^{2+} chelating by EGTA suppresses LTD but not LTP, suggesting the differential Ca^{2+} requirement for the induction of LTP and LTD.

Expression Mechanism of Inhibitory Synaptic Plasticity

Modification of inhibitory synaptic transmission efficacy has been ascribed to alteration of either the presynaptic transmitter release probability or the postsynaptic responsiveness to GABA. In some cases, synaptic plasticity at inhibitory synapses is induced by the postsynaptic Ca^{2+} increase and is accompanied with alteration of the frequency of spontaneous or miniature inhibitory synaptic currents, suggesting the change of presynaptic transmitter release probability (Llano et al., 1991; Pitler & Alger, 1992; McLean et al., 1996; Caillard et al., 1999a,b and 2000; Gubellini et al., 2001). Thus, the sites of induction and expression of plasticity are post- and presynaptic, respectively. To account for this situation, the retrograde messenger molecule released from a postsynaptic neuron and acting on presynaptic terminals has been presumed. At inhibitory synapses in the basolateral amygdala and the hippocampus, LTD caused by heterosynaptic excitatory inputs is mediated by endocannabinoid released from a postsynaptic neuron (Marsicano et al., 2002; Chevaleyre & Castillo, 2003). The endocannabinoid release depends on the postsynaptic activation of mGluR1. Endocannabinoid acts on CB1R at the GABAergic presynaptic terminal. The endocannabinoid-mediated retrograde signaling underlies multiple forms of downregulation of synaptic transmission at both inhibitory and excitatory synapses (Wilson & Nicoll, 2001; Ohno-Shosaku et al., 2001; Kreitzer & Regehr, 2001a,b; Diana et al., 2002; Yoshida et al., 2002; Bodor et al., 2005; Melis et al., 2004; Trettel & Levine, 2003; Yanovsky et al., 2003; Isokawa & Alger, 2005; Di et al., 2005; Marsicano et al., 2002; Chevaleyre & Castillo, 2003; Chevaleyre et al., 2006). We also know that the intracellular Ca^{2+} increase potentiates or depresses the postsynaptic $GABA_AR$ responsiveness in other cases (Stelzer et al., 1994; Wang & Stelzer, 1996; Lu et al., 2000; Morishita & Sastry, 1993 & 1996; Aizenman et al., 1998; Ouardouz & Sastry, 2000; Nusser et al., 1998; Kano et al., 1992 & 1996; Kawaguchi &

Hirano, 2000). Alteration of the number and/or properties of postsynaptic $GABA_AR$s on the plasma membrane underlie synaptic plasticity at some inhibitory synapses.

In a hippocampal neuron, repeated firing of action potentials shifts the reversal potential of GABA-induced Cl^- currents to a positive value and reduces inhibitory action of GABA (Fiumelli et al., 2005). This phenomenon is also brought about by the intracellular Ca^{2+} increase, that downregulates K^+-Cl^- cotransporter KCC2. Suppression of KCC2 function downstream of the spike-triggered Ca^{2+} influx seems to be mediated by protein kinase C (PKC). To summarize, alteration of the presynaptic transmitter release probability, the postsynaptic $GABA_AR$ number/properties, and the driving force for Cl^- flux through $GABA_AR$, are the expression mechanisms for plasticity at inhibitory synapses (Figure 1).

Mechanism to Regulate $GABA_AR$ by Neuronal Activity

Neuronal activity activates Ser/Thr and Tyr kinases including PKC, PKA, CaMKII, protein kinase G (PKG), Akt/PKB, and Src. These protein kinases phosphorylate $GABA_AR$ at the amino acid residues in the cytoplasmic region between 3rd and 4th transmembrane segments (Smart, 1997; Moss & Smart, 1996; Brandon et al., 2002a; Song & Messing, 2005). The functional consequences of $GABA_AR$ phosphorylation are divergent depending on the subunits composition, the kinase and the cell type. CaMKII–mediated phosphorylation of $GABA_AR$ composed of $\alpha1\beta3\gamma2$ but not that of $\alpha1\beta2\gamma2$ augments the responsiveness in HEK293 cells and in cerebellar granule neurons (Houston & Smart, 2006). PKC-mediated phosphorylation of $\beta1$-3 and $\gamma2$ subunits down-regulates the receptor function in both heterologous expression system such as HEK293 cells and *Xenopus* oocyte, and cortical neurons (Kellenberger et al., 1992; Krishek et al., 1994; Brandon et al., 2002a,b; Song & Messing, 2005). On the other hand, phosphorylation of $\beta1$ and $\gamma2$ subunits by catalytic domain of PKC enhances the receptor function in hippocampal neurons and in fibroblasts (Lin et al., 1994 and 1996; Poisbeau et al., 1999; Jovanovic et al., 2004). $GABA_AR$ phosphorylation by PKA also exerts diverse effects on the receptor function (Porter et al., 1990; Kano and Konnerth, 1992; Moss et al., 1992; McDonald et al., 1998; Poisbeau et al., 1999; Nusser et al., 1999; Flores-Hernandez et al., 2000; Brandon et al., 2002a). It has also been suggested that $GABA_AR$ phosphorylation by tyrosine kinases including Src augments the receptor function (Moss et al., 1995; Boxall, 2000), although no apparent effect of Src was reported in another study (Kawaguchi and Hirano, 2006). Thus, the effects of phosphorylation of $GABA_AR$ seem to be determined by the subunits composition, the kinase and the individual neuron-specific mechanism, and there is no consensus rule for the functional consequence of $GABA_AR$ phosphorylation. The activity of protein phosphatases such as protein phosphatase 1 (PP-1), protein phosphatase 2A (PP-2A) and protein phosphatase 2B (calcineurin) is also regulated by the neuronal activity. Calcineurin is involved in LTD at inhibitory synapses in the hippocampus (Wang et al., 2003). Taken together, neuronal activity-dependent regulation of $GABA_AR$ phosphorylation is a candidate mechanism for synaptic plasticity, although the detail is enigmatic.

Proteins directly interacting with $GABA_AR$ have been identified. They are GABARAP ($GABA_A$ receptor-associated protein), PRIP (phospholipase C-related, but catalytically inactive protein), AP2 (adapter protein 2 complex), GODZ (Golgi-specific DHHC zinc finger protein), BIG2 (brefeldin A-inhibited GDP/GTP exchange factor 2), Plic-1, HAP1

(huntingtin-associated protein 1), AKAP (A-kinase-associating protein), RACK1 (receptor for activated C kinase), and Src (Wang et al., 1999; Bedford et al., 2001; Kanematsu et al., 2002; Chen and Olsen 2007). These proteins regulate the intracellular trafficking, endocytosis, and/or properties of $GABA_AR$. For example, AP2 association with $GABA_ARs$ is tightly coupled to the clathrin-dependent endocytosis of $GABA_AR$ (Kittler et al., 2000). GABARAP plays a role in the intracellular trafficking and targeting of $GABA_AR$ toward the plasma membrane through association with γ2 subunit (Kneussel et al., 2000; Kittler et al., 2001; Moss and Smart, 2001; Kneussel, 2002; Nymann-Andersen et al., 2002; Leil et al., 2004; Luscher and Keller, 2004; Chen and Olsen, 2007; Kanematsu et al., 2007). $GABA_AR$ properties such as single channel conductance are also modulated by GABARAP binding (Chen et al., 2000: Everitt et al., 2004; Luu et al., 2006). PRIP interacts with GABARAP and with $GABA_AR$ β subunits, and seems to be implicated in trafficking of $GABA_AR$ (Kanematsu et al., 2002; Terunuma et al., 2004; Kanematsu et al., 2007). Furthermore, PRIP directly binds to PP-1 and PP-2A, regulating the phosphorylation level of $GABA_AR$ (Yoshimura et al., 2001; Kanematsu et al., 2006). Association of Plic-1 with α2, α3, α6, and/or β subunits facilitates the intracellular receptor recycling, leading to the increase in number of cell surface $GABA_ARs$ (Bedford et al., 2001). Thus, it seems that $GABA_AR$-associating proteins play some roles in synaptic plasticity, although the detailed mechanism has not been clarified. Recently, it was shown that GABARAP plays a pivotal role in the plasticity of $GABA_AR$ function in a cerebellar Purkinje neuron, which is discussed in detail below.

Functional Relevance of Modulation of Inhibitory Synaptic Transmission

Although synaptic plasticity at excitatory synapses has been closely related to learning and memory, the role of inhibitory synaptic plasticity *in vivo* remains largely unknown. The long-lasting potentiation of glycinergic synaptic transmission to a Mauthner cell in goldfish contributes to the behavioural adaptation (Oda et al., 1998). Excitation of Mauthner cells by sounds causes escape reaction to avoid a menace. LTP of glycinergic synaptic transmission is induced by the conditioning sound that is below the threshold for escape reaction. As a result, the probability of escape reaction occurrence decreases, suggesting that the augmented glycinergic inhibition of Mauthner cells is responsible for the behavioural habituation to the sensory stimuli. The inhibitory synaptic inputs exert powerful effect on the firing activity of a postsynaptic neuron. Alteration of inhibitory synaptic transmission efficacy should modulate the output pattern of neuronal networks as well as excitatory one.

GABA-mediated inhibition is also critical for the developmental plasticity of the sensory nervous system. In the primary visual cortex, formation and plasticity of ocular dominance columns is regulated by $GABA_AR$-mediated inhibitory synaptic transmission. At birth, the thalamic inputs from both eyes to the layer IV of visual cortex are overlapping. As development proceeds, the area receiving excitatory signals from each eye separates, and the ocular dominance columns are formed. This remodeling process is highly plastic. Deprivation of visual input from one eye in a juvenile animal affects column formation (Wiesel and Hubel, 1963). This plastic change of the neuronal circuits occurs only during the early postnatal development, which is called a critical period. Enhancement of α1 subunit containing $GABA_AR$-mediated inhibition in the visual cortex by infusion of benzodiazepine induces the premature onset of critical period (Fagiolini and Hensch, 2000; Fagiolini et al.,

2004). On the other hand, diminished GABA-mediated transmission in a GAD65 knockout mouse abolishes the critical period (Hensch et al., 1998; Iwai et al., 2003). The onset of critical period is accompanied with alteration of the number and single channel conductance of $GABA_A$Rs in the layer 2/3 neurons (Katagiri et al., 2007). Thus, the cortical network remodeling is controlled by the GABAergic transmission.

Synaptic Plasticity at GABAergic Synapses on a Cerebellar Purkinje Neuron

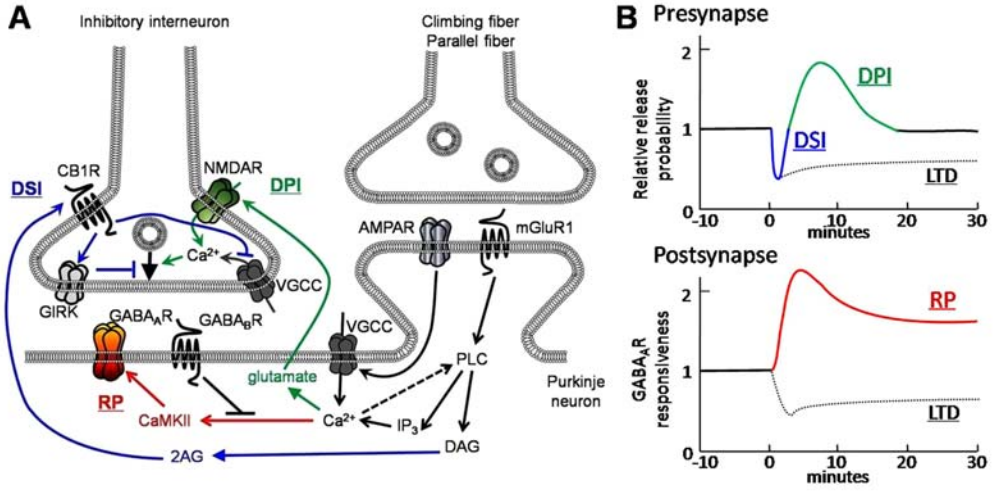

Figure 2. Four forms of plasticity occurring at inhibitory synapses on a cerebellar Purkinje neuron. A, Induction mechanism of DSI, DPI, and RP. B, Time courses of changes of presynaptic release probability and postsynaptic $GABA_A$R responsiveness during DSI, DPI, RP and LTD. How LTD is expressed is not known. DSI is induced as follows. The postsynaptic intracellular Ca^{2+} increase and/or mGluR1 activation caused by excitatory inputs from a climbing fiber and/or parallel fibers produces diacylglycerol (DAG), which is broken down to 2AG by DAG lipase. 2AG reaches the presynaptic terminal and activates CB1R, downregulating the release probability of synaptic vesicles through activation of GIRK and inhibition of VGCC. In the DPI induction, the intracellular Ca^{2+} increase leads to the glutamate release from the postsynaptic Purkinje neuron by an unknown mechanism. The glutamate activates the presynaptic NMDAR, that augments presynaptic release machinery for about 10 minutes. On the other hand, the postsynaptic responsiveness of $GABA_A$R is potentiated for more than tens of minutes after the large increase in postsynaptic intracellular Ca^{2+}, leading to RP. Homosynaptic activity suppresses RP through the postsynaptic $GABA_B$R activation. Thus, dynamic regulation of pre- and postsynaptic function at inhibitory synapses on a Purkinje neuron is brought about by glutamatergic heterosynaptic inputs and the inhibitory presynaptic activity.

Hereafter, we describe the regulatory mechanism of inhibitory synaptic transmission to a cerebellar Purkinje neuron, in which four forms of synaptic plasticity have been reported (see Figure 2) (Llano et al., 1991; Kano et al., 1992; Duguid and Smart, 2004; Mittmann & Hausser, 2007). In the molecular layer of the cerebellar cortex, there are two types of inhibitory interneurons, stellate and basket neurons receiving excitatory inputs from parallel

fibers of granule neurons. Stellate and basket neurons form GABAergic synapses on dendrites and the soma of a Purkinje neuron, respectively. Collaterals of a Purkinje neuron axon also form GABAergic synapses on different Purkinje neurons. The four types of synaptic plasticity reported at the GABAergic synapses on a Purkinje neuron are depolarization-induced suppression of inhibition (DSI), depolarization-induced potentiation of inhibition (DPI), RP, and long-term depression (LTD) (Llano et al., 1991; Kano et al., 1992; Duguid & Smart, 2004; Mittmann & Hausser, 2007). All of them are triggered by activation of a climbing fiber that causes potent depolarization and the resultant increase in intracellular Ca^{2+} concentration in a Purkinje neuron. DSI and DPI are short-term plasticity accompanied by the altered probability of presynaptic transmitter release, and each induction is mediated by a retrograde messenger molecule released from the postsynaptic Purkinje neuron (Llano et al., 1991; Duguid & Smart, 2004). RP is expressed as postsynaptic alteration of $GABA_AR$ properties (Kano et al., 1992; Kawaguchi & Hirano, 2000). Finally LTD is induced when the presynaptic inhibitory interneuron and the climbing fiber are stimulated simultaneously at 1 Hz for 5 minutes (Mittmann & Hausser, 2007), although the mechanism of LTD induction is not known.

Depolarization-Induced Suppression of Inhibition (DSI)

DSI was first reported by Llano and colleagues in 1991. Postsynaptic depolarization induces short-term (lasting for several tens of seconds) depression of GABAergic synaptic transmission. This transient depression is triggered by the postsynaptic Ca^{2+} increase and expressed as the decrease in transmitter release from the presynaptic terminals (Figure 2). Thus, involvement of a retrograde messenger is presumed. DSI is also reported at GABAergic synapses on a hippocampal CA1 pyramidal neuron (Diana and Bregestovski, 2005; Chevaleyre et al., 2006). Similar decrease of transmitter release also occurs at the excitatory synapses in the hippocampus, which was named depolarization-induced suppression of excitation (DSE) (Chevaleyre et al., 2006). Discovery of DSI and DSE prompted the search for retrograde messenger molecules. The candidate molecules were nitric oxide (NO), endocannabinoid and glutamate (Fitzsimonds and Poo, 1998; Chevaleyre et al., 2006; Glitsh et al., 1996). Among these, endocannabinoid was shown to play a critical role in both DSI and DSE (Chevaleyre et al., 2006). 2-arachidonoylglycerol (2-AG) and anandamide (AEA) have been identified as endocannabinoid. Both 2-AG and AEA are produced in neuronal activity-dependently (Chevaleyre et al., 2006). 2-AG production is mediated by PLC and diacyl glycerol (DAG) lipase. Most studies supported the Ca^{2+} dependence of endocannabinoid-mediated DSI and DSE. Increase in the intracellular Ca^{2+} concentration activates or facilitates PLC (Allen et al., 1997; Rebecchi and Pentyala, 2000; Kouchi et al., 2004; Hashimotodani et al., 2005). Activation of PLC produces DAG, which is catalyzed by DAG lipase, resulting in the release of 2-AG from a Purkinje neuron. There are two endocannabinoid receptors CB1R and CB2R. Both of them are seven-transmembrane receptors coupled to heterotrimeric G proteins. CB1R is located on the presynaptic terminal. CB1R activation by endocannabinoid downregulates the voltage-gated Ca^{2+} channel and increases the K^+ conductance through GIRK channel (Chevaleyre et al., 2006). Thus, CB1R suppresses depolarization of a presynaptic terminal and reduces Ca^{2+} influx, depressing the transmitter release.

Production of 2-AG is also triggered by activation of the metabotropic glutamate receptor type 1 (mGluR1)-mediated DAG production through PLC (Chevaleyre et al., 2006). PLC activation also produces IP_3, which binds to IP_3 receptor on ER, causing the Ca^{2+} release to cytosol. Thus, mGluR1 activation has dual effects on the pathway to produce 2-AG; the direct DAG production and the intracellular Ca^{2+} increase that facilitates the PLC activity. Depolarization and mGluR1 activation in a Purkinje neuron cooperatively bring about 2-AG production by their synergistic contribution to the Ca^{2+} increase and DAG production, leading to DSI and DSE (Maejima et al., 2005).

Depolarization-Induced Potentiation of Inhibition (DPI)

DPI is short-term (lasting for about 10 minutes) potentiation of inhibitory synaptic transmission triggered by depolarization of the postsynaptic Purkinje neuron through increase in the intracellular Ca^{2+} concentration (see Figure 2) (Duguid and Smart, 2004). DPI is accompanied by the increased transmitter release from a presynaptic terminal, suggesting that DPI also depends on the retrograde messenger released from a Purkinje neuron. The retrograde messenger other than endocannabinoid released from a Purkinje neuron is glutamate. The glutamate release from a Purkinje neuron was first suggested by Marty and colleagues (Glitsh et al., 1996). Duguid and Smart (2004) found that DPI is induced depending on activation of NMDA-type of glutamate receptor which is located on presynaptic terminals of an inhibitory interneuron. They suggested that glutamate released from the postsynaptic Purkinje neuron in response to depolarization acts on the presynaptic NMDA receptor causing the intracellular Ca^{2+} increase. The detailed molecular mechanism how the Ca^{2+} increase in a presynaptic terminal leads to augmentation of release probability of synaptic vesicles for more than ten minutes remains to be clarified.

Rebound Potentiation (RP)

Postsynaptic depolarization such as that caused by repetitive activation of a climbing fiber potentiates the efficacy of inhibitory synaptic transmission in a Purkinje neuron for longer than tens of minutes (Kano et al., 1992). This long-lasting enhancement of GABAergic synaptic transmission was named rebound potentiation (RP). Direct depolarization through a whole-cell patch pipette can substitute for activation of a climbing fiber to induce RP. RP is expressed as the increased responsiveness of postsynaptic $GABA_AR$, and the RP induction depends on the depolarization-induced increase in intracellular Ca^{2+} concentration (Kano et al., 1992). It was also reported that activation of metabotropic glutamate receptor type 1 (mGluR1) by pharmacological agent causes RP (Hashimoto et al., 1996). mGluR1 is coupled to the Gq-type heterotrimeric G-protein and causes the intracellular Ca^{2+} increase.

CaMKII activation downstream of the Ca^{2+} increase is required for the induction of RP (Kano et al., 1996). However, how CaMKII induces RP has been unclear. CaMKII phosphorylates cytoplasmic region of $GABA_AR$ β and γ subunits (Moss and Smart, 1996; Brandon et al., 2002a). Thus, one possibility is that direct phosphorylation of $GABA_AR$ by CaMKII causes RP. CaMKII–mediated phosphorylation of $GABA_AR$ composed of $\alpha1\beta3\gamma2$ but not that of $\alpha1\beta2\gamma2$ augments the responsiveness (Houston & Smart, 2006). However, in a

Purkinje neuron most $GABA_AR$ contains not β3 but β2 subunit (Pirker et al., 2000), suggesting that another mechanism works to induce RP. Recently, the detailed molecular mechanism to induce RP downstream of CaMKII activation was reported (see Figure 3) (Kawaguchi & Hirano, 2007). The key molecule is GABARAP, which has binding sites for $GABA_AR$ γ subunit and for tubulin, a constituent of cytoskeletal microtubule. Thus, GABARAP might work as a molecular hub for interaction of $GABA_AR$ and microtubule. When association of GABARAP and $GABA_AR$ γ2 subunit is inhibited by a peptide of intracellular region of γ2 subunit, the RP induction is impaired. Importantly, interaction of GABARAP and γ2 subunit is not only required for the induction of RP but also for the maintenance. The peptide application after establishment of RP attenuates the once potentiated $GABA_AR$ response, suggesting that the potentiation of $GABA_AR$ function is mediated by $GABA_AR$ binding to GABARAP (Kawaguchi & Hirano, 2007). Furthermore, the association of GABARAP with tubulin is also required for RP. Exogenous expression of GABARAP lacking N-terminal tubulin binding region in a Purkinje neuron impaired the RP induction through direct binding to γ2 subunit. It was also demonstrated that pharmacological destruction of microtubule inhibits the maintenance of RP. Using imaging study based on fluorescence resonance energy transfer (FRET) technique, it was shown that GABARAP undergoes sustained structural change in response to depolarization of a Purkinje neuron. This long-lasting conformational change of GABARAP depends on the activity of CaMKII. Further, the single amino acid replacement of GABARAP V33E abolishes the susceptibility of GABARAP to undergo the structural change and suppresses the RP induction (Kawaguchi & Hirano, 2007). These results suggest that CaMKII-mediated sustained structural change of GABARAP is critical for the RP induction.

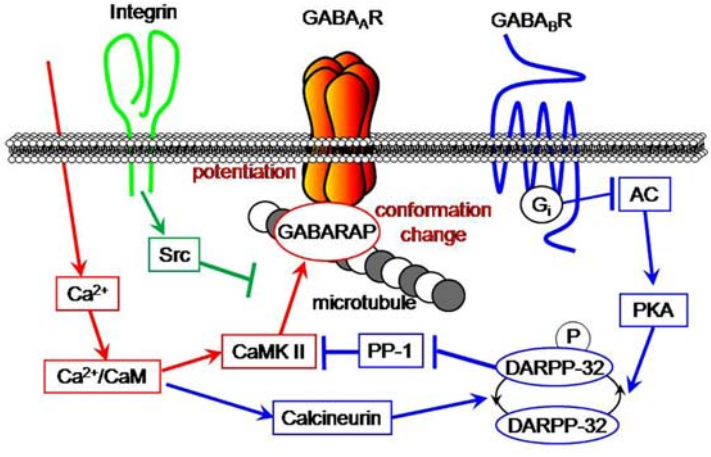

Figure 3. Molecular mechanism to regulate the induction and expression of RP. CaMKII activation leads to the sustained conformation change of GABARAP, which would augment the function of individual $GABA_AR$ though association with $GABA_AR$ γ2 subunit and with microtubule. $GABA_BR$ activation in conjunction with the intracellular Ca^{2+} increase facilitates calcineurin/DARPP-32/PP-1 pathway by decreasing PKA activity via inhibition of adenylyl cyclase (AC). As a result, CaMKII activity is inhibited, and the RP induction is suppressed. A cell adhesion molecule integrin α3β1 negatively regulates the induction of RP through Src-family protein tyrosine kinases.

GABARAP plays a role in trafficking of $GABA_AR$ toward the plasma membrane (Kneussel et al., 2000; Kittler et al., 2001; Moss and Smart, 2001; Kneussel, 2002; Nymann-Andersen et al., 2002; Leil et al., 2004; Luscher and Keller, 2004; Chen and Olsen, 2007). Therefore, CaMKII-mediated structural change of GABARAP might play a role in RP through facilitating the transportation of $GABA_AR$ to the cell surface. However, the amount of surface $GABA_AR$ is not affected by disturbing GABARAP association with $GABA_AR$ γ2 subunit (Kawaguchi & Hirano, 2007), suggesting that involvement of GABARAP in the RP is not through facilitated transportation of $GABA_AR$ to the plasma membrane. Another possible role of GABARAP in RP is to enhance the $GABA_AR$ function. GABARAP alters the properties of $GABA_AR$ such as single channel conductance through associating with γ2 subunit (Everitt et al., 2004; Luu et al., 2006). Thus, the depolarization-induced conformational change of GABARAP might facilitate the function of individual $GABA_AR$ through alteration of single channel conductance or open time. Because GABARAP is widely expressed in various brain regions, GABARAP-mediated alteration of $GABA_AR$ responsiveness might be involved in inhibitory synaptic plasticity not only in a Purkinje neuron but also in other neurons.

Interesting regulatory mechanisms of RP have also been reported. When a presynaptic inhibitory interneuron is activated during depolarization of the postsynaptic Purkinje neuron, the RP induction is suppressed (Kawaguchi and Hirano, 2000). This suppressive effect of presynaptic activation on the RP induction is mediated by activation of postsynaptic $GABA_BR$. This negative regulation of the induction of synaptic plasticity by presynaptic activity is unique, as most forms of synaptic plasticity reported to date are positively regulated by the presynaptic activity. RP is induced not by the homosynaptic activation but by the heterosynaptic excitatory inputs such as those from a climbing fiber (Kano et al., 1992). Taking it into consideration that repetitive activation of a climbing fiber causes the large Ca^{2+} increase in most dendritic regions in a Purkinje neuron, transmission efficacy of most of inhibitory synapses on the Purkinje neurons would be augmented at the same time without synapse-specificity. The suppressive effect of $GABA_BR$ activation on the RP induction is confined to the region where $GABA_BR$ is activated (Kawaguchi and Hirano, 2000). Thus, the presynaptic activity-mediated negative regulation would function as a mechanism to synapse-specifically regulate the induction of RP depending on the pattern of both pre- and postsynaptic neuronal activity.

The molecular mechanism to suppress the induction of RP by presynaptic activity was clarified (Figure 3). Kawaguchi and Hirano (2000) reported that the activity of PKA is required for the induction of RP in addition to CaMKII. The $GABA_BR$ activation inhibits adenylyl cyclase through promoting activation of Gi/o-type of trimeric G protein, reducing the intracellular concentration of cAMP. Reduction of cAMP leads to the decrease of PKA activity, resulting in the impairment of RP induction. How PKA and CaMKII contribute to the induction of RP was then analyzed (Kawaguchi and Hirano, 2002). The role of PKA in the RP induction is to gate activation of CaMKII through an intracellular signaling pathway. Suppression of RP induction by $GABA_BR$ requires the activities of PP-1 and calcineurin. Calcineurin is activated by Ca^{2+} and calmodulin (CaM). Furthermore, knock-down of DARPP-32 (dopamine and cAMP-regulatied phospho-protein 32 kDa) expression using the antisense oligonucleotide impairs the induction of RP (Kawaguchi and Hirano, 2002). DARPP-32 is phosphorylated by PKA and dephosphorylated by calcineurin. When

phosphorylated, DARPP-32 inhibits the activity of PP-1 through direct binding (Hemmings et al., 1984). Thus, calcineurin and PKA oppositely control the PP-1 activity through regulating phosphorylation of DARPP-32. When activated by Ca^{2+}/CaM, CaMKII autophosphorylates at Thr286 residue, which turns CaMKII into a Ca^{2+}-independent active form (Giese et al., 1998; Blitzer et al., 1998). The phospho-Thr286 of CaMKII is dephosphorylated by PP-1. PP-1 activity also counteracts the CaMKII activity, as PP-1 dephosphorylates proteins phosphorylated by CaMKII. It was explained that the reduction of PKA activity together with activation of calcineurin during conjunction of depolarization and $GABA_BR$ activation, results in dephosphorylation of DARPP-32, and then PP-1 is released from the inhibition by phospho-DARPP-32. As a result, CaMKII activity is inhibited by the augmented PP-1 activity, impairing the RP induction.

Interestingly, the gating mechanism similar to the regulation of calcineurin/DARPP-32/ PP-1 pathway by PKA also plays a critical role in the synaptic plasticity at excitatory synapses. Inhibitor-1, a homologous protein of DARPP-32, mediates the role of DARPP-32 in the hippocampus (Malenka, 1994; Malenka and Nicoll, 1999; Lisman and Zhabotinsky, 2001). In the hippocampal CA1 pyramidal neuron, repetitive stimulation of Schaffer collateral results in the LTP or LTD of excitatory synaptic transmission (Bliss & Collinglidge, 1993). LTP induction requires CaMKII activation, whereas the induction of LTD relies on the activity of calcineurin and PP-1. Thus, molecules implicated in switching of LTP and LTD is similar to that regulating the induction of RP. The high frequency presynaptic stimulation augments the phosphorylation level of inhibitor-1 by PKA (Cooper et al., 1995; Wong et al., 1999). This is due to a Ca^{2+}/CaM-mediated activation of type-1 adenylyl cyclase expressed in a pyramidal neuron, which is absent in a Purkinje neuron. As a result, PP-1 activity is inhibited by phospho-inhibitor-1, which enables effective activation of CaMKII and resultant phosphorylation of the substrates including ionotropic glutamate receptor (Blitzer et al., 1995, 1998; Makhinson et al., 1999; Lee et al., 2000; Allen et al., 2000; Otomakhova et al., 2000). In contrast, when low frequency stimulation is applied, the moderate increase of intracellular Ca^{2+} takes place, that preferentially activates calcineurin rather than CaMKII without marked activation of adenylyl cyclase type1 (Malenka, 1994; Malenka & Nicoll, 1999). As a result, the phosphorylation balance of inhibitor-1 shifts to the dephosphorylated state, leading to the release of PP-1 from inhibition. Then, the phosphorylation level of synaptic proteins including glutamate receptor decreases, and LTD is induced. The similarity of switching mechanism of synaptic plasticity at excitatory synapses in hippocampus and that at inhibitory synapses on a Purkinje neuron, suggests that regulation of activity balance of protein kinase/phosphatase determines the induction of plasticity at a variety of synapses in the central nervous system.

It has also been shown that integrins, a kind of cell-adhesion molecule working as a receptor for extracellular matrix proteins, are implicated in regulation of RP (Figure 3) (Kawaguchi & Hirano, 2006). When the activity of integrins is facilitated, the RP induction is impaired, which is abolished by the integrin function blocking antibody against integrin α3 subunit and that against β1 subunit. Only β1 subunit forms an integrin heterodimer with α3. Thus, the activity of integrin α3/β1 subunit seems to negatively regulate the induction of RP. This idea was confirmed by the experiment showing overexpression of integrin α3 subunit by itself suppresses the RP induction (Kawaguchi & Hirano, 2006). Integrin-mediated suppression of RP is rescued by inhibition of Src-family protein tyrosine kinase, suggesting

that Src-family mediates the suppressive effect on RP downstream of integrin activity. This is supported by the result that raising Src activity by itself suppresses the RP induction (Kawaguchi & Hirano, 2006). Taken together, RP is regulated not only by the neuronal activity but also by cell-adhesion molecule integrin. $GABA_AR$ is phosphorylated not only by Ser/Thr kinases but also by Tyr kinases. $GABA_AR$ phosphorylation by Src-family tyrosine kinase has also been suggested to modulate the function of $GABA_AR$ (Wan et al., 1997; Boxall, 2000; Brandon et al., 2001). However, the role of direct phosphorylation of $GABA_ARs$ by Src in the RP remains unknown. $GABA_AR$ phosphorylated by Src might lose the susceptibility to undergo the change during RP.

Functional Relevance of Plasticity at GABAergic Synapses on a Purkinje Neuron

The physiological significance of four forms of plasticity at inhibitory synapses on a Purkinje neuron is unclear. The inhibitory inputs to a neuron shape the firing pattern. Computational simulation studies have suggested that the inhibitory synaptic transmission to a Purkinje neuron is more influential on the firing pattern compared with excitatory one (Jeager et al., 1997; Jeager and Bower, 1999). Indeed, LTD of the inhibitory transmission has been shown to alter the output pattern of a Purkinje neuron, as well as LTD of the excitatory inputs (Mittmann & Hausser, 2007). Thus, plasticity of inhibitory and excitatory synaptic inputs to a Purkinje neuron would synergistically bring about the experience-dependent refinement of information processing in the cerebellar cortex, contributing to the motor coordination, learning and memory.

A mutant mouse lacking glutamate receptor δ2 subunit, which shows ataxia and impairment of LTD at excitatory synapses on a Purkinje neuron, exhibits augmented inhibitory synaptic transmission in the molecular layer of cerebellar cortex and occlusion of the RP induction (Ohtsuki et al., 2004). These results suggest that RP is induced at most of inhibitory synapses in the basal condition *in vivo* probably through increased inputs from a climbing fiber in the mutant mice (Yoshida et al., 2004). This indicates that RP actually takes place *in vivo* and might contribute to the compensation of enhanced excitatory synaptic inputs. Changes of the GABAergic transmission efficacy might also affect the induction of plasticity at excitatory synapses by altering excitability of a neuron. In the hippocampus and amygdala, LTD at inhibitory synapses modulates the induction of excitatory synaptic plasticity (Chevaleyre & Castillo, 2003; Azad et al., 2004). In this way, plasticity at excitatory and inhibitory synapses would affect each other so that the balance of excitation and inhibition would be regulated sophisticatedly. Recently, it was also shown that mGluR1 activity supports the RP induction (Sugiyama et al., 2008). Disturbing the balance of excitation and inhibition in the cerebellar cortex results in ataxia (Watanabe et al., 1998). Clarification of the detailed molecular mechanism of RP, DSI, DPI, and LTD would contribute to the understanding of precise roles of each synaptic plasticity.

Conclusion

Synaptic plasticity at inhibitory synapses has been reported in the central nervous system, and various regulatory mechanisms have been clarified. However, the information is limited compared to that at excitatory synapses. We here overviewed several forms of regulation of inhibitory synaptic transmission and their mechanisms, followed by detailed description of inhibitory synaptic plasticity in the cerebellar cortex. Four forms of plasticity, DSI, DPI, RP, and LTD take place at the GABAergic synapses on a Purkinje neuron. DSI and DPI are transient depression and augmentation of presynaptic GABA release, which are triggered by the postsynaptic depolarization. DSI and DPI are mediated by endocannabinoid and glutamate released from the postsynaptic Purkinje neuron, respectively. RP is also triggered by the postsynaptic depolarization, and expressed as the long-term augmentation of postsynaptic responsiveness of $GABA_AR$. The RP induction is dependent on the activity of CaMKII and the resultant sustained modulation of GABARAP conformation, leading to alteration of the scaffold of $GABA_AR$. Further, the induction is dynamically regulated by the pattern of pre- and postsynaptic activities. The presynaptic activation coupled with the postsynaptic depolarization suppresses the RP induction through $GABA_BR$-mediated facilitation of protein phosphatase pathway. The activity of cell-adhesion molecule integrin is also involved in the regulation of RP induction through Src family protein tyrosine kinase. Taken together, the efficacy of $GABA_AR$-mediated inhibitory synaptic transmission is elaborately regulated by the neuronal activities and cell-cell and/or cell-extracellular matrix interactions. Interestingly, the molecules involved in regulation of inhibitory synaptic plasticity also play critical roles in excitatory synaptic plasticity, suggesting that there are some common molecular processes at both excitatory and inhibitory synapses. Future studies might clarify the interplay of excitatory and inhibitory synaptic plasticity and their synergistic contribution to information processing in the central nervous system.

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Chapter 6

$GABA_AR$ Lateral Mobility and Localization

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Abstract

The mammalian central nervous system (CNS) is a highly complex integrative system, processing a vast number of positive and negative inputs. Proper functioning of the brain depends on a delicate interplay between excitatory and inhibitory neurotransmission among neurons. The major inhibitory neurotransmitter in the vertebrate brain is gamma-aminobutyric acid (GABA). GABA mediates its inhibitory effect through its interaction with a variety of receptors in all areas of the central nervous system. Two major types of GABA receptors have been well described: Type A GABA receptors ($GABA_AR$) and type B GABA receptors ($GABA_BR$). These receptors are quite different, not only from a pharmacological point of view but also structurally and functionally. From the therapeutic point of view the relevance of $GABA_AR$ is determined by the fact that its function is regulated by pharmaceutically significant drugs. The variety of genes discovered to encode the different subunits and subunit isoforms of the $GABA_AR$ and the pentameric stoichiometry imply a tremendous heterogeneity

of $GABA_AR$ subunit composition. This raises the question as to why there is such a structural diversity of $GABA_AR$ subunits and why this exceeds the currently known functional diversity of GABA-mediated currents in neurons. It is conceivable that $GABA_AR$ heterogeneity has evolved so as to meet the specific requirements of distinct neuronal cells. The clustering of $GABA_ARs$ at discrete and functionally significant domains on the nerve surface is an important determinant in the integration of synaptic inputs. The large structural heterogeneity of $GABA_AR$ led to the hypothesis that there could be a link between $GABA_AR$ gene diversity and the targeting properties of the receptor complex. $GABA_AR$ lateral mobility could be a mechanism by which neurons recruit and maintained specific receptors at specific synaptic sites. In fact evidence suggests that the subunit composition of the receptors determines not only different targeting characteristics but also differences in the lateral mobility of the complexes.

The types of subunit isoforms that compose the protein complex could determine the different ways in which these receptors are routed to specific synaptic locations. This might represent an exquisite form of synaptic plasticity.

1. Gaba Receptors

GABA was originally identified as the principal agent in brain extracts capable of inhibiting crayfish stretch-receptor neurons, an effect mediated by an increase in the membrane permeability to Cl^-. This was followed by studies into the inhibitory role of this amino acid in crustacea, in vertebrate, and finally in the mammalian CNS, where it may function at up to 40% of the synapses in brain (for review see Bennett and Balcar, 1999).

GABA mediates its inhibitory effect through its interaction with a variety of receptors in all areas of the central nervous system. Two major types of GABA receptors have been well described: Type A GABA receptor ($GABA_AR$) and type B GABA receptor ($GABA_BR$, Hill and Bowery, 1981). These receptors are pharmacologically, structurally, and functionally quite different.

$GABA_ARs$ are bicuculline-sensitive, ligand-gated Cl^- channels. Following the binding of GABA to the $GABA_AR$, a conformational change results in the opening of an intrinsic Cl^- pore which, in most cases, results in the hyperpolarisation of the recipient neuronal cell leading to stabilisation of the resting membrane potential (Olsen and Venter, 1986; Stephenson, 1988; Farrant *et al.,* 1990).

$GABA_BRs$ bind baclofen, are bicuculline-insensitive (Sivilotti and Nistri, 1988), and their mode of action is metabotropic. $GABA_BRs$ mediate their effects through potassium and calcium channels via a guanine-nucleotide-binding (G) protein (Bowery, 1993). Both $GABA_AR$ and $GABA_BR$ have inhibitory roles in the CNS, although the activation of $GABA_AR$ induces fast inhibitory postsynaptic potentials (IPSP) in contrast with the slow IPSP induced by $GABA_BR$ activation (Nakayasu *et al*., 1995).

In addition, a third type of GABA receptor, the type C GABA receptor ($GABA_CR$, Lukasiewicz, 1994) which is exclusively composed of ρ(1-3) subunits, has been identified in retina. $GABA_CRs$ are also ligand-gated Cl^- channels, however, there are differences in the pharmacological characteristics of $GABA_AR$ and $GABA_CR$ that allow them to be discriminated in a separate type of GABA receptor. $GABA_CRs$ are not sensitive to bicuculline blockade and not modulated by barbiturates, benzodiazepines or neuroactive steroids. These receptors are activated at lower concentrations of GABA and their mean channel open times

in response to GABA are greater (Johnston, 1996; Cherubini and Strata, 1997; discussed in Barnard *et al.*, 1998).

This chapter focuses on GABA$_A$R, their localization and their lateral movement in the plasma membrane.

2. GABA$_A$R Pharmacology

The GABA$_A$R is important not only because of its fundamental role in the regulation of brain excitability but also because its function is allosterically regulated by pharmaceutically significant drugs (Turner and Whittle, 1983; Olsen, 1987; review in Whiting, 2006). The GABA$_A$R response is positively modulated by benzodiazepine agonists such as flunitrazepam and clonazepam (anxiolytics) or negatively modulated by inverse agonists, such as the β-carboline, methyl-4-ethyl-6,7-dimethoxy-β-carboline-3-carboxylate (anxiogenic). GABA$_A$Rs also have binding sites for a variety of other clinically important substances, including barbiturates (anxiolytic and anti-convulsant) certain neuroactive steroids, in either a positive or a negative manner (Majewska *et al.*, 1986, 1990; Akk, 2007) and picrotoxin (convulsant agents) (Bloomquist, 1996). Although both barbiturates and benzodiazepine agonists potentiate the effect of GABA, they do so by different mechanisms. Barbiturates prolong the mean open time of the channel while benzodiazepine agonists increase the frequency of channel opening (McDonald and Olsen, 1994).

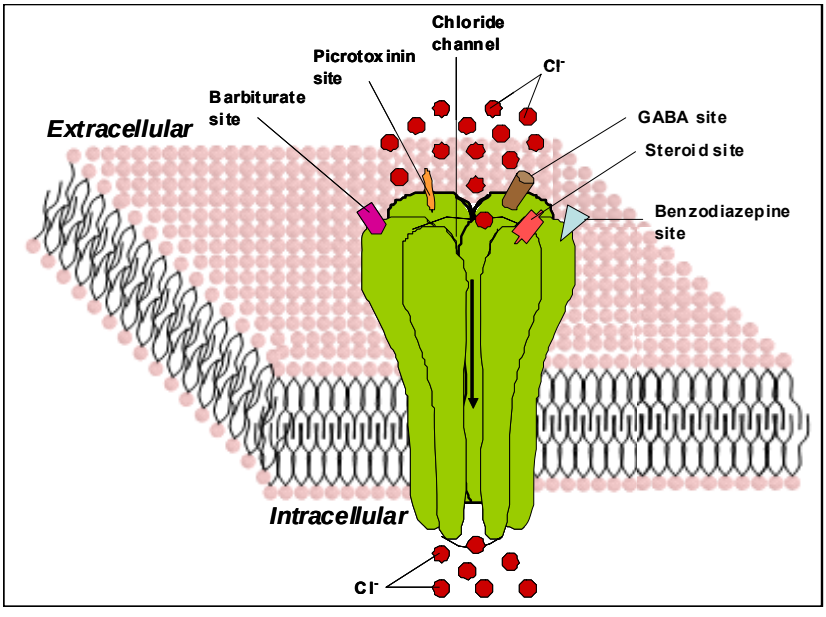

Figure 1. GABA$_A$R Structural model of the GABA$_A$/benzodiazepine receptor-chloride ionophore complex. The cut-away view demonstrates targets for a variety of compounds that influence the receptor complex.

These mentioned compounds, in most cases, do not interact directly with the GABA binding site but exert their action by binding to allosterically-coupled sites on the GABA$_A$ receptors. Thus, binding of these agents induce conformational changes in the GABA$_A$

receptor that may influence its binding properties and modulate GABA-induced chloride ion flux (Sieghart, 1992). $GABA_A$Rs are believed to have at least five different binding sites: the GABA, the picrotoxin/convulsant, the benzodiazepine, the barbiturate and the steroid binding sites (Figure 1).

From binding studies using specific radioligands only three of these different binding sites have been fully characterized, the picrotoxin/convulsant site (Ticku *et al*., 1978; Squires *et al.,* 1983), the benzodiazepine site (Squires and Braestrup, 1977) and the GABA binding site (Zukin *et al*., 1974). However, all the other compounds that interact with $GABA_A$Rs are either not available as radioligands or their potency for modulating $GABA_A$Rs is too low to allow direct binding studies. Thus, their site(s) of action has been investigated by studying their interaction with the three well-characterized binding sites on $GABA_A$Rs previously described.

2.1. The GABA Binding Site

GABA, by binding to $GABA_A$Rs, opens channels that are selectively permeable to Cl^-. Depending on the prevailing electrochemical membrane potential for Cl^-, the effect of GABA can be excitatory or inhibitory. During development, activation of $GABA_A$R in immature neurons results in an efflux of Cl^- that causes a membrane depolarisation (Cherubini *et al.,* 1991). In terminally differentiated neurons, the Cl^- electrochemical membrane potential is such that following $GABA_A$Rs activation Cl^- flows into the cell and induces a slight hyperpolarisation of the membrane and a reduced neuronal excitability of the cells.

The affinity of GABA to $GABA_A$Rs is very low, micromolar concentrations of GABA being required to activate the chloride ion channel in electrophysiological experiments (Segal and Barker, 1984). It has been suggested from electrophysiological experiments that at least two molecules of GABA must bind to the receptor to induce full activation of channel activity (Sakmann *et al*., 1983). Sieghart (1992) discussed the apparent existence of several distinct GABA binding sites on a single $GABA_A$R. This sites show high, low and very low affinity for GABA and its agonist. Up to five GABA binding sites might be present on a single $GABA_A$R. In the unoccupied state, these binding sites might have a similar high affinity for GABA. On increasing occupation of these sites with GABA, the affinity of the remaining unoccupied sites might allosterically become reduced. The high and possibly the low affinity GABA sites are probably constantly occupied under the physiological GABA concentration present in the synaptic cleft. Occupation of these sites does not cause an opening of the chloride channels. Thus, the existence of the remaining binding site with very low affinity ensures that GABA-activated chloride channels can only be opened under conditions of synaptic transmission where a large amount of GABA is released into the synaptic cleft.

2.2. The Benzodiazepine Binding Site

Benzodiazepines, such as diazepam or flunitrazepam, enhance GABAergic function by increasing the frequency of Cl^- channel opening with little effect on channel open time or $GABA_A$R affinity (Edgar and Schwartz, 1992). Benzodiazepines, however, are not able to directly open the $GABA_A$R chloride channel in the absence of GABA (Study and Barker, 1981).

2.3. The Picrotoxin/Convulsant Binding Site

Picrotoxin and cage convulsants are inhibitors of GABA$_A$R activity by directly binding to the GABA$_A$R chloride pore and hence, blocking the channel (Inoue and Akaike, 1988). These substances do not displace benzodiazepines from their high affinity binding sites (Olsen, 1982), but allosterically modulate benzodiazepine receptor binding (Karobath *et al*., 1981).

2.4. Barbiturate Interaction with GABA$_A$R

Barbiturates can exert two different effects on GABA$_A$Rs that are differentially dose dependent. Electrophysiological studies revealed that, at low concentrations these substances enhance the actions of GABA by increasing the mean channel open duration without altering receptor conductance or opening frequency (Study and Barker, 1981). At higher concentrations (>50μM) barbiturates are able to open the GABA$_A$R chloride channel in the absence of GABA (Bormann, 1988).

2.5 Steroid Interaction with GABA$_A$R

Several steroids, at low concentrations (30 to 300 nM), enhance GABA-stimulated chloride conductance (Majewska, 1992; Kokate *et al*., 1994) and at higher concentrations (1μM) produce a direct opening of the Cl^- channel that is inhibited by the GABA$_A$R antagonist bicuculline (Majewska, 1992).

3. GABA$_A$R Gene Diversity

Since the initial cloning in 1987 of cDNAs encoding two different subunits of the GABA$_A$R from bovine brain (Schofield *et al.,* 1987), the molecular biology of this receptor has become increasingly complex (Russek, 1999). The ligand-gated ion channel is proposed to be an hetero-oligomer composed of five subunits (Nayeem *et al.,* 1994). GABA$_A$R subunits are classified with respect to their amino acid sequence homology and designated as α, β, γ , δ, ε, and π (Whiting *et al*., 1997; reviewed in Mehta and Ticku, 1999; Kasparov et al., 2001; Sieghart and Sperk, 2002). Another GABA$_A$R subunit, named as Θ, has been found in rat brain (Chadha *et al*., 1999). The α, β, and γ subunits contain multiple members or isoforms that arise from separate genes that have diversified from an evolutionary origin, so that there are α1–α6, β1–β3 and γ1–γ3 (reviewed in McDonald and Olsen, 1994 and Mehta and Ticku, 1999). The existence of this wide range of subunits and subunit variants implies a vast diversity of potential GABA$_A$R subtypes (review in Picton and Fisher, 2007). Further heterogeneity is introduced by the existence of alternative splice variants for mammalian GABA$_A$R α6, β2 and γ2 subunit genes (McKernan and Whiting, 1996). The γ2 subunit, for instance exists in two forms, γ2 short (γ2s) and γ2 long (γ2l). The latter contains an extra 8 amino acids in its intracellular loop domain (Whiting *et al*., 1990; Kofuji *et al*., 1991; Glencorse *et al.,* 1992), which has been shown to be a target for protein kinase C-mediated

phosphorylation and are responsible for differences in receptor pharmacology (Whiting *et al*., 1990; Wafford *et al*., 1991).

4. Structural Model of $GABA_AR$s

The $GABA_AR$ is proposed to be a heteropentameric glycoprotein of about 275 kDa, composed of combinations of multiple subunits, polypeptides of about 50 kDa. The subunits form a quasi-symmetric structure around the ion channel. Figure 2 represents the predicted structural model of the $GABA_AR$. Each $GABA_AR$ subunit is predicted to have four hydrophobic membrane-spanning domains, designated M1-M4. These putative hydrophobic domains are predicted to span the membrane and form the integral chloride ion channel. It is postulated that the M2 domain contributes to the inner wall of the channel (Schofield *et al.,* 1987, DeLorey and Olsen, 1992). The N-terminal, hydrophilic domain, of each subunit protein is predicted to be located extracellulary. This region contains several potential sites for N-linked glycosylation, thus, *in vivo* carbohydrate attachment would account for the differences between the observed and predicted molecular weights of the natural and cDNA-deduced $GABA_AR$ subunit masses, respectively (Schofield *et al*., 1987). A sequence of 15 amino acids, highly conserved across all the members of the ligand-gated ion channel superfamily receptor, is found in this N-terminal region. This sequence is predicted to form a Cys-Cys β loop. The C-terminal end of the protein follows the M4 domain and, as the N-terminus, is thought to be extracellular.

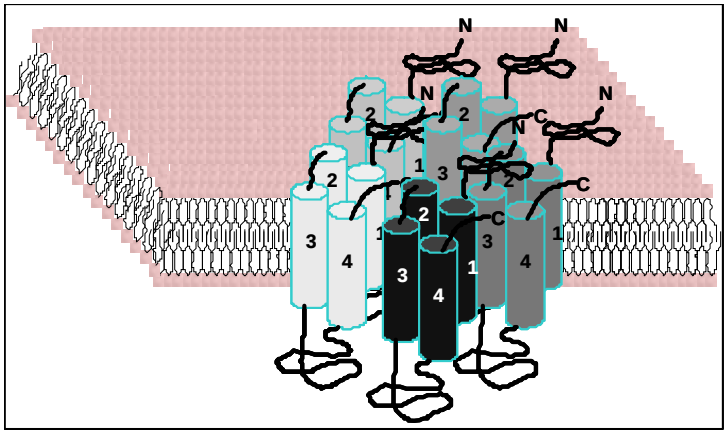

Figure 2. Model of the structure of the $GABA_AR$. Each subunit has four membrane-spanning domains (cylinders numbered 1-4). Modified from DeLorey and Olsen, 1992.

The transmembrane-spanning domains (M1-M4) and defined regions within the N-terminus are highly conserved among different subunit types. In contrast, sequence identity is poorly conserved in the cytoplasmic loop region, which connects M3 and M4. This domain, designated the M3/M4 cytoplasmic loop, is hydrophilic and contains consensus sequences for phosphorylation by various kinases.

5. GABA$_A$R Heterogeneity

The variety of genes that encode the different subunits and subunit isoforms of the GABA$_A$R and the assumption of a pentameric stoichiometry (Nayeem *et al*., 1994), imply a tremendous heterogeneity of GABA$_A$R subunit composition. Permutation analysis predicted that there were 151887 possible different receptor combinations (Burt and Kamatchi, 1991), and new subunits have been discovered since then enhancing this number (Bonnert *et al*., 1999).

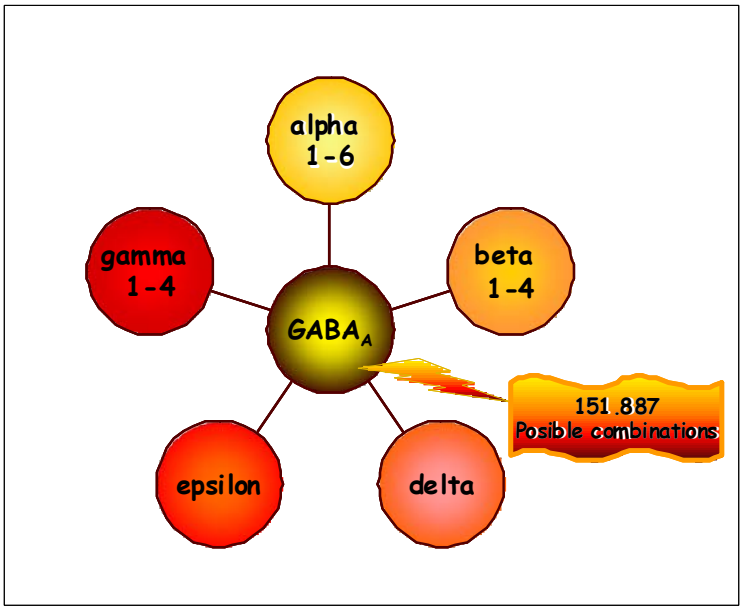

Figure 3. Representation of GABA$_A$R subunit composition heterogeneity.

This theoretical heterogeneity of GABA$_A$Rs has not been experimentally observed, in fact only a restricted number of GABA$_A$Rs have been shown to be functionally expressed (reviewed in Barnard *et al*., 1998). This raises the question as to why there is such a structural diversity of GABA$_A$R subunits and why this exceeds the currently known functional diversity of GABA-mediated currents in neurons. Furthermore, why has such GABA$_A$R structural diversity been created and maintained during evolution?

It is conceivable that GABA$_A$R heterogeneity has evolved so as to meet the specific requirements of distinct neuronal cells. Evidence has emerged that the expression of different GABA$_A$R genes differ in distinct areas of the CNS (Levitan *et al*., 1988a; 1988b; Wisden *et al*., 1992), during development (Killisch *et al*., 1991; Laurie *et al*., 1992; Smith *et al*., 1998) and within individual neurons (Meinecke *et al*., 1989; Mercik *et al*., 2003). In addition, the pharmacological and physiological properties of GABARs are largely determined by their subunit and subtype composition (Korpi et al., 2002).

6. Stoichiometry of $GABA_AR$

Immunoaffinity purification and immunoblotting approaches have been used to identify which of the subunits co-assemble to form native receptors, however different groups have obtained contradictory results. An example of these discrepancies is the arguments both in favour and against whether more than one different α subunit can exist within the same receptor complex. Duggan *et al.* (1991) showed, using Western blots of immunoaffinity-purified $GABA_ARs$ from detergent-solubilised bovine cerebral cortex, that subpopulations of $GABA_ARs$ contained both α1:α2, α2:α3 and α1:α3 subunits in the same receptor complex. McKernan *et al*. (1991) also reported that a subpopulation of rat $GABA_ARs$ contained both α1 and α3 subunits. Immunoprecipitation studies on detergent-solubilised rat cerebellar $GABA_ARs$ showed that in a subpopulation of receptors, both α1 and α6 subunits coexist within the same $GABA_AR$ complex (Pollard *et al*., 1995; Khan *et al*., 1996; Jechlinger *et al*., 1998). However, Quirk *et al*. (1994) reported that the α6 subunit was not co-assembled with any other α subunit. In addition, double-immunolabelling of clustered receptors expressed on the surface of cultured cerebellar granule cells suggested that α1 and α6 subunits do not co-localise in the same receptor complex (Caruncho *et al*., 1993). A similar discrepancy has arisen regarding the γ subunit. Quirk *et al*. (1994) have reported the co-purification, from rat brain, of $GABA_ARs$ that contain both γ2 and γ3 subunits. Tögel *et al*. (1994), however, found no evidence for association of γ2 with γ3 subunits.

These contrasting results obtained from different laboratories may be explained by the sensitivity of detection of the antibodies used. Although the conclusions are confusing most of the $GABA_ARs$ appear to contain at least one α, one β and either one γ or one δ subunit, but receptor stoichiometry has not been unambiguously established. Comparisons of conductances of wild-type and mutated cloned receptors predicted a polypeptide stoichiometry of $(\alpha1)_2(\beta1)(\gamma2)_2$ as more likely than others (Backus *et al.*, 1993). This is in agreement with the data of Khan *et al*. (1994), which also predicted receptors with two α, one β and two γ. Although, once again conflicting data have emerged with reports from several laboratories predicting that the majority of receptors have a subunit stoichiometry of $(\alpha)_2(\beta)_2(\gamma2)_1$ (Chang *et al*., 1996; Tretter *et al*., 1997). Thus, perhaps native $GABA_AR$ do not have an unique subunit stoichiometry (Hevers *et al*., 1998). Rather, subunit assembly may be characteristic of individual neuronal cells, and may be governed by the specific demands made upon that neuron to produce an appropriate inhibitory response to external or internal stimuli. $GABA_AR$ receptor assembly signals have been described and seem to be contained within each subunit, and participate in the final subunit stoichiometry of the complex. But the presence of alternative assembly signal found in some $GABA_AR$ subunit indicate that there might be several assembly pathways that may be determined by subunit availability (Bollan et al., 2003).

7. Brain Distribution of $GABA_ARs$

$GABA_ARs$ are widely distributed throughout the CNS. The study of brain distribution of $GABA_ARs$ have been extensive carried out by Seeburg laboratory, using in-situ hybridisation they have determined the distribution of $GABA_AR$ subunit mRNAs through out the brain

(Laurie et al., 1992a; 1992b; Wisden et al., 1992). These studies have revealed a pattern of expression of GABA$_A$R mRNAs in the adult brain that are summarised in Table 1 together with the results obtained by others research groups. (review in Darlinson and Albrecht, 1995). Since the presence of a mRNA transcript within a cell is not proof that a translated product is actually expressed inmunological assays are necessary to determine the subunits that are finally expressed. In a study carried out by Sieghart laboratory the distribution of GABA$_A$R subunits deriving from 13 different genes was achieved by immunocytochemistry (Sperka et al., 1997). In addition lately and Pirker and cols (2000) have performed a similar studies.

These different approaches concluded that the highest levels of GABA$_A$R subunits found in the brain are the α1, α2, α3, β2, β3 and γ2 subunits, α1 being the most abundant and wide distributed.

Table 1. Regional co-distribution of GABA$_A$ receptor subunits

REGIONAL CO-DISTRIBUTION	SUBUNITS
Cerebral cortex, cerebellum, medulla and cranial nerve nuclei	*α1/γ2*
Cerebral cortex and brainstem	*α3/γ2*
Cerebral cortex, midbrain and pons	*α1/β3*
Cerebral cortex, amygdala and hypothalamus	*α2/β3*
Cerebral cortex	*α3/β3 α6/γ2 α6/δ*
Cerebellum	*α2/γ1 and α6/γ2 and α6/δ*
Forebrain and certain thalamic nuclei	*α4/δ*
Olfactory bulb, hippocampus and dentate gyrus	*α1/α2/β2/γ2*
Olfactory bulb and cerebellum	*α1/β2/γ2*
Olfactory bulb, forebrain, brainstem and cerebellum	*α1/β2/β3/γ2*
Spinal cord	*α2/β3/γ2*
Hippocampus	*α1/α2/α5/β1/β2/β3*
Olfactory bulb	*α1/α2/α5/β1/β2/β3*
Thalamus	*α3/β2/β3/γ2*
Cranial nerve nuclei and medulla	*α1/α4/β2/δ*
Amigdala	*α1/β1/γ2*

8. Why GABA$_A$R Heterogeneity

The existence of different subunit isoforms that have unique but overlapping distributions throughout the mammalian brain, may endow a diversity of physiological properties. From recombinant studies it has been shown that there are slight functional differences between receptors containing distinct subunit isoforms (Verdoorn *et al*., 1990). Levitan and co-workers suggested that there are differences in receptor sensitivity to GABA depending on the α subunit included in the recombinant receptor when expressed in Xenopus oocytes

(Levitan *et al.,* 1988a, 1988b). In addition, on the basis of receptor binding and electrophysiological studies, it has been suggested that recombinant $GABA_AR$s of defined subunit composition have differential sensitivity to allosteric modulation by endogenous molecules found in the brain including neurosteroids (Puia *et al.,* 1990) and Zn^{2+} (Draguhn *et al*., 1990). The macroscopic kinetic properties of the $GABA_AR$ are also determined by subunit composition; in fact outside-out patch recordings showed different receptor responses to GABA depending on the alpha subunit included in the complex (Fisher, 2004).

From a pharmacological point of view, differential expression of the receptor subunit genes generates a great diversity of $GABA_AR$ responses to some psychoactive drugs. While GABA, bicuculline, and barbiturates act on receptors formed from only α and β subunits (Pritchett *et al*., 1988; Levitan *et al*., 1988b), the co-expression of these subunits with the γ subunit is required for the positive or negative allosteric modulation of GABA-evoked Cl^- currents by benzodiazepines (Pritchett *et al*., 1989a). Furthermore, the type of α or γ subunit expressed imparts distinct benzodiazepine pharmacology to the receptors (Pritchett *et al*., 1989a, 1989b; Pritchett and Seeburg, 1990; Herb *et al*., 1992; reviewed in Sieghart, 2000).

In addition to physiological and pharmacological differences, the composition of the receptor might determine its distribution and maintenance on the nerve cell surface. In the CNS, $GABA_AR$s are distributed on cell bodies, dendrites, and in some cells at axon hillocks, at both synaptic and extrasynaptic sites (Somogyi *et al*., 1989; Baude *et al*., 1993; Craig *et al*., 1993, Nusser *et al*., 1996; Scotti et al., 2001). The segregation of $GABA_AR$s to strategic locations on the nerve cell surface is an important determinant in the integration of synaptic inputs (Vu and Krasne, 1992). Therefore, knowledge of the precise localisation of $GABA_AR$ at the cellular and subcellular level may provide insight into inhibitory mechanisms, drug actions, and perhaps disease states involving GABAergic pathways in the CNS. Numerous studies have centred on localising the sites on cells and processes at which these receptors occur with the aid of autoradiographic techniques in which radiolabelled molecules such as GABA, benzodiazepines, and others with high affinity for the $GABA_AR$ are used as markers (Kuhar e*t al*., 1986). While autoradiographic techniques have proved highly useful for the analysis of regional distributions of $GABA_AR$s, the cellular and subcellular localisation of the receptors demands higher resolution techniques. The post-synaptic localisation of $GABA_AR$ has been confirmed using subunit-specific antibodies (Baude *et al*., 1992). In addition, extra-synaptic labelling has also been detected using monoclonal antibodies which are specific for antigenic sites on both the α and β subunits (Baude *et al*., 1992; Nusser *et al.,* 1995).

Immunocytochemistry results demonstrate a perisynaptic localization of delta subunit-containing GABA(A) receptors (Wei et al., 2003). The α1, α2, β2/3 and γ2 subunits were contained in clustered receptors present at GABAergic synapses and in the extrasynaptic membrane on cultured hippocampal neurons. α4, β1 and delta subunits were contained in the extrasynaptic membrane (Mangan et al. 2005*).*

Previous studies employing fluorescently labelled GABA/benzodiazepine receptors on cultured neurons have shown that while $GABA_AR$s are localised in clusters of high density on the cell body and in patches on dendrites, more than 85% of the $GABA_AR$ on processes and 70% on cell bodies are immobile (Velazquez *et al.,* 1989). The results suggest that even in the absence of synaptic contact, there are specific mechanisms that segregate and immobilise GABA/benzodiazepine receptors on the cell body and dendrites.

9. Plasma Membrane Dynamics

Plasma membrane proteins are not totally free to drift randomly on the "lipid sea" were they are embedded, but instead are subjected to restraining influences that restricts their mobility. Three non-exclusive models have been proposed for the transient confinement of membrane proteins (reviewed in Sheets *et al.,* 1995), briefly:

a) The membrane-skeleton fence model

In this model the spectrin and ankyrin based cytoskeleton compartmentalise the membrane into small domains (0.1-1 μm^2) providing a barrier to the free diffusion of membrane proteins. The cytoplasmic domains of membrane proteins interact sterically with the cytoskeletal network beneath the cell surface, thus membrane proteins with large cytoplasmic domains will experience more restrictions in their mobility than proteins with small cytoplasmic regions. The membrane proteins can escape from one domain and move to adjacent compartments as a result of the dynamic properties of the cytoskeleton.

b) Localised high concentration of proteins

High local concentrations of proteins, directly or indirectly bound to the cytoskeleton, can act as obstacles to the free diffusion of membrane proteins. Here, the diffusing proteins interact with other proteins either sterically or specifically through direct chemical interactions. The protein obstacles may form large networks *via cis* interactions forming a physical barrier for diffusion. Such a mechanism may explain the transient confinement of GPI-anchored proteins (GPI: glycosylphosphatidylinositol) which can not interact directly with the cytoskeletal meshwork.

c) Localised lipid domains

Local differences in the lipid composition of the bilayer, forming highly viscous microdomains, may also account for protein confinement.

10. Postsynaptyc Neurotransmitter Receptors Localization

In addition to the restriction in mobility of membrane proteins due to domain confinement, as described above, more specific mechanisms are needed to ensure the total anchoring or immobilisation of some proteins. In fact, neurotransmitter receptors rely on varied complex mechanisms to regulate their surface transport and distribution. Striking examples of how cell surface components are localised and maintained in discrete membrane domains of the neuron are the placement of Ca^{2+} channels at dendritic spines for signal amplification and at nerve endings for neurotransmitter release (Cohen *et al*., 1991), and the sequestration of AMPA and NMDA receptors at dendritic spines (Baude *et al*., 1995).

Postsynaptyc membranes are believed to contain a large number of interconnected scaffolds proteins, which create a net that is known as the postsynaptic density (PSD). Membrane receptors interact with these submembrane proteins to reach an appropriate synaptic localization (Kennedy, 2000; Kneussel and Betz, 2000; Sanes and Lichtman, 2001; Li and Sheng, 2003). Further complexity is added when considered that both receptors and scaffold proteins are subject to constant protein turnover.

The transport of neurotransmitter receptors from the intracellular compartment to the membrane seems to be mediated by microtubule-based motor complexes (Hirokawa and Takemura, 2005). Once the receptors arrive to the cellular membrane, they interact with the scaffold proteins. Synaptic strength is subsequently regulated by the number of postsynaptic scaffold proteins, a larger number of scaffolds proteins imply more binding sites for neurotransmitter receptor anchoring (Maas et al., 2006). The number of receptors at PSDs could in that case depend on turnover of scaffold components, which represents an additional system for synaptic modulation.

Some important scaffold proteins that bind to inhibitory and excitatory neurotransmitter receptors have been described. Here we will summarize some relevant aspect of these interactions.

Inhibitory glycine receptors (GlyR) are clustered opposite the presynaptic terminal by interaction with the anchoring protein gephyrin. The subsynaptic aggregation of gephyrin is induced by calcium influx (Kirsch and Betz, 1998). Gephyrin has been shown to interact with polymerized tubulin *in vitro* (Kirsch *et al*., 1991), and the postsynaptic localisation of gephyrin/GlyR clusters seems to be mediated by microtubules and microfilaments (discussed by Kirsch, 1999).

The β subunit of the GlyRs has been proven to mediate the binding to gephyrin via its cytoplasmic loop region. In overlay and transfection experiments this binding was shown to involve a motif of 33 amino acids in the central region of the M3-M4 cytoplasmic loop of GlyRs β subunit, with an 18- amino acid core sequence harbouring the dominant binding determinants (Meyer *et al*., 1995). Site-directed mutagenesis studies of this binding motif have revealed that the gephyrin binding activity of the GlyRs β subunit can be assigned to hydrophobic amino acid residues located on one side of a potentially imperfect amphipathic helix (Kneussel *et al.,* 1999). Dimeric gephyrin interacts with GlyRß subunits and that multimerization is required to form a hexagonal gephyrin lattice (Kneussel and Betz, 2000; Sola et al., 2004). GlyR and gephyrin interact and are postulated to be cotransported from an intracellular localization to synaptic sites by the dynein motor complex (Maas et al., 2006).

The ionotropic glutamate receptors NMDAR and AMPAR, and the metabotropic glutamate receptor, mGluR, are clustered on the postsynaptic side of an excitatory synapse by PDZ (or functionally PDZ-like) protein-interaction domains (PDZ is named after the proteins in which these domains were first identified: PSD-95, discs large and ZO-1). PDZ domains are motifs of about 90 amino acids that mediate protein-protein interactions by interacting with the C-termini of proteins (Dong *et al*., 1997).

Different anchoring proteins have been shown to interact with each of the three different glutamate receptors. NMDARs are concentrated at specific domains by interaction with PSD-95 proteins (discussed in Sheng, 1997), AMPARs bind the synaptic protein GRIP (glutamate receptor interacting protein, Dong *et al*., (1997)) and mGluRs are clustered *via* interaction with a protein named Homer (Brakeman *et al.,* 1997).

The interaction between the glutamate receptors and the anchoring proteins seems to be mediated by the intracellular domains of some of the subunits that form the receptor complex (reviewed in Dingledine *et al.* 1999). In fact, some specific amino acid sequences contained in the intracellular region of NMDA and AMPA receptors have been implicated in the spatially distinctive clustering of the complex (Ehlers *et al*., 1995; Kornau *et al*., 1995; Dong *et al*., 1997). Ehlers *et al*. (1995) have identified a sequence of amino acids contained in the

C-terminal domain of the NR1 subunit (proposed to be intracellular), that is responsible for the targeting or anchoring of the NR1 to structures associated with the plasma membrane. They determined that the 37-amino acid CI exon cassette (found in some of the four different NR1 splice variants) was necessary for the formation of NR1-enriched domains. Thus, alternative splicing can regulate the subcellular distribution of the NR1 subunits.

Recently, evidence has emerged that some aspects of assembly, trafficking, targeting, and/or expression of extrasynaptic alpha6-containing GABA(A) receptors in cerebellar granule cells are selectively regulated by AMPA receptor-mediated signalling (Payne et al., 2007).

In addition, the use of the two-hybrid system has shown that a tSXV motif (where S is serine, X is any amino acid and V is valine) contained in the C-terminal of the NR2 subunit and certain NR1 splice forms interacts with the PDZ domain of the PDS-95 protein (Kornau *et al*., 1995). Additional studies have shown that the NMDAR NR1 subunit interacts directly with the 68 kDa neurofilament subunit in a manner regulated by alternative splicing. This interaction occurs between the cytoplasmic C-terminal domain of NR1 and the rod domain of the neurofilament and requires the presence of the alternatively spliced C1 exon cassette in the NR1 subunit (Ehlers *et al.,* 1988).

Moreover, interactions between AMPA and GRIP are mediated by the association of the C-terminal motif SVKI* (*denotes a stop codon) of GluR2, GluR3 and possibly GluR4c (Dong *et al*., 1997). The AMPA-type glutamate receptor/GRIP complex interacts with the scaffold protein named as GRASP-1, which is a neuronally enriched protein (Ye et al., 2000; 2007).

The machinery employed to anchor GABA$_A$Rs at postsynaptic specific domains of the membrane is less clear. Gephyrin has been shown to indirectly interact with γ2 subunit-containing GABA$_A$R complexes (Essrich *et al*., 1998). Furthermore, a GABA$_A$R-associated protein (GABARAP) has been identified that interacts selectively with γ2 subunits and colocalises with GABA$_A$R in cultured cortical neurons (Wang *et al*., 1999). Sequence analysis of this protein reveals that is similar to the light chain 3 (LC-3) of microtubule-associated proteins MAP-1A and MAP-1B. Interestingly, the heavy chain of MAP-1B has been shown to interact with the GABA$_C$R (Hanley *et al.,* 1999).

However, GABARAP is exclusively found at intracellular vesicle structures, and is therefore considered as a factor that participates in receptor transport processes but not in receptor anchoring (Kneussel et al., 2000; Kittler et al., 2001). In fact, O'Sullivan et al. (2005) showed that GABARAP-deficient mice did not display alterations in GABA$_A$R γ2 subunit cluster number at synapses. It could be hypothesised that GABARAP increases either the trafficking of vesicles containing the receptors in the Golgi network and in the cytoplasm toward the cell membrane along tracks of microtubules, that it facilitates receptor insertion, that it stabilizes surface receptors, or all of these events. However, the precise molecular mechanisms underlying GABARAP-dependent transport of γ2 subunit-containing GABA$_A$R remain unclear (review in Kanematsu et al., 2007).

Other molecules that associate with the intracellular loops of GABA$_A$R subunits have been identified, for instance the ubiquitin-like protein Plic-1 which associates with α2, α3, α6, and β1 – 3, and facilitates accumulation of these receptors in dendritic membranes (Bedford et al., 2001). Another is the Golgi-specific DHHC zinc finger protein (GODZ), which associates with the γ2 subunit and is also believed to modulate the γ2 subunit-dependent trafficking pathway (Fang et al., 2006). Table 2 summarizes GABA$_A$R intracellular loop-

associated proteins and the $GABA_AR$ subunits which interact with (reviewed in Chen and Olsen, 2007).

Table 2. Identified $GABA_AR$ scaffold proteins

GBA_AR INTERACTING PROTEINS	SUBUNITS
GABARAP ($GABA_AR$-associated protein)	gamma
GODZ (Golgi-specific DHHC zinc finger protein)	gamma
PRIP1-2 (phospholipase C-related, catalytically inactive proteins)	gamma2 / beta
Plic-1 (ubiquitin-like protein)	alpha / beta
Radixin (actin-binding protein)	alpha5
HAP1 (Huntingtin-associated protein-1)	beta1
GRIF-1 ($GABA_AR$ interacting factor-1)	beta2
BIG2 (brefeldin A-inhibited GDP/GTP exchange factor 2)	beta3

11. Membrane Dynamics of $GABA_AR$

How are neurons able to maintain neurotransmitter receptors at specific membrane domains?

As stated above, the molecular heterogeneity of $GABA_ARs$ determines biophysical and pharmacological properties of the receptor (for review see Sieghart, 2000). In addition, there is strong evidence for the localization of $GABA_ARs$ not only at synaptic sites but also at presynaptic and axonal sites (Nusser et al., 1998, Sakatani et al., 1991, Kullmann et al., 2005). However, it is not clear whether these neurotransmitter receptors are edited and routed to their final postsynaptic domains or if a freely mobile pool of receptors is maintained on the cell surface. The large structural heterogeneity of $GABA_ARs$ led to the hypothesis that there could be a link between $GABA_AR$ gene diversity and the targeting properties of the receptor complex. Neuronal demands could recruit these free receptors to specific regions of the neuronal membrane. The lateral movement in the plasma membrane represents a mechanism by which the local $GABA_AR$ concentration might be regulated to adjust the efficacy of synaptic inhibitory transmission (Velazquez et al, 1989). In fact, we have previously shown that the diversity of subunits isoforms also determines distribution and mobility of $GABA_AR$ on the nerve cell surface (Perán et al, 2001, 2004). It has been suggested that $GABA_AR$ synaptic localization seems to be in part encoded by structural determinants present on alpha1, 2, 3 and gamma subunits (reviewed by Lüscher and Keller, 2004). Nevertheless, the same receptor subunits are also abundant at extrasynaptic sites, suggesting that receptors move in and out of the postsynaptic membrane.

The process of neurotransmitter receptor targeting has been explained by hypothesising that receptors enter the neuronal surface membrane at extrasynaptic sites and reach a final synaptic localization by subsequent mediated lateral membrane diffusion (Kneussel, 2005). Clustering of the receptors at appropriate postsynaptic sites is gained by the binding to submembrane scaffold molecules, (Kneussel and Betz, 2000; Kim and Sheng, 2004). Figure 4 shows how receptors are transported laterally within the plane of the cellular surface by motor proteins. To achieve immobilization at their sites of action, cytoplasmic receptor residues bind to submembrane scaffolds proteins, which are coupled to the underlying cytoskeleton

(Kneussel et al., 2007). The interaction with the scaffolds proteins is mediated by the intracellular loop of the GABA$_A$R subunit (Peran et al., 2006).

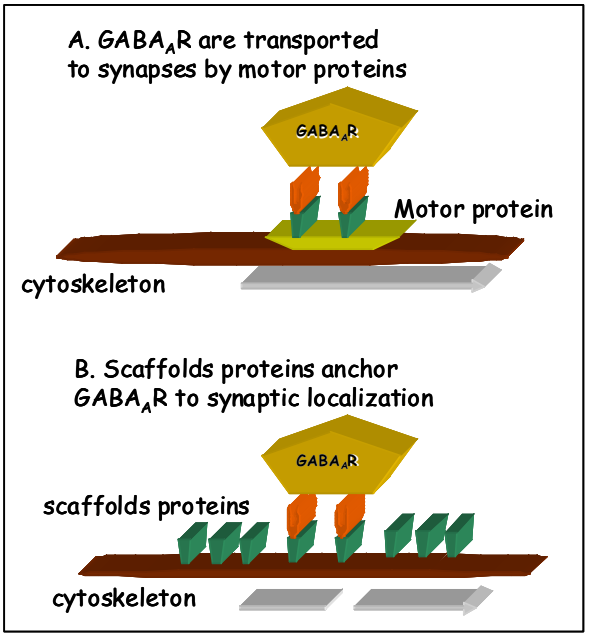

Figure 4. Representation of GABA$_A$R mediated transport and anchoring at synaptic sites. In dark green: scaffolds proteins; in orange: intracellular loop of the GABA$_A$R subunit; in light green: GABA$_A$R complex; in brown: cytoskeleton.

The association of scaffold molecules with neurotransmitter receptors outside the synapse and their participation in the regulation of trafficking have been described elsewhere (DeSouza and Ziff, 2002; Wenthold et al., 2003; Meier and Grantyn, 2004). Further complexity on the processes that localize inhibitory neurotransmitter receptors at postsynaptic sites emerges from GABA$_A$Rs comprising a highly heterogeneous set of membrane proteins that are differentially expressed in the mammalian CNS. New findings in the study of GABA$_A$Rs support the idea that the subunit composition of the complexes determines the type of scaffold proteins that bind to the receptors (reviewed in Lüscher and Keller, 2004). For instance, gephyrin and dystrophin have been described as clustering proteins for GABA$_A$Rs. Although these proteins seem to regulate the synaptic clustering of GABA$_A$Rs, they do so by distinct mechanisms (Knuesel et al., 1999). It has been recently shown that down-regulation of the postsynaptic anchoring protein gephyrin increased the lateral mobility of GABA$_A$R subunits (Jacob et al., 2005), but biochemical data for direct interactions between any GABA$_A$R subunit and gephyrin is still lacking. In fact, Maas et al. (2006) have provided new evidence that gephyrin participates only in intracellular transport. New evidence has shown that a member of the ERM family, a protein called radixin, anchors GABA$_A$Rs at cytoskeletal elements via GABA$_A$R α5 subunit binding (Loebrich et al., 2006).

11.1. The Use of FPR and SPT in the Study of $GABA_AR$ Lateral Mobility

The use of quantum dot labeling of single glycine receptor molecules (Dahan et al., 2003) or single-molecule fluorescence microscopy of AMPA receptors (Tardin et al., 2003) have confirmed that individual neurotransmitter receptor molecules can be observed moving in and out of synapses independent of endocytosis. Several groups are involved in the study of how $GABA_AR$ are anchored and maintained at specific membrane domains (review in Lüscher and Keller 2004). Their approaches, however, depend on biochemical reconstitution of the system. In addition, it is known that the interaction of membrane proteins with the cytoskeleton is complex and can involve interactions or mechanisms of restriction or compartmentalisation that are above interactions at the secondary or tertiary structural levels that are not commonly revealed in reconstituted systems.

To investigate the mechanisms responsible for the membrane dynamics of $GABA_AR$ the use of techniques that allow a direct measure of the receptor lateral mobility is essential. There are two powerful techniques that permit the lateral motions of receptors in the membranes of single, living cells to be examined under physiological conditions, Single particle tracking (SPT) (Kusumi and Sako, 1996; Babcock et al., 2004) and Fluorescence Recovery After Photobleaching (FRAP) (Reits and Neefjes, 2001). In FRAP experiments, fluorescently labelled molecules are irreversibly photo bleached by a high-intensity laser beam that briefly illuminates a small area of the cell. Diffusion of surrounding non-bleached molecules leads to recovery of the fluorescence. A higher mobility of the molecules results in a shorter time of recovery. From the recovery curve, it is possible to obtain estimates of the diffusion coefficient and immobile fraction (Axerold et al., 1976). In SPT, computer-enhanced video microscopy is used to track the motion of proteins or lipids on the cell surface. Individual molecules or small clusters are observed, with a typical spatial resolution of tens of nanometers and a typical time resolution of tens of milliseconds. Single receptor trajectories can be measured (Saxton and Jacobson, 1997).

We have already provided evidence, using SPT and FRAP in transfected cells, that $GABA_ARs$ mobility depend on the subunit composition of the complex (Peran et al., 2001; 2004). Furthermore we have shown that a specific region within the alpha1, the M3/M4 cytoplasmic domain, plays a role in the immobilization of the receptor (Peran et al., 2006). The intracellular loop M3/M4 contributes most of the cytoplasmic domain of these receptors and includes multiple interaction sites for putative trafficking and postsynaptic scaffold proteins as well as phosphorylation sites for diverse serine/threonine and tyrosine kinases (Lüscher and Keller, 2004).

12. Conclusion

The clustering of membrane proteins into discrete and functionally significant domains is an important feature of neuronal cell biology. Synaptic plasticity is increased by the fact that neurotransmitter receptors are localized in and out of synaptic sites and are routed to their specific synaptic localization where they interact with scaffold proteins. These proteins belong to a quite large family with marked differences among the members and are exposes to a continuous turnover which indirectly contribute to synaptic plasticity. The heterogeneity of the described scaffold proteins that bind $GABA_ARs$, and the possible ones which have not

been described yet, make the dynamics of $GABA_AR$ sorting and mobility and incredible complex problem.

The relation between $GABA_AR$ subunit composition and the lateral mobility of the complex on the membrane has not yet been fully determined. Receptors containing different subunit complements appear to have different rates of motion. This property would provide a neuron with a new type of synaptic plasticity, that is, the ability to route specific complexes to different neuronal domains according to their subunit composition, and the ability to have different pools of receptors that are more or less able to be rapidly moved to a different location.

Even though our knowledge of synaptic mechanisms has increased in the last years, further identification of the processes of receptors-scaffold proteins interaction and PSDs dyanmics, as well as functional understanding of postsynaptic protein turnover and receptor lateral mobility is still needed.

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Chapter 7

The Role of NMDA Glutamate Receptors in Pain and Anesthetic-Induced Neurodegeneration during Development

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Abstract

Pain enables the specialized nervous system to detect sources of bodily harm and trigger responses which can prevent or mitigate potential damage. Chronic or intractable pain is the most common reason for a visit to a physician in the United States. Recent studies demonstrate that plastic changes in neurons of the central nervous system (CNS) may contribute to the mechanisms that underlie pain hypersensitivity. The neurotransmitter glutamate and its membrane receptors play significant roles in generating and maintaining a variety of sustained neuronal responses. Activation of NMDA receptors and the consequent influx of calcium are major events that contribute to pain processing. For this reason, antagonists of NMDA receptors, such as ketamine and phencyclidine are commonly used as anesthetics in clinical practice. However, the disturbance of glutamate systems may cause a variety of neuronal toxicities, such as abnormal neuronal development, abnormal synaptic plasticity and neurodegeneration. This review focuses on the role of glutamate receptors, especially NMDA receptors, in pain processing. As tools for clinical interventions, antagonists of NMDA receptors will be discussed with particular emphasis on their ability to induce neurodegeneration during development.

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1. Introduction

As everyone knows, pain is a common bond that links all humanity. Pain is defined as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" [1]. Without the signals of pain as an early warning system, we would suffer severe and possibly irrevocable harm. Many patients exhibit chronic and persistent pain and it becomes one of the most common sources of long term suffering and disability.

It has become increasingly evident that allodynia, referred pain, and widespread hyperalgesia play an important role in chronic or persistent pain states [2]. Hyperalgesia can be produced by tissue injury and by damage to peripheral nerves or the central nervous system. The initiation of primary hyperalgesia is believed to result from the sensitization of primary afferent nociceptors [3-5]. Central sensitization is a mechanism that may underlie secondary hyperalgesia. During central sensitization, the excitability of central neurons is enhanced. Increased background discharges, expanded receptive fields, enhanced responsiveness to peripheral nerve volleys and to innocuous and noxious stimulation can be observed in sensitized central neurons. [4, 6-10]. One of the intracellular transduction pathways that may contribute to central sensitization involves N-methyl-D-aspartate (NMDA) receptors. NMDA receptors and the consequent Ca^{2+} influx caused by their activation are major events that may contribute to central sensitization [11-21]. Therefore, the development of NMDA receptor antagonists has been the goal of research in the treatment of persistent pain. On the other hand, activation of NMDA receptors mediates most of the excitatory neurotransmission in the CNS. Normal neuronal development, differentiation and outgrowth depend on stimulation of NMDA receptors. Long-term blockade of NMDA receptors by anesthetic agents during the synaptogenesis stage in developing brain can cause apoptotic degeneration of neurons.

This review briefly discusses the structure of NMDA receptors, the role of NMDA receptors in pain processing, and characteristics of different classes of NMDA receptor antagonists. This is followed by a discussion of the effects of these antagonists on the developing brain of rodents and nonhuman primates.

2. NMDA Receptors

Intracellular signaling processes are critical for the normal development and function of the nervous system. In particular, glutamate receptors are critical proteins that play a crucial role in generating and maintaining a variety of sustained neuronal responses. Among them, NMDA receptors (NMDAR) and the consequent Ca^{2+} influx that follows activation of NMDA receptors make major contributions to long-lasting pain states.

Structure of NMDA Receptors

Glutamate, a critical excitatory neurotransmitter, modulates neurotransmission, neuroplasticity, neuronal outgrowth and survival via two classes of receptors – fast ligand-

gated ionotropic receptors and slower metabotropic (mGlu) receptors. Ionotropic glutamate receptors (iGluRs) consist of three subtypes: *N*-methyl-*D*-aspartate (NMDA); α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA); and kainate (KA) receptors [22-24]. NMDA-type glutamate receptors (NMDAR) are widely distributed throughout the CNS.

NMDAR are heterodimers that incorporate different subunits of three types: NR1, NR2 and NR3. To form a functional NMDAR channel, the NR1 subunit is required as an essential channel-forming component and at least one NR2 subunit is required. Multiple splice variants of a single gene generate eight different NR1 subunits. There are four different NR2 subunits (A, B, C and D) and two NR3 subunits (A and B), which are encoded by six separate genes [25-27].

As an ion channel, the NMDAR has a large extracellular N-terminus, three transmembrane domains (TM1, TM3 and TM4), a re-entrant membrane pore loop (M2), an extracellular region between TM3 and TM4, and an intracellular C-terminus, which interacts with a number of intracellular proteins and leads to signal transduction.

There are two critical domains in the extracellular region of the NMDA receptor units: the N-terminal domain (NTD), which is important in subunit assembly, and the agonist binding domain (ABD), which contains the ligand binding site. Activation of NMDA receptors requires the simultaneous binding of glutamate and the co-agonist glycine for the efficient opening of the ion channel which is nonselective to cations. The agonist glutamate binds to the ABD of NR2, whereas glycine binds to the ABD of NR1 and/or NR3 [23, 27-30].

The activation and function of NMDA receptors are modulated by a variety of endogenous and exogenous compounds: Mg^{2+} acts as a voltage-dependent channel blocker, whereas Zn^{2+} acts as a noncompetitive and voltage-independent blocker of the NMDA current. The activity of NMDA receptors can be partially inhibited by changes in H^{+} concentration. Through phosphorylation of the receptor, NMDA-activated currents are enhanced by an increase in the probability of channel openings [11, 23, 27].

The Role of NMDA Receptors in Pain Processing

The mechanisms by which tissue injuries and inflammation produce a pain state are being extensively studied. There are numerous data suggesting that inflammation and injury not only initiate a local change in the sensitivity of nociceptors but also trigger central sensitization, an increase in the excitability of neurons in the spinal cord and brain. Glutamatergic activation of NMDA receptors is one possible mechanism for an increase in synaptic strength and may be involved in the induction and development of prolonged pain states. NMDA receptors localize in different levels of the pain processing pathways.

Periphery

Both NMDA and non-NMDA ionotropic glutamate receptors contribute to peripheral nociception [31]. The Fibromylgia syndrome (FMS) is known as a chronic, generalized pain state accompanied by a number of associated symptoms. Many skin biopsy findings from patients with FMS demonstrate increased expression of NMDA 2D subtypes [32, 33]. Electron microscopic analyses have demonstrated that the NR1 subunit of the NMDA

receptor is localized on cutaneous unmyelinated sensory axons innervating the hairy skin of the rat's tail. Immunohistochemical staining has also shown expression of NR1 subunits in unmyelinated fibers in the glabrous skin of the rat hind paw. A glutamate injection into the periphery leads to nociceptive behaviors, such as mechanical allodynia, mechanical hyperalgesia and thermal hyperalgesia. All of these nociceptive behaviors can be blocked by the co-injection of glutamate antagonists [34-37]. In the complete Freund's adjuvant (CFA)-induced inflammation model, there is a significant increase in the proportions of unmyelinated and myelinated axons labeled for NMDAR (NR1) in the cutaneous nerve fibers in the inflamed paw compared to the contralateral non-inflamed rat hindpaw [38]. NMDA receptors are also localized on unmyelinated axons at the dermal-epidermal junction in hairy skin of humans [39]. A local injection of ketamine, a NMDAR antagonist, significantly attenuates masseter mechanical sensitization and muscle pain in healthy young women [40]. Subcutaneous injection of glutamate into the plantar surface of the rat hind paw activates peripheral NMDAR and results in a dose-dependent increase in extracellular adenosine, a molecule that plays a regulatory role in both pain and inflammation. Application of NMDA receptor antagonists blocks this release [41].

A variety of NMDAR subunits are expressed in the majority of the cell bodies of dorsal root ganglion (DRG) primary afferent neurons. Immunohistochemical staining and retrograde tracing techniques show NR2B subunit expression in small-diameter primary afferent DRG neurons that give rise to the sciatic nerve fibers [42]. In the studies by Mayer's group, the predominant NMDAR subunit expression in DRG includes NR1, NR2B and NR2D. NR2B-containing NMDARs are present in both A- and C-fibers, whereas NR2D-containing receptors are present only in C-fibers. Both somatic and visceral tissues of adult rats are innervated by activated NMDARs expressed in DRG neurons and most of these NMDARs are composed predominantly of NR2B and NR1 subunits [43-45]. Experimental colitis in the rat upregulates the activity and expression of NR2B in all DRG neurons innervating the area of inflammation [46]. Immunofluorescence staining demonstrates that the distribution of the NR1-like immunoreactive neurons in the lumbar DRG (L4–5) of normal rats and most of the immunoreactive products are localized in the cytoplasm. In the inflammatory pain model, subcutaneous CFA injection into the rat hindpaw causes a significant increase in the percentage of NR1 labeled neurons compared to the contralateral side of DRG. NR1 subunits of NMDAR in the DRG also have close relationships with the generation and development of neuropathic pain [47, 48]. Activation of NMDAR in DRG by epidural glutamate perfusion induces nociceptive behavior-mechanical hyperalgesia in rats [49].

Spinal Cord

An abundance of evidence suggests that inflammation and injury not only initiate a local change in the sensitivity of nociceptors but also trigger central sensitization, an increase in the excitability of neurons in dorsal horn neurons of the spinal cord. Abundant evidence demonstrates that glutamatergic transmission at NMDA receptors is one possible mechanism for an increase in synaptic strength and is involved in the induction and development of central sensitization.

In the rat spinal cord, the NR1 subunit is commonly expressed in dorsal horn neurons of the spinal cord, whereas NR2B is the most commonly expressed NR2 subunit. Both subunits

are important in pain conditions [50-56]. There is considerable evidence that NMDA receptors in the spinal cord are activated after peripheral inflammation [15, 25, 56-62].

Following peripheral inflammation, the development of hyperalgesia and allodynia depends on NMDA receptor-mediated central changes in synaptic excitability. NR1 and NR2B subunits are involved in the initiation of neuropathic pain due to peripheral nerve injury and spinal cord injury [63]. Application of NMDA receptor antagonists at the time of nerve injury prevents injury-induced changes in spinal NR1 and NR2B subunit expression and attenuates nociceptive behaviors, such as mechanical allodynia and hyperalgesia [64-67]. It has also been demonstrated that spinal NMDA receptors are involved in a rat model of visceral hypersensitivity and partially mediate the processing of both noxious and innocuous colorectal stimuli in colorectal distention in rats [68-69]. Activation of NMDA receptors also contributes to the induction and development of pain in arthritis that is induced by injection of kaolin and carrageenan into the knee joint of rats [70-72]. Studies have demonstrated that delivery of NMDA receptor antagonists inhibits the hypersensitivity of spinal cord nociceptive neurons [73-78]. Additionally, systemic or intrathecal administration of NMDA receptor antagonists alleviates nociception and hyperalgesia [14, 63, 69, 72, 79-89].

As a critical protein in the central nervous system, the sensitivity of NMDA receptors is strictly modulated by protein phosphorylation, a major mechanism by which glutamate receptor function is regulated [90]. The enhanced responsiveness of the NMDA receptor after its phosphorylation is an important means by which central sensitization is initiated and maintained [11, 15]. Through phosphorylation of the receptor, NMDA-activated currents are enhanced by increasing the probability of channel openings. This may cause an increased sensitivity to sensory processing in the dorsal horn of the spinal cord that results in greater firing of spinal efferent systems in response to given noxious inputs and normally innocuous stimuli [15, 57, 91-95]. Since a prolonged time course of central sensitization depends on the activation of signal transduction cascades, central sensitization can be modulated, either up or down, by regulating the phosphorylation status of NMDA receptors. Protein kinases, such as PKC, PKA, PKG and CaMKII, and the opposing enzyme, protein phosphatase, affect the responses of dorsal horn neurons through modulating the phosphorylation of NMDA receptors. The consequences of these modulations can be central sensitization, long-lasting inhibition, and/or changes in gene expression [96-101]. Application of inhibitors of protein phosphatase have been shown to augment and prolong the time course of phosphorylation of NR1 and NR2B subunits of NMDA receptors (NMDARs) in the dorsal horn neurons and lead to prolonged central sensitization [102-105].

Brain

Many experiments in pain research show that pain is processed in several distributed brain regions, instead of in a single cortical area. NMDA receptors are also widely distributed in the brain. During the initiation and development of persistent pain states, both the expression and function of NMDA receptors are changed. In situ hybridization and immunocytochemical studies have demonstrated that each NMDA receptor subunit gene is expressed in the rat brain with regional differences. NR1 expression widely exceeds that of the other subunits, such as NR2A-D and NR3A. Compared with NR1, NR2 subunits are distributed more regionally. NR2A and NR2B are distributed predominantly in forebrain structures, whereas NR2C and NR2D are expressed mostly in selected areas, such as the

cerebellum [106, 107]. In the CFA-induced hind paw inflammation model, NMDA receptor gene expression was investigated in the rostral ventromedial medulla (RVM), a critical structure in pain modulatory circuitry. Due to the CFA injection, mRNAs of NR1, NR2A and NR2B subunits were increased [108]. Recent studies suggest that the anterior cingulate cortex (ACC), a pivotal structure in the central processing of pain, receives ascending sensory signals from the thalamus and plays a critical role in pain discomfort. Transgenic mice over expressing the NR2B subunit in the ACC and insular cortex exhibit enhanced persistent pain and allodynia after peripheral injection of formalin or CFA [109-112]. The amygdala, a part of the limbic system, is involved in emotional and cognitive aspects of behavior. Recent evidence suggests that the amygdala acts as a key player in the emotional-affective component of pain. Persistent changes in synaptic transmission in the amygdala, long-term potentiation (LTP) and long-term depression (LTD) are believed to be initiated by calcium influx through NMDA receptors. In carrageenan-induced arthritis, the expression of phosphorylated NR1 protein was increased in the central nucleus of the amygdala (CeA), which is defined as the 'nociceptive amygdala' [113, 114]. In the same model of arthritic pain, activation of NMDA receptors contributes to pain-related sensitization of amygdala neurons [115]. In spared nerve injury-induced neuropathic pain and cisplatin-induced neuropathy, antagonists of NMDA receptors upregulate the expression of NR2B in the medial prefrontal cortex [116]. The periaqueductal grey (PAG) area is known as a critical structure in the descending modulation of pain. It inhibits nociceptive transmission within neurons in the spinal cord and acts as a connection between diencephalic and forebrain structures involved in pain processing. The expression of NMDA receptors was investigated using in situ hybridization. NR1 mRNA was found to be dominantly expressed through caudal to rostral areas of the PAG, whereas mRNAs of NR2A and NR2B subunit were not detected, and the NR2C gene was rarely expressed in PAG. Recent evidence suggests that inflammatory pain, induced by the subcutaneous injection of formalin, causes changes in excitability in neurons in PAG and injections of NMDA receptor antagonists into dorsal PAG attenuate nociceptive behavior [117-119].

3. Antagonists of NMDA Receptors

Glutamate is generally known as one of primary excitatory neurotransmitters of the CNS. It participates in neuronal migration, differentiation, development and plasticity throughout the entire life span. As a type of glutamate receptors, NMDA receptors play a critical role in fast excitatory synaptic transmission, and dysfunctional or over activation of NMDA receptors may cause disturbances in plasticity and the development of neurotoxicity [23, 120, 121]. Recently, numerous reports have demonstrated that NMDA receptors contribute to physiological processes, such as memory and learning and neuronal development [122-128]. Over activation of NMDA receptors are also involved in a variety of neurodegenerative processes associated with acute and chronic neuropathologies including ischemic stroke, seizure disorders, Alzheimer's disease, schizophrenia, Parkinson's disease, Huntington's disease, hypoxia/ischemia, anxiety disorder and pain perception [23, 112, 121, 127, 128]. Due to the important role of NMDA receptors in the modulation of pain processing, it is conceivable that antagonists of NMDA receptors may be effective analgesics. Therefore, antagonists of NMDA receptors are potential targets for the treatment of persistent pain.

Mechanisms

NMDA receptors are located in the periphery, spinal cord and CNS and are involved in both peripheral and central sensitization. Their antagonists are expected to have effects on persistent pain depending on the access of these compounds to each of these sites of action. In the late of 1980s, broad-spectrum competitive NMDA antagonists were developed. Windup responses in persistent pain were inhibited by these molecules. In the early 1990s, receptor subtype-selective antagonists were identified and a new class of molecule, which is able to selectively inhibit the NR2B subunit was characterized [23, 27, 119, 129-131].

Competitive antagonists, such as D-AP5 and 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), are compounds acting at the glutamate binding site on the NR2 subunits and, they show some selectivity between the different NR2 subunits (affinity ranking NR2A>NR2B>NT2C>NR2D). In the periphery, administration of D-AP5 was shown to reduce bee venom-induced mechanical allodynia and hyperalgesia. Pre-treatment with D-AP5 into the same area of the bee venom injection site reduced the bee venom-increased spontaneous responses of wide-dynamic range (WDR) neurons in the spinal cord. Intrathecal application of D-AP5 alleviates the mechanical allodynia in rats with spinal cord injury. Pain behaviors in both acute and tonic phases of the formalin test were significantly reduced by a D-AP5 injection into the dentate gyrus of the hippocampus and PAG. In an arthritic pain model, infusion of D-AP5 into the amygdala inhibited the increased responses to normally innocuous stimuli in arthritis [63, 115, 132-134]. Other antagonists with the same characteristic include perzinfotel, CPP, selfotel, and WAY-129, a derivative of perzinfotel [129].

Noncompetitive ion channel blockers also do not discriminate between the different NMDAR subtypes since they tend to penetrate the CNS. Channel (pore) blockers including ketamine, which is used as a dissociative anesthetic, shows analgesic activity in a number of preclinical inflammatory and neuropathic pain models [89, 135]. In patients with acute neuropathic pain after oral surgery, pain was relieved with an intramuscular injection of ketamine. Peripheral application of ketamine had no effect on glutamate-evoked masseter muscle pain. Low doses of ketamine are used as an adjuvant drug in current perioperative pain management. Ketamine also plays a role in the treatment of opioid resistant pain by attenuating central sensitization involved in hyperalgesia and allodynia [136-140]. Topical application of ketamine is useful for the treatment of complex regional pain syndrome type I (CRPS I), postherpetic neuralgia, and other neuropathic pain syndromes [141-144]. Intrathecal application of MK801 (dizocilpine), another channel blocker, blocked thermal hyperalgesia, but not tactile allodynia, induced by spinal nerve ligation (SNL). Infusion of MK801 and dextromethorphan attenuate or reverse the development of morphine resistant pain [145-146]. Since function of the NMDA receptor is essential for normal neuronal activity, systemic application of these channel blockers leads to intolerable side effects resembling those induced by phencyclidine (PCP), including hallucinations, dreams, salivation, and an increase in sympathomimetic activity [129, 144]. In order to gain a greater therapeutic index for this class of antagonists, some low-affinity channel blockers have been identified that can inhibit spinal neurotransimission in neuropathic pain yet exhibit fewer side effects. The clinical results for these compounds including dexthromethorphan, amantadine, memantine, bicifadine, CNS-5161 and neramexane are not satisfying. Among them,

memantine is approved for use in the treatment of Alzheimer's disease dementia and attenuates wind-up pain in rodents [119, 129].

As a co-agonist to the NMDA receptor, glycine has its recognition site located on the NR1 subunit. The binding of glycine reduces the rate of desensitization of the NMDA receptor and consequently enhances receptor responses. Therefore, glycine site antagonists potentiate receptor desensitization and inhibit NMDA responses. In central sensitization, the responses of NMDA receptors are usually enhanced. With glycine site antagonists, it is possible to inhibit the enhanced NMDA receptors without affecting normal physiological processes. The compounds in this class include: 5,7-dichlorokynurenic acid (5,7-DCK), L-701,324, (+)-HA966 and GV 196771. These antagonists have proved effective in a variety of animal pain models [119, 129,147]. Recent experiments in Kayser's lab have demonstrated that (+)-HA966 has antinociceptive effects in a rat model of peripheral neuropathy [148-150]. Both L-701,324 and 5,7-DCK inhibited Phase II of formalin-evoked nociceptive behavior, attenuated cold allodynia in the chronic constriction injury model and tactile allodynia in the SNL model, without apparent CNS-mediated side effects [151].

Recently, NR2B antagonists have been the compounds of considerable focus. NR2B receptors are widely distributed in brain, dorsal horn and dorsal root ganglion, and are believed to be the primary sites for the transmission of pain signals and in the initiation of central sensitization. Molecules in this class include ifenprodil derivatives, including eliprodil, Cl-1041, Ro 2506981 and CP-101,606. They are potent NR2B-selective antagonists and are thought to be promising pain killers with less CNS-mediated side effects in a variety of animal pain models and human trials [27, 107, 112, 129, 152-156].

Anesthetic-Induced Neurodegeneration during Development

NMDA receptor antagonists, such as ketamine, MK801 and PCP are all noncompetitive NMDA receptor antagonists. It is well known that activation of NMDA receptors by glutamate plays an important role in brain development. Normal activation of NMDA receptors is critical for neuronal differentiation, establishment and outgrowth. Therefore, continuous blockade of NMDA receptors, especially during a critical time of CNS development, may reduce the establishment of redundant synapses in the developing brain and cause neurotoxicity, neurodegeneration and/or apoptosis [26, 121, 127, 128, 157-159]. This critical period is a time of rapid growth of neurons and synaptogenesis, which is also called the brain growth spurt. It occurs in different species at different times relative to birth. In rats and mice, the brain growth spurt occurs during the first three postnatal weeks, whereas in humans it extends from the sixth month of gestation to several years after birth. During this period, the brain is more susceptible to dysfunction of NMDA receptors. Apoptosis may be observed in the cortex and limbic system of the brain due to the application of NMDA antagonists [159, 160].

PCP is a drug of abuse with psychotomimetic effects. Administration of PCP can cause both positive and negative symptoms of schizophrenia in humans. The chronic application of PCP leads to increases in locomotor activity and stereotypic behavior in female rats, and apoptosis was found in the olfactory tubercle and piriform cortex of these animals. Additionally, the expression of NR1 mRNA in the rat forebrain was upregulated, synthesis of

NR1 and NR2A were increased, and receptor composition was also changed in rats chronically treated with PCP. On the other hand, acute application of PCP in high doses also induces neurotoxicity in rats, and it is characterized by neuronal vacuolization. In perinatal rats, PCP-induced neuronal apoptosis results in long-lasting deficits in spatial learning and sensorimotor and locomotor activity. Superoxide radicals may be involved in the mechanism underlying these long-lasting changes, since the neurodegeneration and associated defects in prepulse inhibition behaviors were attenuated by pretreatment with a superoxide dismutase mimetic, M40403. During the early postnatal period, acute PCP application to rats causes wide spread cortical neurodegeneration, whereas subchronic PCP administration induces selective neurotoxicity in the cortex. Upregulation of NR1 and NR2A subunits following subchronic application of PCP is partly responsible for the noted apoptotic cell death [161-170].

Compared to PCP, MK-801 is a more potent NMDA channel blocker. PCP and MK-801 are similar in their cortical neurotoxic effects. Repeated injection of MK-801 is used as an animal model of psychosis and continuous application of MK-801 often induces stereotypical behavior [157, 169, 171]. In cell death analyses, application of MK-801 combined with lamotrigine causes a significant increase in TUNEL-positive cells in multiple brain regions (striatum, thalamus, and cortical areas) of rat pups [172]. Similar to PCP, oxidative stress plays a role in MK-801-induced neuronal toxicity in the cortex of rats since pathological changes can be attenuated by several antioxidants [173-175]. In neonatal rats, administration of MK-801 during the first two weeks of life causes abnormal axonal arborization in retinal connections to the superior colliculus, resulting in abnormal visual responses [176].

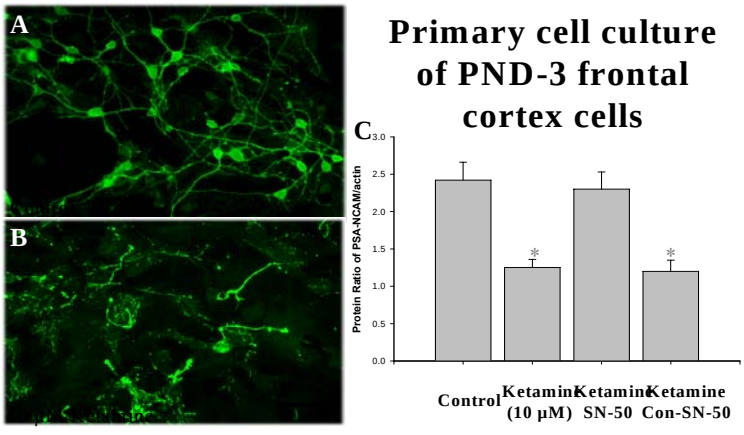

Figure 1. Effects of ketamine and SN-50 on the decrease in PSA-NCAM expression in monkey frontal cortical cultures. PSA-NCAM immunoreactivity was intense on the surface of cell bodies and processes of neurons in the control cultures (A). 10 μM ketamine diminished PSA-NCAM expression. PSA-NCAM expression is weak and characterized by pieces of residue, fragmentation and neuronal shrinking (B); scale bar = 50 μm. Densitometry measurements of Western analysis were used to calculate a ratio of PSA-NCAM to actin (by stripping the membranes) in each lane in tree independent experiments. SN-50 (2.5 μM) effectively prevented PSA-NCAM reduction induced by ketamine. No protective effect was observed from the inactive control peptide for SN-50 (2.5 μM). A probability of $p < 0.05$ was considered significant.

Ketamine is a dissociative anesthetic with a short duration of action that produces a dissociative state, a sense of detachment from one's physical body and the external world. It is widely used in pediatric medicine and has a wide- range of effects in humans, including analgesia, anesthesia, hallucinations, bronchodilation and neurotoxicity. Ketamine is primarily used for the induction and maintenance of general anesthesia, usually in combination with some sedative drug. As with other NMDA channel blockers, ketamine causes neurotoxicity in neonatal rats when given repeatedly during the brain growth spurt period. Suppression of NMDA receptor function by ketamine triggers apoptosis in neurons [121, 127, 157, 158, 160]. Ketamine induces heat shock protein HSP72, a marker of nonlethal neuronal injury, in neurons of the posterior cingulate and retrosplenial cortex of the rat [177]. Administration of ketamine to PND7 infant rats triggers a massive wave of apoptotic neurodegeneration affecting many neurons in several major regions of the developing brain [178]. In the developing brain of neonatal mice, administration of ketamine induces severe degeneration of neurons and significant increases in neuroapoptosis were found in the parietal cortex. At 2 months of age, ketamine treated mice display severe deficits for motor activity and learning performance [179, 180]. In addition to it neurotoxic effects, low concentrations of ketamine produce deficits in dendritic arbor development in immature GABAergic neurons and may potentially inhibit the normal development of neural networks [181, 182].

Since ketamine is widely used in pediatric anesthesia, it is important to know whether the ketamine-induced neurodegeneration observed in infant rodents has scientific relevance to its effects in humans. Nonhuman primates have physiological, metabolic and reproductive systems similar to those of humans, and therefore make a good animal model for assessing the effects of ketamine in humans. The brain growth spurt period in both humans and nonhuman primates extends much longer than in rodents. A monkey model, which studies ketamine exposure during the brain growth spurt is the most appropriate for determining whether ketamine-induced neurotoxicity in young rodents occurs in primates as well [121, 127, 128].

The α-2,8-linked sialic acid polymer on neural cell adhesion molecules (PSA-NCAM) is a neuronal marker and an important regulator of cell-surface interactions [183]. The regulation of PSA-NCAM expression by NMDAergic activity plays a critical role in neuroplasticity during development, particularly in NCAM-mediated cell-cell interactions and synapse formation [184]. According to *in vitro* studies, ketamine treatment of frontal cortical cells collected from the neonatal rhesus monkey (PND-3) caused a substantial decrease in mitochondrial metabolism of MTT along with a concomitant decrease in PSA-NCAM immunostaining (Figures 1A and B). Co-administration of SN-50, a peptide inhibitor of NF-kB transport, dose-dependently protected against the neurotoxic effects of ketamine, indicating NF-κB signaling is involved in the mechanism underlying ketamine-induced neuronal cell death (Figure 1C). In this process, NR1 protein expression is upregulated and NR1 antisense oligonucleotides (ODNs) can protect neurons from ketamine-induced apoptosis [185]. Intravenous application of ketamine in rhesus monkeys for 24 hours produces enhanced neuronal apoptosis, especially in animals at early developmental stages. Electron microscopic analyses indicate that ketamine-induced neuronal cell death is both apoptotic and necrotic in the primate. NR1 subunit mRNA is increased by ketamine in the frontal cortex, which is consistent with the location of the noted enhanced cell death. Short term exposure to ketamine (3 hrs of intravenous aesthesia) does not lead to neuronal cell death, even in monkeys at early developmental stages [26].

Recent data from both the developing rodent and nonhuman primate have demonstrated that the long-term blockade of NMDA receptors with channel blockers in the developing brain may result in a compensatory up-regulation of NMDA receptors, especially NR1 subunits. In addition, the composition of NMDA receptors may also be changed. Neurons that have these upregulated NMDA receptors are thought to be more vulnerable to the toxic effects of glutamate. After washout of the antagonists, upregulation of NMDA receptors leads to enhanced responses and consequently results in the toxic accumulation of intracellular Ca^{2+}, which eventually causes neuronal cell death [170, 185, 186].

4. Conclusion

NMDA receptors play a key role in both physiological and pathophysiological pain states. Some NMDA receptors antagonists are used as analgesics and anesthetics. Based on recent approaches to the treatment of chronic and persistent pain, NMDA antagonists which can selectively inhibit NMDA NR2B subunits may represent potential targets for pain relief with fewer side effects. During the development of the CNS, neuronal migration, differentiation and synaptic plasticity depend on excitatory neurotransmission through NMDA receptors. Continuous blockade of NMDA receptors by channel blockers during development results in neuronal degeneration and apoptosis in rodents and nonhuman primates. The effects in humans are currently unknown.

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Chapter 8

Spinal Processing of Pain: A Review on NMDA Receptor-Mediated Activation of Arachidonic Acid and Nitric Oxide Signalling Pathways

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Abstract

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system. This amino acid activates both metabotropic and ionotropic glutamate receptors. Among the latter class of receptors is the *N*-methyl-D-aspartate (NMDA) receptor, which activation at the level of the spinal cord increases neuronal responsiveness resulting in central sensitisation. This is manifested clinically as altered pain in which non-noxious stimulation is perceived as painful (allodynia) and normally painpul stimuli are felt as even more painful (hyperalgesia). The increased nociceptive response is believed to be due to the entry of Ca^{2+} into the neurons, which triggers different metabolic pathways activating a number of enzymes and ultimately immediate-early genes. The increase in intracellular Ca^{2+} concentration after NMDA receptor stimulation activates phospholipases. Activation of phospholipase A_2 hydrolyses membrane phospholipids increasing the level of free arachidonic acid. Arachidonate, in turn, may be converted by cyclooxygenases into prostaglandins, which activate specific prostanoid receptors. In addition to activating phospolipases, Ca^{2+} entering through NMDA receptors activates calcineurin and binds to calmodulin forming the Ca^{2+}-calmodulin (CaM) complex. Calcineurin desphosphorylates the constitutive nitric oxide synthase (NOS) enzymes, which catalytic activity is usually reduced by protein kinase C-induced phosphorylation, and the CaM complex further activates the enzyme. NOS catalyze the conversion of L-arginine into L-citrulline and NO, and this gas activates guanylate cylases to increase cGMP production. Apart from interacting with each other, retrograde second-

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messengers resulting from these cascades cause presynaptic and interneuron augmentation of neurotransmitter and/or neuropeptide release, as well as the formation and expression of new receptors. All these complex mechanisms involved in the amplification and prolongation of neural responses, which underlie many forms of spinal sensitisation, are discussed in this chapter.

Spinal NMDA Receptors

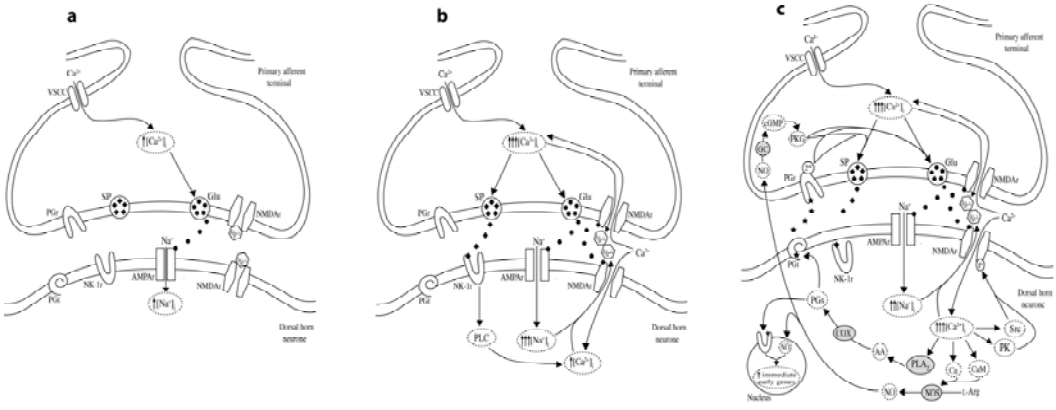

Figure 1. Schematic representation of pain neurotransmission in the spinal cord. **(a)** Under normal conditions, afferent terminal stimulation (mainly that of Aδ-fibres) increases intracellular calcium concentration ($[Ca^{2+}]_i$) allowing depolarisation of the cell membrane and release of stored glutamate (Glu). On dorsal horn neurones, Glu activates α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid receptors (AMPAr) leading to an increase in intracellular sodium concentration ($[Na^+]_i$) and a brief neuronal firing. **(b)** During repetitive stimulation of primary afferent C-fibres, a larger $[Ca^{2+}]_i$ releases more Glu into the synaptic cleft producing a larger depolarisation of the dorsal horn neurone, which releases the magnesium (Mg^{2+}) block from *N*-methyl-D-aspartate receptors (NMDAr). This increases $[Ca^{2+}]_i$ even more in primary afferent terminals with the subsequent release of substance P (SP). Activation of neurokinin-1 receptors (NK-1r) by SP stimulates phospholipase C (PLC) to further increase $[Ca^{2+}]_i$ from intracellular stores in dorsal horn neurones. **(c)** Although NK-1r suffer internalisation, the increased $[Ca^{2+}]_i$ in dorsal horn neurones activates protein kinases (PK) and Src-family of protein tyrosine kinases (Src), which increase the activity of NMDAr by phosphorylating (P) them. The increased $[Ca^{2+}]_i$ can also activate calcineurin (Cn) and bind to calmodulin to form the Ca^{2+}-calmodulin complex (CaM). Both Cn and CaM activate nitric oxide synthase (NOS) to produce nitric oxide (NO) from the precursor L-arginine (L-Arg). NO then diffuses back to activate guanylate cyclases (GC) to increase the intracellular concentration of 3',5'-cyclic guanosine monophosphate (cGMP), which results in activation of cGMP-dependent protein kinases (PKG). PKG may increase the release of Glu and SP. In addition, the increased $[Ca^{2+}]_i$ can activate PLA_2 to produce free arachidonic acid (AA), which is converted into prostaglandins (PGs) by cyclo-oxygenases (COX). A PG transporter (PGt) moves PGs from the intracellular space into the synaptic cleft. PGs then activate specific PG receptors (PGr) in primary afferent terminals, and second messengers (2^{nd}) increase the release of Glu and SP. Both NO and PGs can also act in the nucleus of dorsal horn neurones and may play a role in gene transcription.

The amino acid glutamate is the main excitatory neurotransmitter in the mammalian central nervous system and acts on two different classes of receptors: metabotropic and

ionotropic glutamate receptors. The former receptors function by intracellular signalling through G-proteins and are subdivided into three groups (Group I-III). The latter class of receptors contain integral cation-specific ion channels and are subdivided into kainate, α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA). There are at least seven subunits of NMDA receptors: NR1, NR2 (A, B, C, and D), and NR3 (A and B). The NR1 subunit exists in at leas eight splice variants: 1a, 1b, 2a, 2b, 3a, 3,b, 4a, and 4b. The number of subunits a functional NMDA receptor is composed of is still a matter of debate, both tetrameric and pentameric structures have been proposed (Dingledine *et al.,* 1999). Although the specific functions for most subunits are to be determined, the diversity of subunits and splice variants, and the way they couple in the receptor itself could explain why NMDA receptors have been implicated in many forms of synaptic transmission, plasticity, and neurodegeneration.

It is recognised that spinal glutamate outflow is increased after Aβ- and C-fibre activation. During afferent terminal stimulation, intracellular calcium concentration ($[Ca^{2+}]_i$) increases, which allows depolarisation of cell membrane and release of stored glutamate. Activity of low threshold fibres, such as that required in acute painful stimulus, appears to activate normally only the AMPA receptor, which leads to a brief neuronal firing. The conditions needed for NMDA-receptor activation are much more complex and only achieved by repeated C-fibre activity, such as that gained after induction of peripheral inflammation which is accompanied with increased glutamate release in the dorsal horn of the spinal cord (Yang *et al.,* 1996; Vetter *et al.,* 2001) (Figure 1). Activation of NMDA receptors causes spinal cord neurones to become more responsive to all of its inputs, resulting in central sensitisation (Bennett, 2000). Apart from the release of glutamate and its binding to the N-terminal domain of NR2 subunits (Gardoni and Di Luca, 2006; Chen and Roche, 2007), activation of NMDA receptors can be regulated by other means.

Regulation of Spinal NMDA Receptors

Magnesium Ions

At resting potential the channel is blocked by extracellular Mg^{2+} ions, but activation exceeding a defined threshold, usually through postsynaptic non-NMDA receptor activation, depolarises the membrane removing the Mg^{2+} ion. This permits the opening of the channel and a massive depolarisation of the neurone results from the influx of Ca^{2+} and Na^{+} (Ghosh and Greenberg, 1995; Chaplan *et al.,* 1997). Hence, non-NMDA and NMDA receptors might operate in succession (Neugebauer *et al.,* 1993). In fact, Na^{+} influx through either subtype of ionotropic glutamate receptor or voltage-gated Na^{+} channels potentiates the activity of NMDA receptors (Yu and Salter, 1998). It seems that once Na^{+} enters the postsynaptic neurone it functions as a signalling ion acting directly on the NMDA channel or on a closely associated regulatory site, which agrees with Na^{+} sensitivity of NMDA receptors being set by a Src protein tyrosine kinase (see below) associated with the channel (Yu and Salter, 1998). Nonetheless, removal of the Mg^{2+} block from the NMDA receptor channel is not a Na^{+}-mediated effect *per se*, but a voltage-sensitive one. Hence, Ca^{2+} influx through voltage-gated Ca^{2+} channels also potentiates NMDA-receptor functioning (Ikeda *et al.,* 2003).

Other Divalent Cations

In addition to the effects of Mg^{2+}, Zn^{2+} and Cd^{2+} ions may be endogenous negative modulators of NMDA receptors because they affect the ability of NMDA ligands to operate the channel. Their actions are not competitive with respect to NMDA or glycine (see below), and are not voltage-dependent to the same degree as those of Mg^{2+}, suggesting a different site of action (Reynolds and Miller, 1990; Dingledine *et al.,* 1999). Another cations, Sr^{2+}, Ca^{2+} and Ba^{2+}, may increase or decrease NMDA ligand binding, an effect that seems to be related to their concentration (Reynolds and Miller, 1990).

Glycine

The NMDA receptor is reciprocally coupled to a glycine receptor distinct from the classical receptor in the spinal cord because it is strychnine insensitive. The N-terminal domain of the NR1 subunit contains the binding site for glycine (Gardoni and Di Luca, 2006; Chen and Roche, 2007). Although glycine does not activate the channel itself, it potentiates NMDA responses and is necessary for channel opening (Reynolds and Miller, 1990). Even glycine released from inhibitory interneurones facilitates the activation of spinal NMDA receptors (Ahmadi *et al.,* 2003). Blockade of glycine sites prevents channel opening (Viu *et al.,* 1998).

D-Serine

Similar to glycine, D-serine does not evoke NMDA-receptor responses by itself. This amino acid rather functions as an endogenous co-agonist of NMDA receptors since its depletion in the medium strongly diminishes NMDA-receptor activity (Mothet *et al.,* 2000). D-Serine has been regarded as an endogenous ligand for the glycine site of the NMDA receptor (Mothet *et al.,* 2000).

Phencyclidine

There is a phencyclidine binding site located close to the Mg^{2+} site within the channel, and phencyclidine site ligands, such as ketamine and dizocilpine, reduce NMDA-receptor currents. Phencyclidine site ligands preferentially bind to NMDA receptors that are in a state consistent with channel opening, while ligands bind less well to inactive channels (Reynolds and Miller, 1990).

Polyamines

Polyamines such as spermidine and spermine act within the modulatory site on the NMDA receptor. They can induce a weak voltage-dependent inhibition, a glycine-dependent potentiation, and a voltage- and glycine-independent potentiation of NMDA receptor function (Dingledine *et al.,* 1999).

Protein Phosphorylation

The carboxy-terminal domains of NMDA receptor subunits regulate receptor interactions with a variety of cytosolic proteins, and these protein-protein interactions dictate the precise intracellular trafficking and localisation of NMDA receptors (Gardoni and Di Luca, 2006; Chen and Roche, 2007). These carboxy-termini are also substrates for post-translational modifications such as phosphorylation, which regulates many cellular processes including receptor activity. For instance, activation of protein kinase C (PKC) potentiates the NMDA receptor function by phosphorylating serine and threonine residues (Yu *et al.,* 1997) on the NR1 subunit (Ghosh and Greenberg, 1995). Protein kinase A (PKA) also enhances NMDA receptor function (Dingledine *et al.,* 1999; Westphal *et al.,* 1999), and this is more evident when the NMDA receptor is composed of NR2A subunits. This receptor subunit is coupled to the associated protein yotiao, which anchors PKA and, therefore, an increase of the receptor currents in comparison to NMDA receptors not expressing yotiao is observed (Westphal *et al.,* 1999).

The Src-family of protein tyrosine kinases phosphorylates NMDA receptor subunits NR2A and NR2B (Yu *et al.,* 1997; Tezuka *et al.,* 1999; Velázquez *et al.,* 2007), and the anchor proteins postsynaptic density-95 (PSD-95) (Tezuka *et al.,* 1999) and PSD-93 (Tao *et al.,* 2003) may play an important role in this process. Brain-derived neurotrophic factor, acting through tyrosine kinase B neurotrophin receptors, elicits NR1 and NR2B subunit phosphorylation-dependent enhancement of NMDA-channel functional activity (Levine *et al.,* 1998). Nerve growth factor, acting on tyrosine kinase A neurotrophin receptors, upregulates brain-derived neurotrophic factor which, apart from enhancing NMDA receptor phosphorylation, also induces changes in NR2A and NR2B receptor subunit expression (Thompson *et al.,* 1999). Calcium/calmodulin-dependent protein kinase II, when activated by Ca^{2+} influx through the NMDA receptor, phosphorylates NR1 and NR2B subunits increasing NMDA receptor-dependent Ca^{2+} influx (Leonard *et al.,* 1999).

In general, phosphorylation of the NMDA receptor sensitises the receptor so that its subsequent responsiveness to glutamate is enhanced. This allows subthreshold inputs to reach threshold levels, resulting in allodynia and hyperalgesia.

Protein Desphosphorylation

Interestingly, increased $[Ca^{2+}]_i$ can also produce Ca^{2+}-dependent inactivation of NMDA receptors (Kyrozis *et al.,* 1996). Once in the neurone, Ca^{2+} activates calmodulin which then desphosphorylates NMDA receptors (Tong *et al.,* 1995). Yotiao proteins, in addition to anchor PKA to NR2A subunits, couple type I protein phosphatase to NMDA receptors, and this enzyme desphosphorylates NMDA receptors negatively regulating the receptors' currents (Westphal *et al.,* 1999).

Fluctuation of NMDA receptors between phosphorylated and desphosphorylated forms may depend on the rate of synaptic stimulation and the magnitude of the associated Ca^{2+} influx through them. Calmodulin may also act as a Ca^{2+} sensor contributing in breaking the recruitment of NMDA receptors during sensory synaptic input. Nevertheless, activation of NMDA receptors responds almost immediately with stimulation of different metabolic

pathways and the formation of retrograde messengers, which may account for the NMDA receptor-mediated excitability.

The above sites and regulatory mechanisms are potential targets for reducing the activity of NMDA receptors and could be exploited for the pharmacological control of pain.

NMDA Receptors and the Spinal Nociceptive Process

NMDA Receptor Expression

Both mRNA and protein of NMDA receptors are constitutively expressed in spinal and dorsal root ganglion (DRG) neurones (Liu *et al.,* 1994; Tölle *et al.,* 1996; Parada *et al.,* 2003). The expression is high in DRG and the superficial layers of the dorsal horn of the spinal cord (Table 1), which are important areas for the transmission of nociceptive information. Interestingly, chronic monoarthritis did not affect the spinal staining of NMDA mRNA, but did increase the spinal binding of [^{3}H]PDBU, a molecule that binds and activates the diacylglycerol binding site on PKC (Tölle *et al.,* 1996). This suggests that arthritic pain may indirectly increase NMDA-receptor function by a PKC-mediated phosphorylation action.

Table 1. Expression and anatomical distribution of mRNAs encoding for the different NMDA receptor splice variants in the lumbar spinal cord of the rat.*

Splice variant	Subtype	Dorsal horn	Lamina X	Ventral horn	Reference
NR1		√	√	√	(Tölle *et al.,* 1996)
		√			(Yung, 1998)
	a	√	√	√	(Tölle *et al.,* 1996)
	b	√ (few)	√	√ (few)	(Tölle *et al.,* 1996)
	1	√	nd	√ (few)	(Tölle *et al.,* 1996)
	2	√	√	√	(Tölle *et al.,* 1996)
	3	√ (few)	nd	nd	(Tölle *et al.,* 1996)
	4	√	√	√	(Tölle *et al.,* 1996)
NR2	A	nd	nd	nd	(Tölle *et al.,* 1996)
		nd			(Yung, 1998)
	B	nd	nd	nd	(Tölle *et al.,* 1996)
		√			(Yung, 1998)
	C	√ (few)	nd	nd	(Tölle *et al.,* 1996)
		nd			(Yung, 1998)
	D	√	√	√	(Tölle *et al.,* 1996)

* Abbreviations: √ = present; nd = not detected.

NMDA Receptors and Pain

Direct activation of spinal NMDA receptors after intrathecal administration of NMDA produce nociceptive behaviours, allodynia, and hyperalgesia in different models of pain

(Björkman *et al.,* 1996; Dolan and Nolan, 1999; Eguchi *et al.,* 1999; Kawamata and Omote, 1999; Fairbanks *et al.,* 2000; Tao and Johns, 2000; Minami *et al.,* 2001; Parada *et al.,* 2003; Muscoli *et al.,* 2004; Shimoyama *et al.,* 2005). The increased nociceptive response is believed to be due to the entry of Ca^{2+} into the neurone, which triggers different metabolic pathways activating a number of enzymes and ultimately immediate-early genes (Ghosh and Greenberg, 1995; Muscoli *et al.,* 2004). Retrograde second messengers resulting from this cascade cause presynaptic augmentation of neurotransmitter and neuropeptide release, which can result in amplification and prolongation of the neural response, underlying many forms of central sensitisation. Candidates include the neuromediators arachidonic acid (AA), prostaglandins (PGs) and nitric oxide (NO) (Dumuis *et al.,* 1988; Herrero *et al.,* 1992; Dawson and Dawson, 1995; Chaplan *et al.,* 1997; Kawamata and Omote, 1999; Minami *et al.,* 1999).

Supporting evidence for the AA/PG/NO model comes from studies showing that non-steroidal anti-inflammatory drugs (NSAIDs, which inhibit PG synthesis) (Malmberg and Yaksh, 1992b; Björkman *et al.,* 1996; Dolan and Nolan, 1999; Parada *et al.,* 2003) and inhibitors of the enzyme NO synthase (NOS, which catalyses the formation of NO) (Dolan and Nolan, 1999; Kawamata and Omote, 1999; Fairbanks *et al.,* 2000) inhibited the increased nociceptive response induced after intrathecal administration of NMDA.

Directly or indirectly, NMDA receptors may contribute to release neuromodulators other than AA, PGs and NO, and substances can differ depending on the kind of stimulus. Some of these neuromodulators include glutamate, substance P (SP), γ-aminobutyric acid (GABA), opioid peptides, monoamines, and superoxide, just to mention a few.

NMDA receptor-mediated events are considered to be postsynaptic, but the existence of active presynaptic NMDA receptors (autoreceptors) in the dorsal horn of the spinal cord has been demonstrated (Liu *et al.,* 1994). Although presynaptic NMDA receptors seem to contribute to the release of glutamate (Robert *et al.,* 1998; Parada *et al.,* 2003) and SP (Liu *et al.,* 1997; Malcangio *et al.,* 1998), there is controversy on the nociceptive role of these receptors (Urban and Nagy, 1997).

NMDA Receptor Blockers and Pain

Electrophysiological studies have showed that infusion of the NMDA receptor channel blocker, ketamine, reduced NMDA receptor-mediated transmission in a neonatal rat spinal cord preparation (Lizarraga *et al.,* 2006). Likewise, blockade of spinal NMDA receptors reduced the excitability of dorsal horn neurones to electrical stimulation of the dorsal root at C-fibre strength (Ikeda *et al.,* 2003) or to mechanical stimulation of an inflamed joint (Neugebauer *et al.,* 1993). In accordance, there is strong evidence that intrathecal administration of NMDA receptor antagonists inhibited intrathecal NMDA-induced behaviours (Kawamata and Omote, 1999; Fairbanks *et al.,* 2000), as well as thermal (Fairbanks *et al.,* 2000; Tao and Johns, 2000; Yaksh *et al.,* 2001) and mechanical (Dolan and Nolan, 1999; Nishimura *et al.,* 2004) hypersensitivity. These antagonists also reduced hyperalgesia in animal models of inflammatory and neuropathic pain (Chaplan *et al.,* 1997), and produced postoperative analgesia in human beings (Koinig *et al.,* 2000; Ozyalcin *et al.,* 2004).

NMDA Receptor Deficiency and Pain

Deletion of spinal NR1 receptor subunit by use of antisense oligonucleotides abolished nociceptive behaviours and thermal hyperalgesia observed after intrathecal injection of NMDA or intraplantar formalin in rats (Garry *et al.,* 2000; Shimoyama *et al.,* 2005). Injection in the spinal cord dorsal horn of vector-derived short interfering RNAs targeted and knocked down the NR1 subunit at the same time that reduced mechanical allodynia in mice administered complete Freund's adjuvant into a hind paw (Garraway *et al.,* 2007). Similarly, gene disruption techniques in mice showed that NR1-1 receptor subunits were involved in allodynia induced by intrathecal administration of PGE_2, and NR1-4 receptor subunits in that induced by intrathecal injection of $PGF_{2\alpha}$ (Minami *et al.,* 1999). Decreased spinal excitatory transmission along with antihyperalgesia in models of neuropathic and arthritic pain accompanied the reduced spinal expression of NR2A and NR2B subunits after disruption of the *psd-93* gene in mice (Tao *et al.,* 2003). Even knocking down of spinal *psd-93* (Zhang *et al.,* 2003) and *psd-95* (Tao *et al.,* 2000a), without affecting NMDA subunit expression, reduced thermal and mechanical hyperalgesia in rats.

However, not all NMDA receptor subunits seem to have an excitatory action. Knockout of the NR2A or NR2D subunit produced no difference in withdrawal thresholds as compared to wild-type mice in a postoperative pain model (Nishimura *et al.,* 2004). Compared to wild-type mice, mice lacking the NMDA receptor NR1-1A subunit gene had an enhanced nociceptive response to SP administered by the intrathecal route, which suggested an inhibitory role for the NR1-1A receptor subunit (Inoue *et al.,* 2000).

It seems that each NMDA receptor subunit plays a specific role in different types of pain. Although most NMDA receptor subunits are pronociceptive, some are antinociceptive; the net effect may depend on the subunit composition of the NMDA receptor. Blockade of spinal NMDA receptors may help to prevent or reduce the development of central sensitisation.

NMDA Receptors and the Arachidonic Acid Pathway

The increase in $[Ca^{2+}]_i$ after NMDA receptor stimulation activates phospholipases. Activation of phospholipase A_2 (PLA_2) can make membrane phospholipids more susceptible to hydrolytic processes that are associated with an increasing level of free AA. Arachidonate, in turn, may be converted by cyclo-oxygenases (COX) to prostanoids. Supporting evidence for this model comes from: *(i)* increased AA release after administration of glutamate and NMDA to cultured cerebellar granule cells (Viu *et al.,* 1998) and astrocytes (Strokin *et al.,* 2003); and *(ii)* increased spinal release of PGs after spinal treatment with NMDA (Dirig and Yaksh, 1999) and after activation of high-threshold nociceptive afferent inputs (Ramwell *et al.,* 1966).

Phospholipases A_2 and the Spinal Nociceptive Process

Arachidonic Acid Generation and Functions

Arachidonate concentrations in the cytoplasm are low under physiological conditions, and formation of AA is generally considered to be the rate-limiting step in prostanoid synthesis. Arachidonic acid is found in the *sn*-2 position of membrane phospholipids, and PLA_2 hydrolises the phospholipid *sn*-2 ester bond to generate free AA and lysophospholipids (Balsinde *et al.,* 2002). It is theorised that AA diffuses back to the primary afferent terminal to amplify pain signals, but this is unlikely to happen due to AA's physicochemical properties. AA certainly potentiates NMDA receptor currents by itself, probably by altering the receptor's lipid environment (Miller *et al.,* 1992), or by functionally converting K^+ channels from non-inactivating delayed rectifiers into rapidly inactivating A-type channels (Oliver *et al.,* 2004). Both increased release and decreased glial uptake of glutamate have also been proposed as possible excitatory mechanisms for AA (Dumuis *et al.,* 1988; Herrero *et al.,* 1992). Recently, Sung *et al.* (2007) provided convincing evidence for AA as the mediator that reduced spinal glutamate uptake and therefore increased regional glutamate concentration in a rat model of neuropathic pain.

PLA_2 Isozymes and Functions

In mammals, 19 proteins possessing PLA_2 activity have been identified. Based on biological properties, the PLA_2 superfamily can be divided into three distinct classes: cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), cytosolic Ca^{2+}-independent PLA_2 ($iPLA_2$), and secretory PLA_2 ($sPLA_2$) (Balsinde *et al.,* 2002).

$cPLA_2$ are found in the cytosol of most cells and are translocated to nuclear/endoplasmic reticulum membranes in response to increased $[Ca^{2+}]_i$; their activity is enhanced if phosphorylated by mitogen-activated protein kinases (Balsinde *et al.,* 2002). These isozymes possess a preference for phospholipids containing AA and in fact, AA release from astrocytes has been linked to $cPLA_2$ activity (Strokin *et al.,* 2003). $iPLA_2$ are a group of related isozymes that require mM concentrations of Ca^{2+} for activation and are not selective for AA-containing phospholipids. These isozymes are released into the extracellular space and can induce phospholipid cleavage in adjacent cells. $sPLA_2$ are involved in AA remodelling rather than AA liberation, incorporating and exposing AA and other fatty acids into membrane phospholipids (Balsinde *et al.,* 2002).

PLA_2 Expression

Both PLA_2 mRNA and protein are constitutively expressed in the spinal cord, and they can be induced after lipopolysacharide, interleukin-1β and zymosan treatment (Svensson and Yaksh, 2002), but not after carrageenan injection (Lucas *et al.,* 2005).

PLA_2 Inhibitors and Pain

Corticosteroids, which among other actions inhibit PLA_2, have been used to study the role of these enzymes in the spinal processing of pain. Single intrathecal administration of methylprednisolone and triamcinolone did not affect flinching behaviour in rats submitted to a paw formalin test, and the former even induced mechanical allodynia (Abram *et al.,* 1994). Similarly, a single intravenous dose of dexamethasone did not increase mechanical nociceptive thresholds in normal and sensitised (due to footrot) sheep (Chambers *et al.,* 1995). These data suggest that corticosteroids have no central analgesic or antihyperalgesic acitions.

Intrathecal administration of the type-unspecific PLA_2 inhibitors arachydonyl trifluoromethylketone ($AACOCF_3$) and methyl arachydonyl fluorophosphanate (MAFP) (Lucas *et al.,* 2005), and the preferring $cPLA_2$ inhibitor AX048 (Yaksh *et al.,* 2005) produced thermal antihyperalgesia in rats injected carrageenan into one hind paw. Intrathecal injection of $AACOCF_3$ and AX048 also prevented both increased spinal release of PGE_2 and thermal hyperalgesia induced by intrathecal administration of NMDA or SP, respectively (Lucas *et al.,* 2005; Yaksh *et al.,* 2006). The intrathecal injection of $AACOCF_3$ attenuated the development of both thermal hyperalgesia and mechanical allodynia in a rat model of chronic constriction nerve injury (Sung *et al.,* 2007). Although it could be argued that the spinal antihypersensitive effects of $AACOCF_3$ and MAFP might be due to possible interaction with the cannabinoid (CB) system (Ates *et al.,* 2003), the spinal antihyperalgesic actions of AX048 were not blocked by intrathecal pre-treatment with the CB_1 receptor antagonist SR141716 (Yaksh *et al.,* 2006).

The presence and biological activity of PLA_2 suggest a role for this family of enzymes in the spinal processing on nociceptive information, but the lack of more selective inhibitors has left open the issue regarding the relative contribution of these enzymes. This is an area where further research is warranted.

Cyclo-Oxygenases and the Spinal Nociceptive Process

COX Isozymes and Functions

The membrane-bound enzymes COX exist in at least two isoforms: COX-1 and COX-2. Both COX isozymes, however, use the same endogenous substrate and form the same products by the same catalytic reaction. This is due to the highly conserved homology (>90%) of the COX and peroxidase enzyme regions between isozymes (Conn and Pin, 1997). Cyclooxygenation of AA produces PGG_2, which is reduced to PGH_2 by COX-mediated peroxidase activity. PGH_2 is converted to final active products via individual prostaglandin synthases (see below) (Appleton *et al.,* 1996).

The major difference in COX isoenzymes lies in their pathophysiological functions. COX-1 is constitutively expressed in most tissues at a different constant level, and it is believed to be responsible for the formation of PGs with physiological functions. COX-2 also produce PGs with physiological effects, but it is normally undetectable in most tissues and can be induced at high levels in a variety of cells by pro-inflammatory stimuli (Appleton *et*

al., 1996; Conn and Pin, 1997). The COX-2 mRNA contains consensus sequences for several nuclear transcription factors including nuclear factor-κB (NF-κB), providing potent mechanisms for induction. The gene also displays repeated motifs in the untranslated regions common to immediate early genes rendering the mRNA less stable, whereas during catalysis COX-2 undergoes auto-inactivation (Svensson and Yaksh, 2002). Such properties indicate an effective regulation of enzyme expression and activity.

The presence of additional COX enzymes was recently demonstrated in various tissues from both human beings and dogs. Canine neuronal tissues expressed three new identified mRNAs derived from the COX-1 gene. The enzyme products of these mRNAs were termed COX-3 protein (subsequently renamed to COX-1b since it is a splice variant of COX-1), partial COX-1a (PCOX-1a) and PCOX-1b (Chandrasekharan *et al.,* 2002). In transfected insect cells, canine COX-1b was selectively inhibited by various NSAIDs, including paracetamol, in comparison to COX-1 and COX-2; PCOX-1a had no COX activity (Chandrasekharan *et al.,* 2002). These data suggested that COX-1b could be the elusive target of paracetamol and therefore to be involved in pain transmission. However, PGI_2 production in rat brain and cerebellum, which also expressed COX-1b mRNA, was not particularly sensitive to inhibition by paracetamol compared to other COX inhibitors (Warner *et al.,* 2004), and even a functionally active human COX-1b construct transfected to COS-7 cells was not inhibited by paracetamol, but it was by lysine acetyl salicylic acid (Censarek *et al.,* 2006). It is possible that the dog COX-1b functions differently than that from the rat or human. For instance, although COX-1b was constitutively expressed in the rat spinal cord, its amino acid sequence was completely different than the known COX and it lacked COX activity in transfected COS-7 cells (Snipes *et al.,* 2005). Contrary to this, the mutant COX-1b reported by Censarek *et al.* (2006) contained all predicted amino acids from the human COX-1 intron 1 sequence and was functional, but its natural occurrence remains to be demonstrated. Various methodological issues in the original report describing the existence of COX-1b (Chandrasekharan *et al.,* 2002) have been recently highlighted and the conclusions questioned (Kis *et al.,* 2005).

COX Expression

In the spinal cord, COX-1, COX-2 and COX-1b mRNAs and proteins are constitutively expressed, and their predominance may depend on the anatomical localisation, the age, and the species (Table 2). In the rat, COX-1 immunoreactivity was detected in small to medium sized cell bodies in the DRG and the dorsal roots, and to a lesser extent in the dorsal horn of the spinal cord. COX-2 immunoreactivity was not present in DRG neurones, but it was observed in the superficial (laminae I-II) and deep (laminae V-VI) dorsal horn, around the central canal (lamina X), and in individual motor neurones in the ventral horn (Willingale *et al.,* 1997). Distribution of COX-1 and COX-2 in the dorsal horn was consistent with COX products being involved in the processing of nociceptive input, but COX metabolites may also be involved by stimulating afferent fibres.

Table 2. Constitutive expression of cyclo-oxygenase isozymes in the spinal cord.[*]

Isozyme / Method	Species	Localisation				Cell type	Reference
		Dorsal horn	Lamina X	Ventral horn	DRG		
COX-1							
IHC	Mouse	√		√		Glial cells	(Maihofner *et al.*, 2000)
IHC	Rat	√		√		Neurones	(Zhu *et al.*, 2003)
IHC	Rat	√(few)	nd	nd	√	Neurones	(Willingale *et al.*, 1997)
ISH	Sheep	√	√	√		Neurones	(Dolan *et al.*, 2003)
RNA	Mouse	√[†]		√[†]			(Ballou *et al.*, 2000)
RT-PCR	Mouse	√[†]		√[†]			(Gühring *et al.*, 2000)
RT-PCR	Mouse	√[†]		√[†]			(Ballou *et al.*, 2000)
RT-PCR	Sheep	√[†]		√[†]			(Dolan *et al.*, 2000; Dolan *et al.*, 2003)
WB	Rat	√[†]		√[†]	√[†]		(Yaksh *et al.*, 2001)
WB	Rat	√		√			(Seybold *et al.*, 2003)
WB	Rat	nd[†]		nd[†]			(Wen *et al.*, 2001)
WB	Rat	√[†]		√[†]			(Dirig and Yaksh, 1999)
WB	Sheep	√[†]		√[†]			(Dolan *et al.*, 2003)

Table 2. Continued

Isozyme / Method	Species	Localisation				Cell type	Reference
		Dorsal horn	Lamina X	Ventral horn	DRG		
COX-2							
IHC	Mouse	nd	√	√		Neurones	(Maihofner *et al.*, 2000)
IHC	Rat	√	√	√	nd	Neurones	(Willingale *et al.*, 1997)
ISH	Sheep	√		√		Neurones	(Dolan *et al.*, 2003)
RNA	Mouse	√†		√†			(Ballou *et al.*, 2000)
RT-PCR	Mouse	√†		√†			(Gühring *et al.*, 2000)
RT-PCR	Mouse	√†		√†			(Ballou *et al.*, 2000)
RT-PCR	Sheep	√†		√†			(Dolan *et al.*, 2000; Dolan *et al.*, 2003)
WB	Rat	√†		√†	√†		(Yaksh *et al.*, 2001)
WB	Rat	√		√			(Seybold *et al.*, 2003)
WB	Rat	√†		√†			(Wen *et al.*, 2001)
WB	Rat	√†		√†			(Dirig and Yaksh, 1999)
WB	Sheep	√†		√†			(Dolan *et al.*, 2003)
COX-1b							
WB	Rat	√†		√†			(Snipes *et al.*, 2005)

* Abbreviations: √ = present; † = homogenate of whole spinal cord; COX-1 = cyclooxygenase-1; COX-2 = cyclooxygenase-2; IHC = immunohistochemistry; ISH = *in situ* hybridisation; nd = not detected; RNA = RNAsa protection assay; RT-PCR = reverse transcription-polymerase chain reaction; WB = western blotting.

Induction of peripheral inflammatory hyperalgesia in rats increased the immunoreactive staining of spinal COX-2 mRNA and protein, but not that of COX-1, 2 days after injecting complete Freund's adjuvant into the hid paw (Seybold *et al.,* 2003). However, in a model of peripheral inflammatory hyperalgesia in which formalin was injected into the hind paw, increased spinal release of PGs during 60 min post-injection was accompanied by increased expression of COX-2 mRNA, but not protein, suggesting that this process is largely independent of the induction of spinal COX-2 (Tegeder *et al.,* 2001). Interestingly, Zhu *et al.* (2003) demonstrated that rats submitted to an incisional model of postoperative pain showed increased COX-1 immunoreactivity in the dorsal horn and the gracile nucleus of the spinal cord on postoperative days 2 to 5. Spinal COX-1 immunoreactivity also increased in the superficial laminae (I-II), but decreased it in the deep dorsal horn (IV-VI), ventral horn, and white matter of rats after 2 weeks of peripheral nerve injury (Zhu and Eisenach, 2003). Concurrently, these data may suggest that both constitutive and inducible spinal COX isozymes play a role in the development and maintenance of spinal sensitisation, and that the expression of COX in the spinal cord is not static, but changes in a time- and laminar-dependent manner.

In addition to determine its biological significance, the spinal anatomical distribution and regulation of COX-3 remain to be characterised.

COX Inhibitors and Pain

Administration of COX inhibitors into the spinal canal induced antihyperalgesia as assessed using different algesiometric tests (Table 3). Support for the inhibition of spinal COX enzymes as a means of reducing this altered pain state comes from: *(i)* antihyperalgesic potency of NSAIDs injected intrathecally correlating with their rank order potencies as COX inhibitors (Malmberg and Yaksh, 1992a; Dirig *et al.,* 1998; Yaksh *et al.,* 2001); *(ii)* antihyperalgesia produced at the same time that inhibition of spinal PG occurred (Muth-Selbach *et al.,* 1999); and *(iii)* antihyperalgesia achieved after intrathecal injection of PG receptor antagonists (Malmberg *et al.,* 1994; Nakayama *et al.,* 2002).

COX-2 seems to be the major isozyme involved in analgesia since spinal administration of non-selective COX inhibitors and COX-2, but not COX-1, selective inhibitors reduced thermal hyperalgesia (Yamamoto and Nozaki-Taguchi, 1996; Smith *et al.,* 1998a; Yaksh *et al.,* 2001). Importantly, the spinal antihyperalgesic actions of COX-2 inhibitors can be shown under conditions and at times when enzyme induction has probably not occurred, stressing the importance of the constitutive COX-2 isozyme in spinal nociception. Recently, the importance of spinal COX-1 in analgesia has been highlighted by the intrathecal delivery of a non-selective COX inhibitor and a COX-1, but not a COX-2, selective inhibitor reducing both mechanical hyperalgesia in a model of incisional post-operative pain (Zhu *et al.,* 2003) and mechanical hyperalgesia and allodynia in a model of neuropathic pain (Hefferan *et al.,* 2003), as well as reversing the effects of abdominal surgery on exploratory behaviours in rats (Martin *et al.,* 2006). Similarly, oral administration of a non-selective COX inhibitor or a COX-1, but not COX-2, selective inhibitor reduced flinching behaviour in rats injected formalin in a hind paw (Tegeder *et al.,* 2001) and restored exploratory behaviours in a rat model of post-operative thoracic surgical pain (Kroin *et al.,* 2006) at the same time that inhibited spinal PG release. It may be possible that selective inhibition of spinal COX-1 and COX-2 are necessary for induction of mechanical and thermal antihyperalgesia, respectively.

Table 3. Effects of spinal administration of cyclo-oxygenase inhibitors on different algesiometric tests.

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
Intrathecal					
(Devoghel, 1983)	Humans	Cancer pain sufferers	Lysine-acetylsalicylate	60 mg in 1 mL = 33 mg acetylsalicylic acid in 1 mL = 5.55 M	Quick (within 15 minutes) and log-lasting (1-22 days) pain relief.
(Seybold *et al.*, 2003)	Rats (spinalized)	C-fibre reflex recorded from the hamstring muscles after electrical stimulation of the sural nerve	S(+)-ibuprofen	1-100 nM	No effect on reflex response.
			R(-)-ibuprofen	100 nM	No effect on reflex response.
		Subcutaneous injection of Complete Freund's Adjuvant + C-fibre reflex recorded from the hamstring muscles after electrical stimulation of the sural nerve	S(+)-ibuprofen	10 and 100 nM	Dose-dependent reduction of the reflex response.
			R(-)-ibuprofen	100 nM	No effect on reflex response.
(Carlsson *et al.*, 1986)	Rats (intact)	Radiant thermal tail-flick test	Dipyrone	50-400 μg in 5 μL = 30-240 mM	Dose-dependent increase of tail withdrawal latencies.
	Rats (spinalized)	Radiant thermal tail-flick test	Dipyrone	400 μg in 5 μL = 240 mM	No effect on tail withdrawal latencies.
		C-fibre reflex recorded from the ipsilateral tibialis anterior muscle after electrical stimulation of the sural nerve	Dipyrone	100 and 400 μg in 5 μL = 60 and 240 mM	No change on flexor reflex activity with the lower dose but increased it with the highest dose.
		Ascending axon activity in response to C-fibre stimulation of the sural nerve	Dipyrone	100 and 200 μg in 5 μL = 60 and 120 mM	About 150% increase in the reflex response.

Table 3. Continued

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
(Yaksh *et al.*, 2001)	Rats	Intrathecal SP + radiant thermal stimuli of the hindpaws	S(+)-ibuprofen	8 – 80 nmol	Dose-dependent increase of paw withdrawal latencies.
			R(-)-ibuprofen	80 nmol	No effect on paw withdrawal latencies.
			SC-384	23 – 230 nmol	Dose-dependent increase of paw withdrawal latencies.
			SC-58125	5-50 nmol	Dose-dependent increase of paw withdrawal latencies.
			SC-236	Not specified	Dose-dependent increase of paw withdrawal latencies.
			SC-560	280 – 2800 nmol	No effect on paw withdrawal latencies.
		Intrathecal NMDA + radiant thermal stimuli of the hindpaws	SC-58125	50 nmol	Increase on paw withdrawal latencies.
			SC-560	Not specified	No effect on paw withdrawal latencies.
		Intraplantar carrageenan + radiant thermal stimuli of the hindpaws	Ibuprofen	80 nmol	Increase on paw withdrawal latencies.
			SC-58125	50 nmol	Increase on paw withdrawal latencies.
			SC-560	280 nmol	No effect on paw withdrawal latencies.
(Malmberg and Yaksh, 1992)^	Rats	Intrathecal NMDA + hot plate test	Acetylsalicylic acid	100 nmol	Increase on paw withdrawal latencies.
			Ketorolac	27 nmol	Increase on paw withdrawal latencies.

Table 3. Continued

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
			S(+)-ibuprofen	27 nmol	Increase on paw withdrawal latencies.
			R(-)-ibuprofen	270 nmol	No effect on paw withdrawal latencies.
		Intrathecal AMPA + hot plate test	S(+)-ibuprofen	27 nmol	Increase on paw withdrawal latencies.
			R(-)-ibuprofen	270 nmol	No effect on paw withdrawal latencies.
		Intrathecal SP + hot plate test	S(+)-ibuprofen	27 nmol	Increase on paw withdrawal latencies.
			R(-)-ibuprofen	270 nmol	No effect on paw withdrawal latencies.
(Malmberg and Yaksh, 1992)^	Rats	Subcutaneous formalin into one hindpaw	Acetylsalicylic acid	27 nmol (18 – 41 nmol)*	Dose-dependent inhibition of phase 2; 70% inhibition with the highest dose.
			Indomethacin	1.9 nmol (1.2 - 4.0 nmol)*	Dose-dependent inhibition of phase 2; 80% inhibition with the highest dose.
			Flurbiprofen	2.1 nmol (1.0 - 4.3 nmol)*	Dose-dependent inhibition of phase 2; 70% inhibition with the highest dose.
			Ketorolac	5.2 nmol (3.2 - 8.3 nmol)*	Dose-dependent inhibition of phase 2; 80% inhibition with the highest dose.
			Zomepirac sodium	5.9 nmol (3.9 - 8.9 nmol)*	Dose-dependent inhibition of phase 2; 75% inhibition with the highest dose.
			S(+)-ibuprofen	15.7 nmol (6.7 – 36 nmol)*	Dose-dependent inhibition of phase 2; 60% inhibition with the highest dose.
			Racemic ibuprofen	18.9 nmol (9.3 – 38 nmol)*	Dose-dependent inhibition of phase 2; 55% inhibition with the highest dose.
			Acetaminophen	257 nmol (163 – 405 nmol)*	Dose-dependent inhibition of phase 2; 50% inhibition with the highest dose.
			R(-)-ibuprofen	>270 nmol	No effect on phase 2.
		Hot plate test	Acetylsalicylic acid	100 nmol	No effect on paw withdrawal latencies.
			Flurbiprofen	80 nmol	No effect on paw withdrawal latencies.
			Ketorolac	80 nmol	No effect on paw withdrawal latencies.
			Zomepirac sodium	80 nmol	No effect on paw withdrawal latencies.
			Indomethacin	30 nmol	No effect on paw withdrawal latencies.

Table 3. Continued

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
(Malmberg and Yaksh, 1993)	Rats	Subcutaneous formalin into one hindpaw	Ketorolac	2.7 – 80 nmol	Dose-dependent inhibition of phase 1 and 2; 52% and 80% inhibition, respectively, with the highest dose.
(Pelissier *et al.*, 1996)	Rats	Paw pressure test	Acetaminophen	100 and 200 μg in 10 μL = 66 and 132 mM	About 600% and 800% increase in the area under the nociceptive threshold curve.
(Lorenzetti and Ferreira, 1996)	Rats	Intraplantar PGE_2 + paw pressure test	Dipyrone	2.5 – 80 μg in 20 μL = 0.375 – 12 mM	Dose-dependent reduction of escape behaviours.
(Akman *et al.*, 1996)	Mice	Radiant heat tail-flick test	Dipyrone	50 – 400 μg in 5 μL = 30 – 240 mM	No effect on tail withdrawal latencies.
		Hot plate test	Dipyrone	50 – 400 μg in 5 μL = 30 – 240 mM	No effect on paw withdrawal latencies.
		Abdominal constriction test	Dipyrone	100 and 200 μg in 5 μL = 60 and 120 mM	About 50% and 65% reduction in the number of stretches.
(Yamamoto and Nozaki-Taguchi, 1996)	Rats	Subcutaneous formalin into one hindpaw	Indomethacin	0.03 – 30 μg in 10 μL = 8.3 μM-8.3 mM	Dose-dependent reduction in the number of flinches in both phase 1 and 2.
			NS-398	0.03 – 30 μg in 10 μL = 9.5 μM-9.5 mM	Dose-dependent reduction in the number of flinches in both phase 1 and 2.
		Hot plate test	Indomethacin	30 μg in 10 μL = 8.3 mM	No effect on paw withdrawal latencies.
			NS-398	30 μg in 10 μL = 9.5 mM	No effect on paw withdrawal latencies.

Table 3. Continued

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
(Willingale *et al.*, 1997)[&]	Rats (anaesthetised)	Mechanical stimulation of the ankle joint	Meclofenamic acid	10 and 100 μM	No effect on dorsal horn neurone responses.
		Mechanical stimulation of the knee joint	Indomethacin	1 and 100 μM	No effect on dorsal horn neurone responses.
		C-fibre reflex recorded from the ipsilateral biceps femoris muscle after electrical stimulation of the sural nerve	Indomethacin	10 and 100 μM	No change with the lower dose. About 67% reduction of the reflex response with the higher dose.
(Dirig *et al.*, 1998)	Rats	Subcutaneous carragennan into one hindpaw + radiant thermal stimuli	S(+)-ibuprofen	27 and 80 nM	Dose-dependent increase on paw withdrawal latencies.
			R(-)-ibuprofen	80 nM	No effect on paw withdrawal latencies.
			SC58125	50 nM	Dose-dependent increase on paw withdrawal latencies.
(Zhu *et al.*, 2003)	Rats	Paw incision + von Frey filaments	Ketorolac	150 μg in 10 μL = 39.5 mM	Increase on paw withdrawal thresholds.
			SC560	100 μg in 10 μL =	Increase on paw withdrawal thresholds.
			NS-398	50 μg in 10 μL = 15.8 mM	No effect on paw withdrawal thresholds.
(Bustamante *et al.*, 1997)	Rats (anaesthetised)	C-fibre reflex recorded from the ipsilateral biceps femoris muscle after electrical stimulation within the area of the sural nerve	Acetylsalicylic acid	100 and 500 μg in 10 μL = 55 and 277 mM	About 35% and 80% reduction of the reflex response.
			Indomethacin	200, 300 and 500 μg in 10 μL = 55, 83 and 139 mM	About 36%, 62% and 89% reduction of the reflex response.
			Ketoprofen	300 and 500 μg in 10 μL = 112 and 196 mM	About 23% and 40% reduction of the reflex response.
			Lysine clonixinate	300, 500 and 1000 μg in 10 μL = 73, 122 and 244 mM	About 30%, 48% and 63% reduction of the reflex response.

Table 3. Continued

Reference	Species	Nociceptive test	COX inhibitor	Dose	Effects
(Dolan and Nolan, 1999)	Sheep	Foreleg mechanical stimulation.	DFU	200 nmol in 200 μL = ?? mM	No effect on mechanical thresholds.
		Intrathecal NMDA + foreleg mechanical stimulation.	DFU	200 nmol in 200 μL = ?? mM	Increase on mechanical thresholds.
(Eisenach *et al.*, 2002)[@]	Humans	Thermal heat stimuli of the skin of the volar forearm and the lateral calf.	Ketorolac	0.25 – 2 mg	No effect on withdrawal thresholds.
Epidural					
(Lauretti *et al.*, 1998)	Humans	Cancer pain sufferers	Diclofenac	75 mg	Pain relief (VAS[#] = 0) for 48 h.
			Tenoxicam	20 mg	Pain relief (VAS = 2) for 22.5 h
(Aldrete, 2003)[@]	Humans	Postlaminectomy syndrome	Indomethacin	2 injections 2 weeks apart, 1 or 2 mg in 3 mL 0.5% bupivacaine	Reduced pain intensity as evaluated by the Pain progress Score 2 weeks after each injection
(Masue *et al.*, 1999)	Rats	Intrathecal nitroglycerin + tail-flick test	S(+)-ibuprofen	52 μg (38-66 μg) /30 μL = 8.4 mM (6.1-10.7 mM)*	Dose-dependent increase of tail withdrawal latencies.
			Indomethacin	5 μg (2.8-7.3 μg) /30 μL = 460 μM (258-672 μM)*	Dose-dependent increase of tail-flick latencies.
			Diclofenac	6.3 μg (5.7-7.6 μg) /30 μL = 660 μM (597-796 μM)*	Dose-dependent increase of tail-flick latencies.
			R(-)-ibuprofen	>1000 μg/ 30μL = >161 mM*	No effect on tail-flick latencies.
		Subcutaneous formalin into one hindpaw	S(+)-ibuprofen	1000 μg/ 30μL = 161 mM	Inhibition of paw-lifting behaviour in the phase 2.
			R(-)-ibuprofen	1000 μg/ 30μL = 161 mM	No effect on paw-lifting behaviour.

*ID_{50} values and 95% confidence intervals.

^ Administration volume = 10 μL.

& Administration volume = 40-50 μL.

@ Administration volume not specified.

VAS = visual analogue scale.

From information in Table 3, it is evident that COX inhibitors produced spinal analgesic effects in models of inflammatory pain and hyperalgesia, but lacked analgesic action in models of acute pain, suggesting that COX inhibitors produced analgesia at the spinal level only when an ongoing afferent barrage was established (central sensitisation). However, Pelissier *et al.* (1996) reported hypoalgesia after intrathecal injection of mM concentrations of paracetamol in rats with no inflammation submitted to a paw pressure test, suggesting an analgesic action in the absence of spinal facilitated states. Spinal administration of smaller concentrations, one or two orders of magnitude, of COX inhibitors did not increase mechanical nociceptive thresholds in animals with no inflammation (Willingale *et al.,* 1997; Dolan and Nolan, 1999; Lizarraga and Chambers, 2006). Most COX inhibitor-mediated spinal antihyperalgesic effects have been reported with drugs being administered in mM concentrations (Table 3). Since nM to μM drug concentrations are necessary to inhibit COX isozymes (Giuliano and Warner, 1999), it is possible that mechanisms other than, or complementary to, prostanoid synthesis inhibition may mediate the spinal antihyperalgesic action of COX inhibitors (see McCormack, 1994 for review).

COX Isozyme Deficiency and Pain

COX-2 deficient, COX-1 homozygous, and wild-type mice showed similar latency times in a hot plate test, which agrees with the lack of analgesic action of NSAIDs in this test. Surprisingly, COX-1 heterozygous mice showed longer reaction times. In an abdominal stretching test in which acetic acid was intraperitoneally injected, fewer writhes were observed in COX-1 deficient mice and COX-2 heterozygous female mice, but not in COX-2 homozygous females or COX-2 deficient mice, as compared to wild-type mice. Although no increase in COX-2 mRNA was detected in the spinal cords of COX-1-null animals, a compensatory increase in COX-1 mRNA in the spinal cords of COX-2-null animals was noticed (Ballou *et al.,* 2000), which makes difficult to interpret these results.

Intrathecal injection of an oligonucleotide antisense targeted to the *Ptgs-2* gene reduced spinal COX-2, but not COX-1, expression in rats and prevented thermal hyperalgesia induced by intrathecal NMDA injection, but not acute thermal nociception (Svensson and Yaksh, 2002).

In summary, a modulatory role for COX isozymes in the processing of pain is suggested by the spinal distribution and expression of these enzymes. This is further supported by the antihyperalgesic action of NSAIDs injected intrathecally, although these drugs may also interfere with other systems involved in nociception.

Prostaglandins and the Spinal Nociceptive Process

PG Formation

Prostaglandins are formed by cyclooxygenation of AA and peroxidation of PGH_2. Individual PG synthases convert PGH_2 into prostanoids, including PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2. Prostanoids are synthesised upon demand and are rapidly metabolised with biological half-lives of approximately 1 min (Appleton *et al.,* 1996). In the normal rat spinal cord, PGD_2 and PGE_2 are the predominant PGs, followed by PGI_2 and $PGF_{2\alpha}$ (Willingale *et al.,* 1997).

Table 4. Constitutive expression of prostaglandin and thromboxane synthases in the spinal cord.[*]

Synthase / Method	Species	Localisation			Cell type	Reference
		Dorsal horn	Lamina X	Ventral horn		
PGFS I						
IHC	Rat	√		√	Neurones and endothelial cells	(Suzuki-Yamamoto *et al.*, 2000)
PGFS II						
WB	Rat				Spinal cord[†]	(Suzuki-Yamamoto *et al.*, 2003)
IHC	Rat		√		Ependimal cells, tanacytes, endothelial cells	(Suzuki-Yamamoto *et al.*, 2003)
mPGES1						
NB	Human				Spinal cord[†]	(Jakobsson *et al.*, 1999)
mPGES2						
NB	Human				Spinal cord[†]	(Tanikawa *et al.*, 2002)
TxS						
IHC	Ovine	√	√	√	Neurones, astroglial and ependimal cells	(Husted *et al.*, 2003)
L-PGDS						
IHC	Mice	√	√	√	Leptomeninges and oligodendrocytes of the spinal cord	(Eguchi *et al.*, 1999)

* IHC = immunohistochemistry; L-PGDS = lipocalin-type pgrostaglandin D synthase; mPGES1 = microsomal pgrostaglandin E synthase 1; mPGES2 = microsomal pgrostaglandin E synthase 2; NB = northern blotting; PGFS I = prostagalndin F synthase I; PGFS II = prostagalndin F synthase II; TxS = thromboxane synthase; WB = western blotting; † homogenate of whole spinal cord.

PG Synthase Expression

Isozymes of specific PG synthases have been identified. PGF synthase (PGFS) exists in two variants: PGFS I (Suzuki-Yamamoto *et al.,* 2000) and PGFS II (Suzuki-Yamamoto *et al.,* 2003); whereas PGE synthase (PGES) subsists as two microsomal-bound forms (mPGS1 and mPGS2) and one cytosolic form (cPGES) (Trebino *et al.,* 2003; Scholich and Geisslinger, 2006; Zeilhofer and Brune 2006). The physiological role of mPGS2 is unclear, while cPGES and mPGES1 couple preferentially with COX-1 and COX-2, respectively (Scholich and Geisslinger, 2006; Zeilhofer and Brune 2006). In addition, the glutathione-*S*-transferases GSTμ2 and GSTμ3 might also produce PGE_2 from PGH_2 (Trebino *et al.,* 2003; Zeilhofer and Brune 2006). There are three PGD synthases (PGDS), but lipocalin-type PGDS (L-PGDS) is believed to be responsible for the biosynthesis of PGD_2 in the central nervous system (Eguchi *et al.,* 1999). The mRNA and protein expression for some PG synthases in the spinal cord is summarised in Table 4.

PG Synthase Deficiency and Pain

In a model of abdominal pain, mPGES1 knockout mice exhibited the same pain behaviour as NSAID-treated wild-type mice, whereas untreated $mPGES^{+/+}$ mice developed the full classic response. However, withdrawal latencies of mPGES-null and wild-type mice in a hot plate test were not different (Trebino *et al.,* 2003), which agrees with the lack of antinociceptive action of NSAIDs in this test. However, the development of mechanical and thermal allodynia in mPGES1-deficient mice was abolished in a spinal nerve ligation model of neuropathic pain (Mabuchi *et al.,* 2004), which contrast with the lack of clinical efficacy of COX inhibitors to control this kind of pain.

L-PGDS seems to play a major spinal role in allodynia. After intrathecal injection of PGE_2, wild-type mice developed allodynia and hyperalgesia, but mice lacking L-PGDS developed hyperalgesia only. Similarly, intrathecal injection of the $GABA_A$ receptor antagonist bicuculline (increases neuronal excitability by decreasing Cl^- influx) produced allodynia in wild-type mice, but not in $L\text{-}PGDS^{-/-}$ mice (Eguchi *et al.,* 1999).

PG Release

In the spinal cord, PGs can be released by activation of high-threshold nociceptive afferent inputs (Ramwell *et al.,* 1966), peripheral inflammatory stimuli (Yang *et al.,* 1996; Smith *et al.,* 1998a; Dirig and Yaksh, 1999; Muth-Selbach *et al.,* 1999; Tegeder *et al.,* 2001; Nakayama *et al.,* 2002; Kroin *et al.,* 2006) or direct spinal administration of neurotransmitters (Chaplan *et al.,* 1997; Dirig and Yaksh, 1999; Yaksh *et al.,* 2001). These events suggest certain level of repetitive stimulation of the primary afferent for the release of PGs and support the NMDA-receptor activation for their generation. Nonetheless, PG generation can also occur by co-activation of AMPA/kainate and mGluR receptors (Meller *et al.,* 1996; Bezzi *et al.,* 1998; Vane *et al.,* 1998), and PGs can even be released from neuronal and non-neuronal elements (Buritova *et al.,* 1996; Willingale *et al.,* 1997; Bezzi *et al.,* 1998). The release of PGs from their generating cells is mediated by the active carrier transporter

multidrug resistance protein 4 (Reid *et al.,* 2003), but its function in the spinal modulation of pain remains to be elucidated.

PGs and Pain

The intrathecal injection of PGs and PG analogues increase nociceptive responses. In mice, for instance, PGE_2 and $PGF_{2\alpha}$ produced allodynia by co-activating AMPA and NMDA receptors and AMPA and mGluR receptors, respectively (Minami *et al.,* 1994b). The constitutive presence of spinal PGD_2 was required for PGE_2, but not $PGF_{2\alpha}$, to evoke this altered pain sensation (Eguchi *et al.,* 1999). In rodents, PGE_2 given intrathecally produced allodynia and hyperalgesia (Taiwo and Levine, 1988; Minami *et al.,* 1995; Minami *et al.,* 2001; Parada *et al.,* 2003). The exact mechanisms by which PGs enhance nociception are not clear, but may include enhanced Ca^{2+}-dependent glutamate (Bezzi *et al.,* 1998), SP (Malcangio *et al.,* 1996; Southall and Vasko, 2001) and calcitonin gene-related peptide (CGRP) release (Andreeva and Rang, 1993; Southall and Vasko, 2001); noradrenaline release inhibition from the bulbospinal noradrenergic pathway (Taiwo and Levine, 1988); up-regulation of the neurokinin$_1$ receptor (NK_1; binding site for SP) in DRG sensory neurones (Segond von Banchet *et al.,* 2003); direct depolarisation of wide dynamic range neurones in the deep dorsal horn of the spinal cord (Baba *et al.,* 2001); and reduction of the inhibitory tone of glycine onto neurones in the superficial laminae of the dorsal horn (Harvey *et al.,* 2004).

Although hypothesised that PGs may diffuse back to the primary afferent terminal to amplify pain signals, it is more likely that they use a specific carrier-mediated transport (PG transporters) (Kanai *et al.,* 1995) since they are charged anions at physiological pH and diffuse poorly across biological membranes. Thus, not just the release (Reid *et al.,* 2003), but also the up-take of prostanoids may be mediated by carrier transporters.

Interestingly, not all PGs increase pain sensation. In mice, for instance, intrathecal administration of PGD_2 blocked nociceptin-induced allodynia (Minami *et al.,* 1997), and even PGE_2 played a physiological inhibitory role in the appearance of thermal hyperalgesia (Minami *et al.,* 2001).

In general, PGs are synthesised upon demand by specific PG synthases, and their release is increased in the spinal cord during development of central sensitisation. Intrathecal injection of some PGs induces allodynia and/or hyperalgesia. Preventing the formation of PGs may be useful for treating some types of pain, although inhibition of specific PG formation and subsequent redirection of eicosanoid synthesis has been recently questioned for such a treatment (Scholich and Geisslinger, 2006).

Prostaglandin Receptors and the Spinal Nociceptive Process

PG Receptors

Prostaglandins act as extracellular and intracellular messengers by activating specific G protein-coupled membrane receptors. Receptors for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2 are

denoted as DP, EP, FP, IP and TP receptors, respectively. For some of them, receptor subtypes and isoforms have been identified. After activation, the signal transduction pathway a given prostanoid receptor follows depends on the G protein species the receptor is coupled to. In general, there are changes in concentrations of the second messengers 3',5'-cyclic adenosine monophosphate (cAMP), inositol-(1,4,5) triphosphate ($InsP_3$) or Ca^{2+} (Table 5).

Table 5. Classification and signal transduction pathways of prostanoid receptors.*

Receptor			G protein	Second messenger and (effect)
Type	**Subtype**	**Isoform**		
DP			G_s	cAMP (↑)
EP	EP_1		Unidentified	Ca^{2+} (↑)
	EP_2		G_s	cAMP (↑)
	EP_3	EP_{3A}	G_i	cAMP (↓)
		EP_{3B}	G_s	cAMP (↑)
		EP_{3C}	G_s	cAMP (↑)
		EP_{3D}	G_i, G_s, G_q	cAMP (↓), cAMP (↑), $InsP_3$ (↑)
	EP_4		G_s	cAMP (↑)
FP			G_q	$InsP_3$ (↑)
IP			G_s, G_q	cAMP (↑), $InsP_3$ (↑)
TP		TP_α	G_q, G_i	$InsP_3$ (↑), cAMP (↓)
		TP_β	G_q, G_s	$InsP_3$ (↑), cAMP (↑)

* Abbreviations: (↓) = decrease; (↑) = increase; Ca^{2+} = calcium; cAMP = 3',5'-cyclic adenosine monophosphate; $InsP_3$ = inositol-(1,4,5) triphosphate.
Elaborated with information from (Coleman *et al.,* 1994; Narumiya *et al.,* 1999)

PG Receptor Expression

Both mRNA and protein for several prostanoid receptors are expressed in the spinal cord, especially in the superficial layers of the dorsal horn, and in DRG neurones (Table 6). In DRG from normal mice, mRNAs for EP_1, EP_3, EP_4 and IP receptors were expressed in about 30%, 50%, 20% and 40% of neurones, respectively. Co-expression of EP_1, EP_3 or EP_4 receptor mRNAs with IP receptor mRNA occurred in 25%, 41% and 24% of IP receptor mRNA-positive DRG neurones, respectively (Oida *et al.,* 1995). Further, mRNA for the SP precursor preprotachykinin A was present in 30% of total DRG neurones, and 70% of these neurones co-expressed IP receptor mRNA and corresponded to C and Aδ fibres (Oida *et al.,* 1995). These data may suggest a nocimodulator role for IP receptors.

The four EP receptor subtypes were also expressed in nuclear membranes from porcine newborn brain cortex (Bhattacharya *et al.,* 1998), suggesting that they may play a role in gene transcription.

PG Receptors and Pain

Allodynia and hyperalgesia can develop after activation of PG receptors in sensory fibres (Bley *et al.,* 1998; Smith *et al.,* 1998b) by way of neurotransmitter and/or neuropeptide release from primary afferent fibres (Andreeva and Rang, 1993; Oida *et al.,* 1995; Buritova *et*

al., 1996; Malcangio *et al.,* 1996; Bezzi *et al.,* 1998; Southall and Vasko, 2001). This may be initiated in part by: *(i)* activation of adenylate cyclase, which increases cAMP levels and thus stimulates PKA; or *(ii)* activation of phospholipase C, leading to enhanced PKC activity and increased $InsP_3$ levels (Coleman *et al.,* 1994; Smith *et al.,* 1998b; Narumiya *et al.,* 1999). However, activation of some PG receptors has an inhibitory action —reducing cAMP production (Table 5). In fact, spinal activation of EP_{3A} receptors reduced the hypersensitivity of dorsal horn neurones in the spinal cord of rats suffering from peripheral inflammation, and decreased PGE_2-mediated increased responses in both spinal and DRG neurones (Bär *et al.,* 2004). Interestingly, spinal EP_1 receptors seemed to play an inhibitory role in the appearance of hyperalgesia induced by intrathecal injection of PGE_2 in both mice and rats (Minami *et al.,* 2001; Nakayama *et al.,* 2004).

The activation of spinal EP_1, EP_2 and EP_4 receptors was pharmacologically involved in the development of spinal hypersensitivity similar to that observed after knee inflammation in rats (Bär *et al.,* 2004). EP_1 receptors and EP_2 and EP_3 receptors (at high and low doses of PGE_2, respectively) were involved in PGE_2-evoked allodynia and hyperalgesia in mice, respectively (Minami *et al.,* 1994a). Accordingly, in mice intrathecal injection of the EP_1 receptor antagonist ONO-NT-012 (also has TP receptor antagonist and EP_3 receptor agonist activities) reduced PGE_2-induced allodynia (Minami *et al.,* 1995) and that of the EP_3 receptor agonist ONO-AE-248 produced hyperalgesia (Minami *et al.,* 2001). Conversely, intrathecal administration of selective EP_1 receptor antagonists (SC-51089 and SC-51234A; Malmberg *et al.,* 1994) (ONO-8711; Nakayama *et al.,* 2002) produced antihyperalgesia in rats injected carrageenan or formalin into a hind paw. The discrepancy between antinociceptive effects (antiallodynia *vs*. antihyperalgesia) at the EP_1 receptor level could be due to differences in species (mice *vs*. rats) or experimental paradigms (intrathecal PGE_2 *vs*. inflammatory pain).

Activation of nuclear EP_1 receptors with 17-phenyltrinor PGE_2 increased the intranuclear concentration of Ca^{2+} and the transcription of the immediate-early gene *c-fos* (Bhattacharya *et al.,* 1998). These data support the hypothesis that prostanoids signalling from the cell membrane/cytoplasm to the nucleus may enhance spinal c-Fos protein expression (a nuclear protein encoded by *c-fos*, which, although not purely involved in nociception, is extensively used as an indirect marker of neurones involved in nociceptive processes) (Buritova *et al.,* 1996).

Blockade of spinal DP receptors with BW A868C (or lack of L-PGDS, see above) produced allodynia (Minami *et al.,* 1996; Minami *et al.,* 1999). On the contrary, over-activation of DP receptors with BW 245C reversed nociceptin- and PGE_2-induced allodynia (Minami *et al.,* 1996; Minami *et al.,* 1997), suggesting complex interactions between opioid-like, EP and DP receptor systems.

The lack of more selective ligands has precluded the pharmacological exploration of possible physiological and pathophysiological functions of prostanoid receptor subtypes on the spinal processing of pain.

Table 6. Expression of prostanoid receptors in the spinal cord and dorsal root ganglia neurones.*

Receptor / Method	Species	Localisation			Cell type	Reference
		Dorsal horn	Ventral horn	DRG		
DP						
ISH		√	√		Neurones	(Wright *et al.*, 1999)
EP						
[^{3}H]PGE_2 AR	Rat	√		√ (few)	Neurones	(Bley *et al.*, 1998)
EP_1						
[^{3}H]iloprost AR	Rat	√		√ (few)	Neurones	(Bley *et al.*, 1998)
ISH	Mice			√ (30%)	Neurones	(Oida *et al.*, 1995)
ISH	Rat			√ (32%)	Neurones	(Nakayama *et al.*, 2004)
RT-PCR	Rat	√ (few)		√		(Lin *et al.*, 2006)
EP_2						
WB	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Sheep	√ (few)	√(few)			(Dolan *et al.*, 2000)
RT-PCR	Rat	√ (few)		√		(Lin *et al.*, 2006)
EP_3						
[^{3}H]iloprost AR	Rat	√		√ (few)	Neurones	(Bley *et al.*, 1998)
RT-PCR	Sheep	√	√			(Dolan *et al.*, 2000)
RT-PCR	Rat			√		(Lin *et al.*, 2006)
ISH	Mice			√ (50%)	Neurones	(Oida *et al.*, 1995)
EP_{3C}						
WB	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Rat			√		(Southall and Vasko, 2001)

Table 6. Continued

Receptor / Method	Species	Localisation			Cell type	Reference
		Dorsal horn	Ventral horn	DRG		
EP_4						
ISH	Mice			√ (20%)	Neurones	(Oida *et al.*, 1995)
ISH	Rat			√	Neurones	(Lin *et al.*, 2006)
IMH	Rat			√	Neurones	(Lin *et al.*, 2006)
RT-PCR	Rat	√ (few)		√		(Lin *et al.*, 2006)
WB	Rat	√ (few)		√		(Lin *et al.*, 2006)
WB	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Rat			√		(Southall and Vasko, 2001)
IP						
ISH	Mice	√		√ (40%)	Neurones	(Oida *et al.*, 1995)
WB	Rat			√		(Southall and Vasko, 2001)
RT-PCR	Rat			√		(Southall and Vasko, 2001)
[^{3}H]iloprost AR	Rat	√		√ (few)	Neurones	(Bley *et al.*, 1998)

*Abbreviations: AR = autoradiography; DP = prostaglandin D receptor; EP = prostaglandin E receptor; IP = prostaglandin I receptor; ISH = *in situ* hybridisation; IHC = immunohistochemistry; RT-PCR = reverse transcrption-polymerase chain reaction; WB = western blotting.

PG Receptor Deficiency

Mice deficient in EP_2 receptors lacked spinal PGE_2-evoked hyperalgesia, but responded normally to peripheral PGE_2. In addition, the initial pain sensitisation (first 6 hours) after injection of zymosan A into a hind paw was similar between wild-type and EP_2-deficient mice, but the latter animals recovered from sensitisation much faster (within 24 hours) than the formers (~7 days) (Reinold *et al.,* 2005). Knocking down the EP_4 receptor with intrathecally delived short hairpin RNA attenuated both thermal and mechanical hyperresponsivness induced by intraplantar injection of complete Freund's adjuvant in the hind paw of rats (Lin *et al.,* 2006). Deletion of both EP_{3C} and EP_4 receptors using antisense oligonucleotides reduced the PGE_2-evoked increased release of neuropepides from DRG neurones (Southall and Vasko, 2001). Mice lacking the EP_3 receptor ($EP_3^{-/-}$) developed allodynia after intrathecal injection of PGE_2, but mice lacking the EP_1 receptor ($EP_1^{-/-}$) did not (Minami *et al.,* 2001). $EP_3^{-/-}$ mice also showed hyperalgesia after high, but not low, doses of PGE_2 and did not develop hyperalgesia after intrathecal injection of the selective EP_3 receptor agonist ONO-AE-248 (Minami *et al.,* 2001). These data confirm the pharmacological findings (see above) that the EP_1 receptor and the EP_2 and EP_3 receptors (at high and low doses of PGE_2, respectively) are involved in PGE_2-induced allodynia and hyperalgesia, respectively. Interestingly, saline-treated $EP_1^{-/-}$ mice were in hyperalgesia and this was reversed by intrathecal PGE_2 (Minami *et al.,* 2001). In mice, pain induced by intraplantar injection of formalin was not ascribed to either EP_1 or EP_3 receptor subtype since wild-type, $EP_1^{-/-}$ and $EP_3^{-/-}$ animals developed similar behaviours after injection of the irritant (Segond von Banchet *et al.,* 2003). Together, these data suggest that intrathecal PGE_2 and peripheral inflammation may induce spinal sensitisation by activating different EP receptors.

The comparable tail-flick and hot-plate latencies, but not writhing responses, between wild-type and IP receptor knockout mice did not support the role of IP receptors in spinal nociceptive transmission (Murata *et al.,* 1997) —the first two tests are sensitive to central acting drugs and the writhing test to both central and peripheral acting drugs.

Prostanoid receptors are present in DRG neurones and superficial layers of the spinal cord dorsal horn and, in general, their activation induces allodynia and/or hyperalgesia. They may do so, at least in part, by altering the concentrations of cAMP, $InsP_3$ or Ca^{2+}. Although, at present, there are few highly selective ligands for PG receptors, the active research in this field will certainly expand our knowledge on the role of these receptors under normal and increased nociceptive spinal transmission. In the near future, it may even be possible to treat pain by targeting specific PG receptor sybtypes.

Protein Kinases A and C and the Spinal Nociceptive Process

Protein Kinases A and C Isoforms and Functions

The mammalian PKA is a tetramer composed of a regulatory subunit dimer, which contains the cAMP binding sites, and a single catalytic subunit bound to each regulatory subunit. At least four regulatory (RIα, RIβ, RIIα, and RIIβ) and two catalytic (Cα and Cβ)

subunits have been characterised. The subunit composition of a holoenzyme contributes to its activation properties by cAMP (Brandon *et al.,* 1997).

PKC represents a family of al least 12 serine/theorine kinases and they are divided in four groups: (*i*) conventional PKCs (α, βI, βII, and γ), which are Ca^{2+}-dependent and activated by phosphatidylserine (PS) and diacylglycerol (DAG); (*ii*) novel PKCs (δ, ε, η, and θ), which are Ca^{2+}-independent and regulated by PS and DAG; (*iii*) atypical PKCs (ζ and ι/λ), which are activated by PS and phosphatidylinositol-2,3,4-triphosphate; and (*iv*) recently described PKCs (μ and ν), which are activated by DAG and phospatidylinositol-4,5-diphosphate (Battaini, 2001; Velázquez *et al.,* 2007).

Once activated, PKA and PKC may phosphorylate relevant substrates that control intracellular events permitting cell-to-cell communication.

Protein Kinases A and C Isoforms and Expression

The α isoforms of the PKA subunits are ubiquitously expressed in neural and non-neural tissues, but the β isoforms are more restricted in the nervous system (Brandon *et al.,* 1997; Malmberg *et al.,* 2003). Immunocytochemistry studies have shown the presence of phosphorylated-PKA in the superficial laminae of the dorsal horn of the mice spinal cord, and immunoreactivity increased 7 days after injection of complete Freund's adjuvant into a hind paw (Yajima *et al.,* 2003).

Most PKC are widely distributed throughout the body, but the γ isoform is restricted mainly to the brain and the spinal cord (Battaini, 2001). Binding studies with [^{3}H]PDBU, a phorbol ester which binds to the DAG binding site on PKC, demonstrated that PKCs were constitutively expressed in lamina I-II of the rat spinal cord (Tölle *et al.,* 1996). In mice and using immunocytochemistry techniques, the presence of phosphorylated-conventional PKCs has been demonstrated in superficial laminae of the dorsal horn of the spinal cord (Yajima *et al.,* 2003), which is consistent with PKC α, βI, and βII being distributed rather homogeneously in the DRG and all superficial layers of the dorsal horn of the spinal cord, and PKC γ only being found in interneurones of lamina II (Malmberg *et al.,* 1997; Velázquez *et al.,* 2007).

An increased binding of [^{3}H]PDBU in lamina I-II of the rat spinal cord demonstrated up-regulation of PKC following 4 and 14 days of monoarthritis induced by injection of complete Freund's adjuvant into the tibio-tarsal joint of one hind limb (Tölle *et al.,* 1996). Immunoreactivity of phosphorylated-conventional PKCs was also up-regulated in the superficial laminae of the spinal cord at 7 days after sciatic nerve ligation in mice (Yajima *et al.,* 2003). Acute (within 24 hours) spinal up-regulation of PKC γ protein also occurred after intrathecal administration of kainic acid in rats (Wen *et al.,* 2001).

Protein Kinases A and C and Pain

In mice, inflammatory thermal hyperalgesia was attenuated by chronic intrathecal treatment for 7 days with the selective PKA inhibitor KT-5720, but not by the selective PKC inhibitor RO-320432. The opposite was true in a model of neuropathic thermal hyperalgesia (Yajima *et al.,* 2003).

The possible mechanisms by which PKA increases nociception may include phosphorylation of NMDA receptors coupled to yotiao proteins resulting in enhancement of current flow through the receptor (Westphal *et al.,* 1999), phosphorylation of spinal glycine α_3 receptors resulting in decreased receptor's activity (Harvey *et al.,* 2004), up-regulation of NK_1 receptors in DRG neurones (Segond von Banchet *et al.,* 2003), and increased AA release from astrocytes (Strokin *et al.,* 2003). Hence, PKA may blunt the inhibition and facilitate the transmission of pain signals through the spinal cord.

For PKC, the increase in channel kinetics and time spent open of the spinal NMDA receptor NR2B subunit is mediated by convergence of multiple excitatory neurotransmitter and neuropeptides released at the primary afferent-spinal cord synapse. For instance group I mGluR receptors and NK_1 receptors facilitate the activation of PKC enhancing NMDA receptor activity through phosphorylation. This is an indirect process that occurs for mGluR5 via the formation of a signalling complex that includes the postsynaptic density protein Shank. This protein is associated with mGluR receptors and contributes to phosphorylation of NR2B subunits by tyrosine kinase Src, a process in which PSD-95 plays an important role (Velázqez et al., 2007).

Protein Kinases A and C Deficiency

As compared to wild-type mice, transgenic mice lacking the RIβ subunit of PKA exhibited less nociceptive responses to either intraplantar formalin and intrathecal PGE_2 injections, but neurophatic pain was not affected (Malmberg *et al.,* 2003). On the contrary, mice lacking the γ isoform of PKC were less prone to develop neuropathic pain than their naïve counterparts (Malmberg *et al.,* 1997).

Collectively, these data suggest that spinal activation of PKA and PKC may be involved in inflammatory and neuropathic pain, respectively. Targeting prostanoid receptor signal transduction pathways may serve to control some types of pain.

NMDA Receptors and the Nitric Oxide Pathway

In addition to activating phospholipases, Ca^{2+} entering through NMDA receptors activates calcineurin and binds to calmodulin forming the Ca^{2+}-calmodulin complex (CaM). Calcineurin desphosphorylates the enzyme NOS, the catalytic activity of whose is usually reduced by PKC-induced phosphorylation, and the CaM complex further activates the enzyme (Dawson and Dawson, 1995). NOS catalyses the conversion of L-arginine to L-citrulline and NO. The physical properties of NO [*e.g.*, small, diffusible, membrane permeable, reactive (Dawson and Dawson, 1995)] allow it to diffuse back to presynaptic terminals where it increases the release of neurotransmitters and/or neuropeptides, which augment the nociceptive input. Supporting evidence for this model comes from: *(i)* increased spinal release of NO after intrathecal perfusion of glutamate or NMDA (Kawamata and Omote, 1999; Rivot *et al.,* 1999); *(ii)* increased spinal release of citrulline, a marker of NO synthesis (Yang *et al.,* 1996), and NO metabolites nitrite/nitrate (Gühring *et al.,* 2000; Vetter *et al.,* 2001) in states of facilitated processing; *(iii)* development of allodynia and hyperalgesia after intrathecal administration of L-arginine (Eguchi *et al.,* 1999; Minami *et al.,* 2001) and

NO donors (Minami *et al.,* 1995; Eguchi *et al.,* 1999; Tao and Johns, 2000; Minami *et al.,* 2001); and *(iv)* increased spinal release of glutamate, SP and CGRP after activation of NMDA receptors or NOS with the consequent release of NO (Aimar *et al.,* 1998; Kawamata and Omote, 1999).

Nitric Oxide Synthases and the Spinal Nociceptive Process

NOS Isozymes and Functions

Three isoforms of NOS have been identified, two are constitutive enzymes: neuronal NOS (nNOS) and endothelial NOS (eNOS); and one is inducible (iNOS) (Dawson and Dawson, 1995; Appleton *et al.,* 1996). Apart from cell type, the NOS isoform synthesised is dependent on cell activation. Both nNOS and eNOS depend on CaM for catalytic activity, whereas iNOS is Ca^{2+}-independent and has calmodulin tightly bound to the enzyme. Calmodulin is considered a protein subunit of iNOS, which accounts for its resistance to Ca^{2+} activation (Dawson and Dawson, 1995; Appleton *et al.,* 1996). In contrast, induction of iNOS requires of protein tyrosine kinase activity (Kleinert *et al.,* 1998). Despite these differences, all NOS isoforms perform the same catalytic reaction: conversion of L-arginine to L-citrulline and NO.

NOS Expression

Both protein and mRNA for all three NOS isozymes are present in the spinal cord of many mammal species, and are found predominantly in small- to medium-sized neurones of the superficial layers of the dorsal horn and around the central canal. The intermediolateral cell column from thoracic and sacral segments and DRG neurones are important sources of NOS also. Scattered expression of NOS in the ventral horn corresponds mainly to motoneurones (Table 7).

The distribution of NOS isozymes in dorsal horn and DRG neurones confers a nocimodulator role to these enzymes. In fact, up-regulation of nNOS (Maihofner *et al.,* 2000a; Maihofner *et al.,* 2000b; Wu *et al.,* 2001) and iNOS (Maihofner *et al.,* 2000a; Wu *et al.,* 2001) protein expression in the superficial layers of the spinal cord occurred after peripheral inflammatory hyperalgesia. Likewise, spinal nNOS protein expression increased after intrathecal kainate injection (Wen *et al.,* 2001), and nNOS protein (Qian *et al.,* 1996) and mRNA expression (Luo *et al.,* 1999) were up-regulated in rat DRG, but not in spinal cord, neurones after peripheral nerve injury.

Interestingly, intrathecal injection of SP(1-7), the major metabolite of SP, decreased nNOS mRNA expression and NOS activity in the rat spinal cord (Kovacs *et al.,* 2001), suggesting a possible feed-back mechanism.

Table 7. Constitutive expression of nitric oxide synthase isozymes in the spinal cord.*

Isozyme / Method	Species	Localisation					Cell type	Reference
		Dorsal horn	Lamina X	IML cell column	Ventral horn	DRG		
nNOS								
NADPH-d	Rat	√	√	√	√ (few)	√	Neurones	(Valtschanoff *et al.*, 1992)
WB	Rat	√			√	√		(Qian *et al.*, 1996)
IHC	Rat	√	√	√	√	√	Neurones	(Terenghi *et al.*, 1993)
IHC	Human	√	√	√	√	√	Neurones	(Terenghi *et al.*, 1993)
NADPH-d	Sheep	√		√	√ (few)			(Xu *et al.*, 1996a)
IHC	Sheep	√	√		√ (few)		Neurones	(Dolan *et al.*, 2000)
NADPH-d	Sheep	√			√ (few)		Neurones	(Dolan *et al.*, 2000)
RT-PCR	Sheep	√†			√†			(Dolan *et al.*, 2000)
IHC	Cat	√	√	nd	√		Neurones	(Pullen *et al.*, 1997)
IHC	Macaque	√	√	√ (few)	√		Neurones	(Pullen *et al.*, 1997)
IHC	Human	√	√	√	√		Neurones	(Pullen *et al.*, 1997)
WB	Rat	√†			√†			(Wu *et al.*, 2001)
RT-PCR	Rat	√†			√†	√		(Kovacs *et al.*, 2001)
IHC	Mouse	√	√				Neurones	(Maihofner *et al.*, 2000)
WB	Rat	√†			√†			(Wen *et al.*, 2001)
IHC	Mouse	√					Neurones	(Maihofner *et al.*, 2000)
NADPH-d	Cat	√	√	√		√	Neurones	(Vizzard *et al.*, 1994)
NADPH-d	Mouse	√	√		nd		Neurones	(Bruning, 1992)
IHC	Rat, mouse, cat, sqirrel monkey	√	√	√	√ (few)		Neurones	(Dun *et al.*, 1994)

Table 7. Continued

Isozyme / Method	Species	Localisation					Cell type	Reference
		Dorsal horn	Lamina X	IML cell column	Ventral horn	DRG		
iNOS								
IHC	Sheep	√	√		√ (few)		Neurones	(Dolan *et al.*, 2000)
RT-PCR	Sheep	√†			√†			(Dolan *et al.*, 2000)
IHC	Cat		√ (few)		nd		Neurones	(Pullen *et al.*, 1997)
WB	Rat	√†			√†			(Wu *et al.*, 2001)
WB	Rat	nd			nd			(Wen *et al.*, 2001)
IHC	Mice	√					Astrocytes	(Maihofner *et al.*, 2000)
eNOS								
IHC	Sheep	√	√		√ (few)		Neurones	(Dolan *et al.*, 2000)
RT-PCR	Sheep	√†			√†			(Dolan *et al.*, 2000)
IHC	Cat	√	√		√ (few)		Astrocytes	(Pullen *et al.*, 1997)

*Abbreviations: √ = present; † = homogenate of whole spinal cord; eNOS = endothelial nitric oxide synthase; IHC = immunohistochemistry; iNOS = inducible nitric oxide synthase; NADPH-d = nicotinamide adenine dinucleotide phosphste-diaphorase staining; nd = not detected; nNOS = neuronal nitric oxide synthase; RT-PCR = reverse transcriptase-polymerase chain reaction; WB = western blotting.

NOS Inhibitors and Pain

In rodents, electrophysiological data have showed that intrathecal infusion of NOS inhibitors reduced the increased responsiveness of dorsal horn neurones to noxious and innocuous stimulation of an inflamed peripheral tissue (Wu *et al.,* 2001), and genetic information has indicated that blockade of spinal NOS reduced c-Fos labelling in laminae I-II of the spinal cord (Roche *et al.,* 1996). Behavioural evidence has demonstrated that selective or non-selective pharmacological inhibition of spinal NOS activity reduced hyperalgesia induced by intrathecal administration of glutamate or NMDA (Ferreira *et al.,* 1999; Kawamata and Omote, 1999; Fairbanks *et al.,* 2000; Park *et al.,* 2000), or by injection of irritants into the paw (Roche *et al.,* 1996; Larson *et al.,* 2000; Park *et al.,* 2000; Wu *et al.,* 2001) or the abdominal cavity (Larson *et al.,* 2000).

Interestingly, the analgesic effects of intrathecally injected NOS inhibitors were still observed well after the spinal activity of NOS had been re-established (Larson *et al.,* 2000), which suggests that transient changes in the spinal synthesis of NO may produce long-term changes in nociceptive processing, probably at the level of synthesis (up- or down-regulation) or activity (sensitisation or desensitisation) of proteins (Larson *et al.,* 2000). Hence, spinal NO seems to be an important modulator in the plasticity of nociceptive pathways.

Analgesia induced after intrathecal administration of non-selective inhibitors of NOS, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME), cannot be solely attributed to inhibition of NOS in neurones. They also induce vasoconstriction by inhibiting eNOS, which may affect interpretation of results. The use of selective nNOS inhibitors, such as 7-nitroindazole, may overcome this pitfall, and they have been shown to reduce C-fibre evoked spinal responses in both normal and inflamed anaesthetised rats (Stanfa *et al.,* 1996).

It is also possible that NOS inhibitors have a dual antinociceptive action by inhibiting the synthesis of NO and by augmenting the levels of L-arginine, which has been shown to induce antinociception by an opioidergic mechanism independent of the NO pathway (Kawabata *et al.,* 1992). L-Arginine could follow alternative pathways to the NOS pathway: *(i)* by arginase to ornithine and urea, and *(ii)* by arginine decarboxylase to agmatine. Ornithine and agmatine, in turn, can be converted into the polyamines putrescine, spermidine and spermine (Reis and Regunathan, 2000). Agmatine and sometimes polyamines can negatively modulate NMDA receptors (Appleton *et al.,* 1996; Dingledine *et al.,* 1999; Reis and Regunathan, 2000), contributing so to reduce facilitated states. In fact, intrathecal administration of agmatine reduced inflammatory, chemical, and mechanical hyperalgesia in rodents (Fairbanks *et al.,* 2000).

NOS Isozyme Deficiency

As compared to wild-type mice, NMDA-stimulated glutamate release was reduced in nNOS knockout mice, and NMDA-stimulated GABA release was reduced in eNOS knockout mice (Kano *et al.,* 1998), suggesting a stimulatory and inhibitory role for nNOS and eNOS isoenzymes, respectively. Wild-type mice and nNOS knockout mice exhibited similar pain behaviours after intraplantar injection of formalin, but analgesia with intraperitoneal L-NAME occurred in wild-type mice only, which suggested that NO was involved in, but was not necessarily required for, the development of noxious stimulation-induced central sensitisation

(Crosby *et al.,* 1995). In contrast, iNOS knockout mice showed reduced thermal hyperalgesia in a zymosan model of pain (Gühring *et al.,* 2000). Antihyperalgesia was attributed to lack of spinal iNOS since intrathecal, but not intraperitoneal, injection of the iNOS selective inhibitor L-NIL reduced thermal hyperalgesia in wild-type mice to a similar level to that observed in $iNOS^{-/-}$ mice. The role of nNOS and eNOS seemed to be minimal since intrathecal injection of the non-selective NOS inhibitor L-NAME into $iNOS^{-/-}$ mice had no effect on thermal hyperalgesia (Gühring *et al.,* 2000). However, a recent study that simultaneously compared nNOS-, iNOS, and eNOS-deficient mice injected with complete Freund's adjuvant into a hind paw, showed a significant reduction in both thermal hyperalgesia and mechanical allodynia only in nNOS-deficient mice as compared to wild-type animals (Boettger *et al.,* 2007).

The above data evidence the prominent role of NOS isozymes in the development and/or maintenance of central sensitisation, and highlights the relevance of different isozymes according to the experimental paradigm. Inhibiting the activity of NOS enzymes in the spinal cord could be an important target for treating some types of pain.

Nitric Oxide and the Spinal Nociceptive Process

NO Formation and Release

NO is formed by oxidation of L-arginine. NO is not stored, and, on the contrary, it is released and utilised immediately; it can travel about 50 μm before degradation (Gühring *et al.,* 2000). In the spinal cord, NO can be released by peripheral inflammatory stimuli (Gühring *et al.,* 2000), peripheral nerve damage (Luo *et al.,* 1999) or direct administration of glutamate and NMDA (Kawamata and Omote, 1999; Rivot *et al.,* 1999). These events suggest certain level of repetitive stimulation of the primary afferent for the release of NO, and support the NMDA-receptor activation theory for its generation.

NO and Pain

Once formed, NO exerts a direct action on postsynaptic ion channel proteins or diffuses to the presynaptic terminals and astrocytes where it activates the enzymes guanylate cyclases to increase the concentration of 3',5'-cyclic guanosine monophosphate (cGMP) and the release of neuroactive substances (Fukuto and Chaudhuri, 1995; Kawamata and Omote, 1999; Gühring *et al.,* 2000).

It is clear that NO plays a neuromodulator role on the spinal nociceptive process, but its precise role is still a matter of debate. On the one hand, support for its nociceptive action at the spinal level comes from reports of intrathecal administration of NO donors promoting allodynia and hyperalgesia (Masue *et al.,* 1999; Tao and Johns, 2000; Sousa and Prado, 2001), probably by enhancing the release of glutamate, SP and CGRP (Aimar *et al.,* 1998; Kawamata and Omote, 1999; Masue *et al.,* 1999). Further support comes from the antinociceptive effect achieved by inhibiting the enzyme NOS with L-arginine analogues (Roche *et al.,* 1996; Ferreira *et al.,* 1999; Kawamata and Omote, 1999; Fairbanks *et al.,* 2000; Larson *et al.,* 2000; Park *et al.,* 2000; Wu *et al.,* 2001). On the other hand, spinal administration of NO donors (Sousa and Prado, 2001) and L-arginine (Masue *et al.,* 1999) can

produce antinociception also. Possible antinociceptive mechanisim for NO could include s-nitrosylation and therefore inactivation of the NMDA receptor by NO or NO-derived compounds (Dawson and Dawson, 1995; Aizenman and Potthoff, 1999; Dingledine *et al.,* 1999), and interaction with noradrenaline and acetylcholine to form 6-nitro-noradrenaline and 6-nitro-acetylcholine, respectively, which inhibit both the reuptake and metabolism of these inhibitory neurotransmitters (Xu *et al.,* 1996b; Chiari *et al.,* 2000).

The opposite nociceptive effects of NO have been pharmacologically and physiologically characterised. After increasing NO concentration with NO donors in the spinal cord of neuropathic rats, both antiallodynic and proallodynic effects were induced, and these effects were attributed to low and high NO concentrations, respectively (Sousa and Prado, 2001). This agrees with electrophysiological studies in new born rat spinal cord preparations in which low concentrations of NO gas-containing medium or NO donors inhibited electrically evoked nociceptive spinal reflexes, but higher concentrations evoked depolarisation of ventral roots (Kurihara and Yoshioka, 1996). However, other electrophysiological studies have demonstrated that NO-mediated excitation and inhibition occurred in different neurones. For instance, NO had an excitatory effect in the majority of rat spinal cord neurones tested in lamina X (93% of neurones), and predominantly inhibited neurones in lamina I-II (49% of neurones) while still excited some (28% of neurones) (Pehl and Schmid, 1997). Similarly, NO produced both mechanical excitation and inhibition of dural nociceptors in urethane-anaesthetised rats (Levy and Strassman, 2004). A difference between these last two studies was that neurones that were excited showed grater spontaneous activity than those that were inhibited in the former study (Pehl and Schmid, 1997), whereas a tendency towards the contrary was observed in the latter one (Levy and Strassman, 2004). Also in this latter study, mechanical activation thresholds of excited neurones were higher than those of inhibited neurones (Levy and Strassman, 2004). Hence, the opposite effects could depend on the level of excitability of the nociceptor (Levy and Strassman, 2004).

NO-mediated opposite effects have also been attributed to the existence of a reduced and an oxidised form of this gas (Machelska *et al.,* 1997). Another hypothesis is that NO may be a modulator of different types of spinal neurones, including excitatory neurones and inhibitory interneurones; NO-induced activation of excitatory neurones could result in hyperalgesia whereas NO-induced activation of inhibitory interneurones could result in hypoalgesia (Luo and Cizkova, 2000). Experimental conditions (*e.g.*, type of afferent fibres activated, intensity and duration of input) may also determine the outcome (Sousa and Prado, 2001). Hence, the circuitry of NO in the spinal processing of pain remains to be fully characterised.

Guanylate Cyclases and the Spinal Nociceptive Process

Guanylate Cyclase Isozymes and Function

Guanylate cyclases exist in at leat five different isoforms: four heterodimers ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha1\beta2$, $\alpha2\beta2$) and one homodimer ($v\beta2$) (Gibb *et al.,* 2003). In the rat spinal cord, NO produced after subdural infusion of NMDA activated guanylate cyclases to increase cGMP concentrations (Kawamata and Omote, 1999).

Guanylate Cyclase Expression

In the dorsal horn of the rat spinal cord, guanylate cyclase α1 subunit, but not β1 subunit, was constitutively expressed and up-regulated 2 to 4 days after intraplantar injection of formalin (Tao and Johns, 2002).

cGMP and Pain

The hyperalgesic actions of NO are mediated by spinal activation of guanylate cyclases as these effects were mimicked by membrane-permeable cGMP analogues (Ferreira *et al.,* 1999) and blocked by guanylate cyclase inhibitors (Ferreira *et al.,* 1999; Kawamata and Omote, 1999; Tao and Johns, 2002). Immunohistochemical studies demonstrated the constitutive presence of cGMP in the superficial laminae of the dorsal horn of the spinal cord, and up-regulation occurred after activation of the NMDA-NO pathway (Morris *et al.,* 1994). It is well accepted that the increased concentration of cGMP in the presynaptic terminal serves to release neuroactive agents. Although the exact mechanisms that take part in this spinal nociceptive process are poorly understood, these may include direct effect on ion channels, stimulation or inhibition of phosphodiesterase activity, and activation of protein kinases (Fukuto and Chaudhuri, 1995).

Interestingly, similar to NO, cGMP also can produce dual nociceptive effects. Pharmacological manipulation of spinal cGMP levels with 8-bromo-cGMP showed that low cGMP concentrations reduced, but high concentrations increased, the hyperalgesic response to injection of formalin into the hind paw of rats (Tegeder *et al.,* 2002), and spinal inhibition of gyanylate cyclase with 1*H*-[1,2,4]oxadiazolo[4,3-α]qinoxalin-1-one (ODQ) reduced both NO-mediated antiallodynic and proallodynic effects in neuropathic rats (Sousa and Prado, 2001)

Electrophysiological data have also demonstrated that both NO-induced spinal excitation and inhibition were mimicked by the cGMP analogue 8-bromo-cGMP (Kurihara and Yoshioka, 1996; Pehl and Schmid, 1997). However, only the excitatory affects were also mimicked by the cAMP analogue 8-bromo-cAMP (Kurihara and Yoshioka, 1996; Pehl and Schmid, 1997). In fact, the membrane-permeable cGMP analogue 8-para-clorophenylthio cGMP (8-pCPT-cGMP) inhibited, but not excited, dural nociceptors in anaesthetised rats, and the gyanylate cyclase blocker ODQ prevented NO-mediated inhibition (Levy and Strassman, 2004).

These data suggest that NO-mediated inhibition depends completely, and excitation partially, on cGMP mechanisms, and that NO-induced production of cGMP alone does not allow any prediction about an excitatory or inhibitory effect on spinal neurones.

cGMP-Dependent Protein Kinases and the Spinal Nociceptive Process

cGMP-Dependent Protein Kinase Isoforms and Function

Two isosymes of cGMP-dependent protein kinases (PKG) are recognised in mammals: cytosolic PKG-I and membrane-bound PKG-II. Further, PKG-I exists in two isoforms: Iα and Iβ (Tao *et al.,* 2000b). In the spinal cord, PKG serve as major effectors for NO and cGMP.

cGMP-Dependent Protein Kinase Expression

Immunohistochemical studies demonstrated that DRG neurones and axonal processes of laminae I and II of the spinal cord are rich sources of PKG-I, with small- and medium-sized neurones being intensely stained (Qian *et al.,* 1996). In neurones of the superficial laminae of the spinal cord, however, isoform Iα was the most abundant and isoform Iβ was scarcely expressed (Tao *et al.,* 2000b). DRG neurones expressing PKG-I also showed SP and CGRP immunoreactivity (Qian *et al.,* 1996). Up-regulation of spinal PKG-I, in particular that of isoform Iα, occurred after induction of peripheral inflammation (Tao and Johns, 2002; Tegeder *et al.,* 2002). Interestingly, in rats injected formalin into a hind paw, spinal PKG-I was further increased after intrathecal treatment with a high dose of cGMP analogue 8-bromo-cGPM, but low doses completely prevented any increase at all (Tegeder *et al.,* 2002).

cGMP-Dependent Protein Kinases and Pain

Facilitation of rat tail-flick latencies after intrathecal administration of NMDA or the NO donor NOC-12 was prevented by intrathecally injecting the selective PKG-Iα inhibitor Rp-8-p[(4-Chlorophenyl)thiol]-cGMPS triethylamine (Rp-8-p-CPT-cGMPS) (Tao and Johns, 2000). Intrathecal injection of the non-selective PKG inhibitor Rp-8-bromo-cGMPS and the PKG-Iα inhibitor Rp-8-p-CPT-cGMPS also reduced the hyperalgesia induced by injecting formalin into the hind paw of rats (Tao *et al.,* 2000b; Tegeder *et al.,* 2002). Together, these data suggest that PKG-Iα may be involved in the generation of spinal hyperalgesia, though the presynaptic targets for PKG-Iα that participate in the sensory processing remain uncertain.

Conversely, the PKG inhibitor KT5823 blocked both NO- and cGMP-mediated inhibition of electrically evoked nociceptive spinal reflexes, but had no effect on the excitatory response of NO (Kurihara and Yoshioka, 1996).

Further studies are necessary to better characterise the role of PKG in the spinal processing of nociceptive information and determine the effect of altering NO signal transduction pathways to control some types of pain.

Poly(ADP-ribose) Synthase and the Spinal Nociceptive Process

NO can also produce postsynaptic effects by activating the nuclear enzyme poly(ADP-ribose) synthase. Interestingly, spinal inhibition of poly(ADP-ribose) synthase after intrathecal injection of benzamide into rats, reduced both mechanical and thermal neuropathic hyperalgesia as well as mechanical neuropathic allodynia in a similar way to that observed with the intrathecal administration of the NOS inhibitor L-NAME (Mao *et al.,* 1997). These data suggest that NO may facilitate the spinal nociceptive process not only by indirectly rising neurotransmitter/neuropeptide release from presynaptic terminals, but also by mechanisms within the same neurone from which is synthesised.

Spinal Interactions Between Arachidonic Acid and Nitric Oxide Pathways

The products of both COX and NOS enzymes can modulate the activity of the other enzyme, but the interactions are complex as both stimulatory and inhibitory actions have been ascribed.

Effects of Cyclo-Oxygenase Pathway Modulation on Nitric Oxide Synthesis

In rat spinal cord slices, PGE_2 and $PGF_{2\alpha}$ (but not PGD_2) further increased the release of NO induced by stimulation of the NMDA receptor-nNOS pathway (Sakai *et al.,* 1998). Accordingly, non-selective COX inhibitors reduced the increased release of NO and the increased protein expression of nNOS and iNOS induced by lipopolysacharide in rat cerebellar slices (DiGirolamo *et al.,* 2003). However, in lipopolysacharide-stimulated microglial cells, PGE_2 and PGD_2, and their cyclopentenone derivates PGA_2, 15-deoxy-$\Delta^{12,14}$-PGA_2, PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2, suppressed iNOS activity and/or expression (Petrova *et al.,* 1999). Moreover, opposite effects on zymosan-induced iNOS protein expression in the rat spinal cord were observed after a single oral administration of the COX-2 selective inhibitor rofecoxib, with low doses increasing and high doses decreasing iNOS expression (Niederberger *et al.,* 2003). The decreased expression of iNOS was attributed to a simultaneous inhibition of NF-κB and activation of AP-1 transcriptor factors (iNOS transcription is stimulated by NF-κB, but inhibited by AP-1) (Niederberger *et al.,* 2003). Discrepancies may also depend on specific prostanoids, most likely PGE_2, determining what biochemical pathway L-arginine follows, or on the particular experimental conditions (Appleton *et al.,* 1996).

Effects of Nitric Oxide Pathway Modulation on Prostaglandin Synthesis

The NO donor sodium nitroprusside increased PGE_2 release from cultured astroglial cells in a similar way to that by incubation of cells with NMDA or cytokines, and incubation with the NOS inhibitor L-NAME prevented the increased release of PGE_2 (Mollace *et al.,* 1995; Mollace *et al.,* 1998), wich suggests that the NO pathway may enhance COX-2 activity. In the mouse spinal cord, NO, specially derived from iNOS, modulated the catalytic activity of COX-1 and/or COX-2 and increased PGE_2 concentration (Gühring *et al.,* 2000). In cultured rat DRG cells, NO mediated the release of SP by indirectly modulating (probably through NF-κB) the expression of COX-2 at the level of gene transcription (Morioka *et al.,* 2002). Behaviourally, inhibition of spinal NO synthesis with L-NAME diminished allodynia (Minami *et al.,* 1995) and hyperalgesia (Park *et al.,* 2000) induced by intrathecal administration of PGE_2 in mice and rats, respectively. The stimulatory and inhibitory actions of NO on PG production could be explained by the relative concentrations of NO. Low levels of NO may reduce PG formation, and thus act as an antinociceptive agent, whereas high levels of NO may raise PG production, therefore acting as a pronociceptive agent (Appleton *et al.,* 1996; Machelska *et al.,* 1997).

Nitric Oxide Pathway and Spinal Cyclo-Oxygenase Inhibition-Mediated Analgesia

Modulation of the NO pathway can affect the spinal analgesic effects of COX inhibitors. On the one hand, pharmacological blockade of the NO pathway reduced the spinal analgesic effect of COX inhibitors. For instance, paw treatment with the NOS inhibitor L-NMMA or the guanylate cyclase inhibitor methylene blue prevented the spinal antihyperalgesic action of the non-selective COX inhibitor dipyrone in rats injected PGE_2 into the paw (Lorenzetti and Ferreira, 1996). Similarly, the spinal antihyperalgesic effects of the COX-2 selective inhibitor lumeracoxib in rats injected formalin into a hind paw were prevented by the intrathecal administration of the NOS inhibitor L-NAME, the NO-sensitive soluble guanylyl cyclase inhibitor ODQ, and the ATP-sensitive K^+ channel inhibitor glibenclamide (Lozano-Cuenca *et al.,* 2005). Together, these data suggest that peripheral or spinal activation of the NO-cGMP pathway may be involved in the antihyperalgesia induced at the spinal cord level by some COX inhibitors. However, a spinal synergistic depression of NMDA receptor-mediated transmission was observed with the COX inhibitor ketoprofen and the NOS inbiditor L-NAME (Lizarraga *et al.,* 2008).

On the other hand, stimulation of the NO pathway also reduced the spinal analgesic effects of COX inhibitors. The NO precursor L-arginine antagonised the analgesic responses of the COX inhibitors diclofenac and S(+)-ibuprofen, all given through the intraperitoneal route, in rats intrathecally injected NMDA (Björkman *et al.,* 1996). However, when combined in the same molecule, intravenous administration of NO donors linked to COX inhibitors (also known as nitro-NSAIDs) can produce synergistic analgesic effects mediated at the spinal level (Romero-Sandoval *et al.,* 2002).

Modification of the NO pathway also had no effect on the spinal analgesic effects of some COX inhibitors. Pre-treatment with the NOS inhibitor L-NAME or the NO precursor L-arginine, either by the intrathecal or the intracerebroventricular routes, did not modify the analgesic effects of S(+)-ketoprofen given orally to arthritic rats (Díaz-Reval *et al.,* 2004). Also, the spinal/supraspinal analgesic effect of the COX-2 preferential inhibitor nimesulide in human beings persisted after administration of a hyperalgesic dose of the NO donor nitroglycerine, suggesting that COX-2 is necessary for NO-mediated hyperalgesia (Sandrini *et al.,* 2002). Clearly, the spinal nociceptive interaction between the NO pathway and COX inhibitors is complex and further studies are required to determine how, and under which experimental conditions, they affect each other.

In summary, activation of spinal NMDA receptors permits Ca^{2+} influx, which in turns activates AA and NO pathways resulting in the production of messengers that could act on presynaptic and postsynaptic neurones, as well as non-neural structures. The effects of these molecules include the release of neurotransmitters and/or neuropeptides from the presynaptic site and inhibitory interneurones, as well as the formation and expression of new receptors. All these complex mechanisms are involved in the development and/or maintenance of spinal sensitisation.

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Chapter 9

Fragrant Compounds in Foods and Beverages Enhance the $GABA_A$ Receptor Responses

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Abstract

The γ-aminobutyric acid type A ($GABA_A$) receptors are ligand gated chloride ion channels in the central nervous system (CNS). These receptors are hetero-oligomeric proteins composed of the different subunit peptides, which are differentially expressed in the different regions of the brain and are responsible for the major fast inhibitory signal transmission in the neuronal circuits, which in turn prevent the brain from overwhelm in stressful situations. Enhancement of inhibitory signals through $GABA_A$ receptors is a potential therapeutic strategy for the treatment of anxiety disorders, sleep disturbances, seizure disorders and muscle spasms. Mood-defining drugs such as benzodiazepine derivatives, barbiturates, neurosteroids, and general anesthetics act on $GABA_A$ receptors. Various components of foods and beverages such as ethanol in liquors potentiate the responses of both synaptic and extra-synaptic $GABA_A$ receptors. Reportedly, behavioral consumptions of alcohol as low as 3 mM potentiate the response of extra-synaptic $GABA_A$ receptors. However, synaptic $GABA_A$ receptors, which consist of γ-subunit with combination of other subunits, are being potentiated only at high concentration of ethanol (>50 mM). Various fragrant compounds in foods and beverages, i.e., cineol, eugenol, geraniol, linalool, *cis*-jasmone, methyl jasmonate, 1-octen-3-ol, myrcenol, terpinen-4-ol, ethyl phenylpropanoate may have calming effect on mood or consciousness because they also strongly enhance the inhibitory signal transmission through $GABA_A$ receptors similar to ethanol, and mood-defining drugs. It has already been revealed that alcohols with long carbon chain, esters with short carbon chain, and phenol derivatives with low numbers of phenolic groups strongly enhance $GABA_A$ receptor responses. These

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fragrant compounds also increase the pentobarbital-induced sleeping time or delay pentetrazole-induced convulsions in mice when they are injected to mice prior to intraperitoneal administration of pentobarbital or pentetrazole. Moreover, fragrant compounds also decrease restriction-stress-induced increases in the plasma adrenocorticotropic hormone (ACTH) levels in rats, as does diazepam, a benzodiazepine derivative. Furthermore, behavioral studies, i.e., elevated plus maze, vogel's anti-conflict test, ambulatory activity in mammals proved that fragrant compounds have anxiolytic effects, which is mediated through $GABA_A$ receptors. Thus such fragrant compounds in foods and beverages may have calming effects on brain, affecting moods or consciousness.

Introduction

Fragrant compounds are inseparable part of modern life. Modern foods, drinks, beverages, commodities, and cultural life as well are consisting of various nice flavors. Without nice flavors, no foods and beverages are plausible. Plausible essence linked with plausible taste attracts consumers' demand of the food products. Possibly, essence is the ultimate result of interaction of fragrant compound(s) not only with the taste system but also with the hippocampus, olfactory and limbic systems in the brain. Food flavors activate the olfactory cortex, parahippocampal gyrus, anterior fusiform gyrus, insula, striatum and cingulate (Shepherd, 2006). The olfactory cortex and amygdale have associations with the desirability of foods, with pleasurable foods activate the medial olfactory and unpleasant foods activate the lateral olfactory (Rolls et al., 2003). Reportedly, along with opioid and dopamine D_1 receptors, $GABA_A$ receptors are involved in the consumption of hedonically positive taste stimuli (Shimura et al., 2006). Small et al. (2005) have shown in human that how smell and taste pathways function, separately and together. However, fragrant compounds not only associated with taste but also enhance texture, and nutritional quality of food products. Fragrant compounds also used in medication because they show various beneficial activities on human body and mind such as antibiotic, antineuralgic, relaxing, sedative etc. Various fragrant compounds, which are components of essential oils used in aromatherapy that has spread worldwide shows promise as an alternative medicine (Balchin, 1997; Buckle, 1999), despite the absence of a scientific basis for its effectiveness (Umezu & Morita, 2003). The medicinal use of essential oils (EOs) originated in ancient Egypt has continued until the present day world. People in Europe have been using EOs as therapeutics to achieve tranquility in their mind since about 70 years ago. On the other hand, the long history of EOs in aromatherapy suggests that they indeed have psychoactive effects. The effects of aromatherapy, inducing tranquility in the human mind, have been thought to come from the stimulation of the olfactory system by the fragrance, which will in turn stimulate the limbic system, the hippocampus or the hypothalamus, the pituitary gland and the autonomic nervous system, the endocrine system or the immune system (Hayashi, 1998). Though the targets of fragrances have not been identified yet but it has also been proposed that fragrances produce direct effects in the brain. Reportedly, the fragrant compounds that potentiate the responses of $GABA_A$ receptors also induce anxiolytic, anticonvulsant and sedative activity (tranquility) in the human mind.

Recently, receptors and channels have been studied extensively and not only the olfactory system (Shepherd, 2006) but also the taste system (Scott, 2005) have been clarified in molecular level. Moreover, receptors for capsaicin, menthol and allyl isothiocyanate have also

been studied in molecular level (Calixto et al., 2005), which also respond to temperature. It has been reported that components of foods or beverages act on receptors, channels or enzymes in the brain and modulate human consciousness (Kanarek & Marks-Kaufman, 1991). For examples, nicotine in tobacco binds to nicotinic acetylcholine receptors in the brain. Ethanol in liquors potentiates the response of $GABA_A$ receptors (Wafford et al., 1990), while it inhibits that of NMDA receptors (Lovinger et al., 1989). It also opens G-protein-coupled inwardly rectifying K^+ channels (Lewohl et al., 1999; Kobayashi et al., 1999). Capsaicin in hot chili peppers opens heat-activated ion channels (warm receptors) (Caterina et al., 1997), whereas menthol in spearmint opens cold receptors (McKemy et al., 2002). Caffeine, the major alkaloid in tea, and coffee acts as a central nervous system stimulant (Kanarek & Marks-Kaufman, 1991), significantly affects cognitive performance, mood, and thirst (Smit & Roges, 2000). The psychostimulant action of caffeine comes from the blocked of adenosine A_{2A} receptors (Fredholm et al., 1999), due to the involvement of DARPP-32 and its phosphorylation (Lindskog et al., 2002). Various reports have shown that many components of foods, beverages, EOs, and phytoncids are well absorbed into the human body, enter into blood circulation, and may reach the central nervous system, can modulate neural signal transmissions via acting on neurotransmitter-receptors, especially the $GABA_A$ receptors because aromatherapy, phytoncids or drinking of beverages modulate the moods of humans. It is well established that many mood-defining drugs target $GABA_A$ receptors in the brain (Chebib & Johnston, 2000). Therefore, it is obvious that after taking foods or beverages their mood-defining components, especially many fragrant compounds modulate $GABA_A$ receptors-responses, which in turn determine the mental state. Generally, fragrant compounds are lipophilic in nature that could easily pass the blood-brain barrier and thereby modulate the responses of neuroreceptors, especially $GABA_A$ receptors. Here, we will describe the fragrant compounds in foods and beverages that enhance the responses of $GABA_A$ receptors in the brain.

The $GABA_A$ Neuroreceptors

The γ–aminobutyric acid type A ($GABA_A$) receptors are ligand-gated chloride ion channels, which are expressed in the central nervous system (CNS). The cDNAs of $GABA_A$ receptor subunits of bovine and human brain were cloned by Schofield et al. (1987) and Schofield et al. (1989) respectively. Sequence homology analyses of both human and bovine $GABA_A$ receptors revealed that they were more than 90% similar. The $GABA_A$ receptors have been evolved from the common ancestral gene (Nicholls, 1994) of the various ionotropic neurotransmitter receptors, which include nicotinic acetylcholine (nACh), glycine, and serotonin-3 (5HT3) receptors. The $GABA_A$ receptors are hetero-oligomeric proteins composed of different combinations of subunit peptides, which are differentially expressed in the different regions of the brain. To date, at least 15 subunits of human $GABA_A$ receptors have been described, and these have been classified into five distinct subfamilies of the subunits termed α, β, γ, δ and ε (Nicholls, 1994). The pentameric $GABA_A$ receptors containing the γ-subunit are expressed synaptically, are the site of action of classical benzodiazepines derivatives. The δ-subunit containing $GABA_A$ receptors have a modified benzodiazepine-binding site that is insensitive to classical benzodiazepines derivatives, are expressed in cortex, hippocampus, thalamus, striatum and cerebellar granule cells. A part of

δ-subunit containing $GABA_A$ receptors are expressed in extrasynaptically, producing tonic inhibitory currents in the brain. However, each subunit has a large N-terminal extracellular domain of approximately 200 amino acids in length, four transmembrane-spanning domains (M1 through M4), a large intracellular loop between M3 and M4, and a small extracellular C-terminal tail. Generally five subunits proteins that make up $GABA_A$ receptors, are symmetrically transverse the post-synaptic membrane to form a central pore. Binding of the γ–aminobutyric acid (GABA) to the GABA binding sites of the receptors, the configuration of the receptors is changed, which leads to the central pore to be opened. Then the chloride ions rush into the post-synaptic neurons, which initiate an inhibitory post-synaptic potential. These inhibitory post-synaptic potentials depress excitatory depolarization, which ultimately decrease the ability of the neurons to fire action potential. Therefore, these receptors are involved in major fast inhibitory neuronal signal transmission in the CNS. The enhancement of neuronal-firing-inhibition-signals through $GABA_A$ receptors is a common therapeutic strategy for the treatment of CNS diseases such as anxiety disorders, sleep disturbances, muscle spasms and seizure disorders.

The $GABA_A$ receptors have a complex pharmacology (Chebib & Johnston, 2000) with binding sites for direct GABA agonists and antagonists together with multiple allosteric sites. Muscimol, a psychoactive compound from *Amanita muscaria* mushrooms, is a potent agonist at $GABA_A$ receptors. Bicucculine inhibits the $GABA_A$ receptor-mediated response competitively, possibly through its binding to GABA binding sites. Picrotoxin inhibits the response noncompetitively through its binding to the ion channel domain of the receptors. A number of structurally diverse compounds enhance the action of GABA; these include benzodiazepines, barbiturates, neuro-steroids (Rupprecht & Holsboer, 1999), general anesthetics (Olsen, 1998) and ethanol (Wafford et al., 1990). In animal behavioral experiments, these compounds exhibit anxiolytic, anticonvulsive and sedative activity (Lister, 1987; Toubas et al., 1990; Reddy & Kulkarni, 1997).

Preparation of the *Xenopus* Oocyte

Foreign genes of receptors, channels or transporters bound in the cell membrane are generally expressed in the membrane of *Xenopus* (*Xenopus laevis*) oocytes. *Xenopus* oocytes are round and have a diameter of > 1.0 mm, and are larger, more stable and simpler in shape than neurons, which make their handling and manipulation easy, and have become popular model in electrophysiological measurements repetitively for a long period of the responses of the receptors expressed. Oocytes produced in the ovarian lobes of adult female frog are classified into stages I to VI depending on their developmental state. Oocytes at the stages of V and VI are harvested usually by survival surgery, and the layer of follicle cells over the oocytes can be left intact or removed either manually or by treatment with collagenase. In our experiments, oocytes were dissected from the ovaries of adult female frogs that had been kept in ice for 1 h. They were manually detached from the inner ovarian epithelium and follicular envelope after incubation in a collagenase (type I, 1 mg/ml; Sigma) solution for 1 h (Kusano et al., 1982). Then these oocytes were kept in a modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$ and 0.82 mM $MgSO_4$ in 5 mM Tris at p^H 7.6] containing 25 mg/L of penicillin and 50 mg/L streptomycin at 17.5 ^{0}C, and were used for the expression of neuroreceptors.

Expression of $GABA_A$ Receptors in the *Xenopus* Oocytes

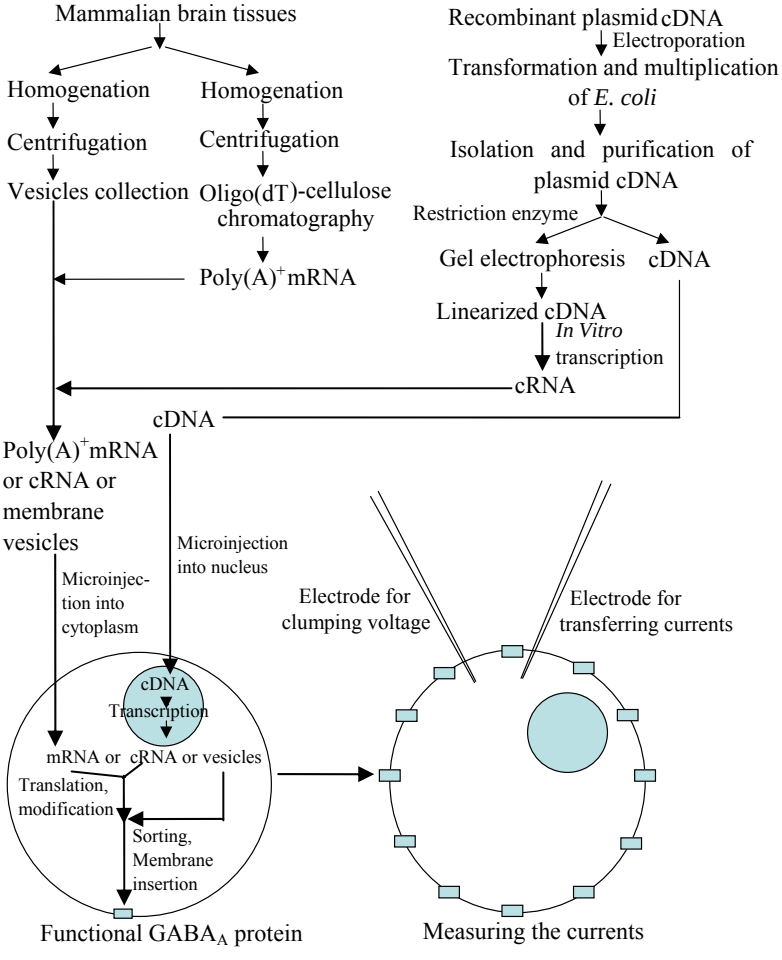

Figure 1. Ways of expression of $GABA_A$ receptors in *Xenopus* oocyte and measurement of currents (modified from Hossain et al., 2007).

The $GABA_A$ receptors can be expressed in *Xenopus* oocyte cells by the microinjection of cDNA prepared from the cloned $GABA_A$ receptors (Figure 1). Double-stranded cDNA is prepared, and is directly microinjected into the nucleus of the oocyte. Detection of nucleus in the oocyte cells is difficult and microinjection into the nucleus can damage them as well as the cells. However, injected cDNAs of $GABA_A$ receptors in the nucleus of oocyte, immediately control over the biosynthetic machinery of the cells, export to cytoplasm a well prepared processed mRNA by transcription, capping and polyadenylation. To generate cDNA, subcloning of cDNA is essential with correct orientation into a suitable expression vector having a eukaryotic promoter (Swick et al., 1992.). There is also a shortcut PCR-based method to generate cDNAs, which starts with RNA that is reverse-transcribed (Cestari et al., 1993). Moreover, the receptors can also be expressed by injecting cRNA prepared from the

cloned cDNA of $GABA_A$ receptors or poly(A)$^+$mRNA or membrane vesicles prepared from the brain's tissue. cRNA, poly(A)$^+$mRNA or membrane vesicles is microinjected into the cytoplasm of the oocyte cells, which is the common route of expression of $GABA_A$ receptors. Since RNA is easily degraded, it is essential to have a good quality RNA, which can be easily checked on a gel (Theodoulou & Miller, 1995). Addition of a poly(A)$^+$ tail to the mRNA transcripts improved their stability in the cytoplasm of the oocyte after the injection (Miller & Zhou, 2000). The capping structure, 5′ 7-methyl guanosine residue protects the mRNA from degradation, serves in protein synthesis initiation process, essential for expression in oocyte (Krieg & Melton, 1984). Microinjection of oocyte cells with mRNA extracted from the brain cells or tissue leads to the synthesis of various functional receptors and ion channels that incorporate into the membrane of oocytes. Membrane vesicles prepared from human brain tissue or transfected cultured cells expressing neurotransmitter receptors, have potentially been used to microinjection into oocyte cells to micro-transplantation of membranes of the oocytes with functional neurotransmitter receptors embedded in native lipids (Miledi et al., 2002, Palma et al., 2003). This approach will help to elucidate the biophysical and pharmacological properties of many neurotransmitter receptors as they occur in the human brain, because they are incorporated into host oocyte membrane while still in their native cell membrane, and these receptors presumably retain their original subunit stoichiometry, structural features, and complement of associated proteins and lipids (Miledi et al., 2002).

Electrophysiological Measurements

Two to six days after the injection of oocyte cells with cDNAs, cRNAs or mRNAs, and only a few hours after membrane vesicles injection, membrane currents are recorded from voltage-clamped oocytes by using two microelectrodes filled with 3 M KCl, one for monitoring the membrane potential and other for passing the current for clamping the membrane potential (Miledi et al., 2002, Palma et al., 2003). An oocyte is placed on the net of a small chamber (0.3 mL) and impaled with two microelectrodes (Fig. 1). The oocyte placed on the net is continuously perfused from the bottom with frog normal Ringer's solution (with components) by the gravity feed system, at a flow rate of 2 mL/min (Kobayashi & Aoshima, 1986).

Measurements of the $GABA_A$ Receptor Response

GABA is dissolved in frog normal Ringer's solution. To examine the action of fragrant compounds on the GABA-elicited response, each test compound is added to the solutions. The solutions are changed by switching a cock of the flow system. The control response is obtained by perfusing the GABA solution without any compound and is taken as 100%. The effect of a given fragrant compound on the response of the receptors is measured by using a mixture of GABA and the compound. Sometimes the compound is added 1-1.5 min before coapplication with GABA when desensitization of the receptors is significantly induced before equilibrium of the compound binding is attained (Aoshima et al., 1992). To eliminate the desensitization of the receptors, the oocyte is washed for > 10 min in frog normal Ringer's solution before the next measurement, because desensitization of the $GABA_A$ receptors is a

reversible process and the receptors usually recover after 10 min of washing (Aoshima et al., 1987).

Effects of Fragrances in Foods & Beverages on $GABA_A$ Receptor Response

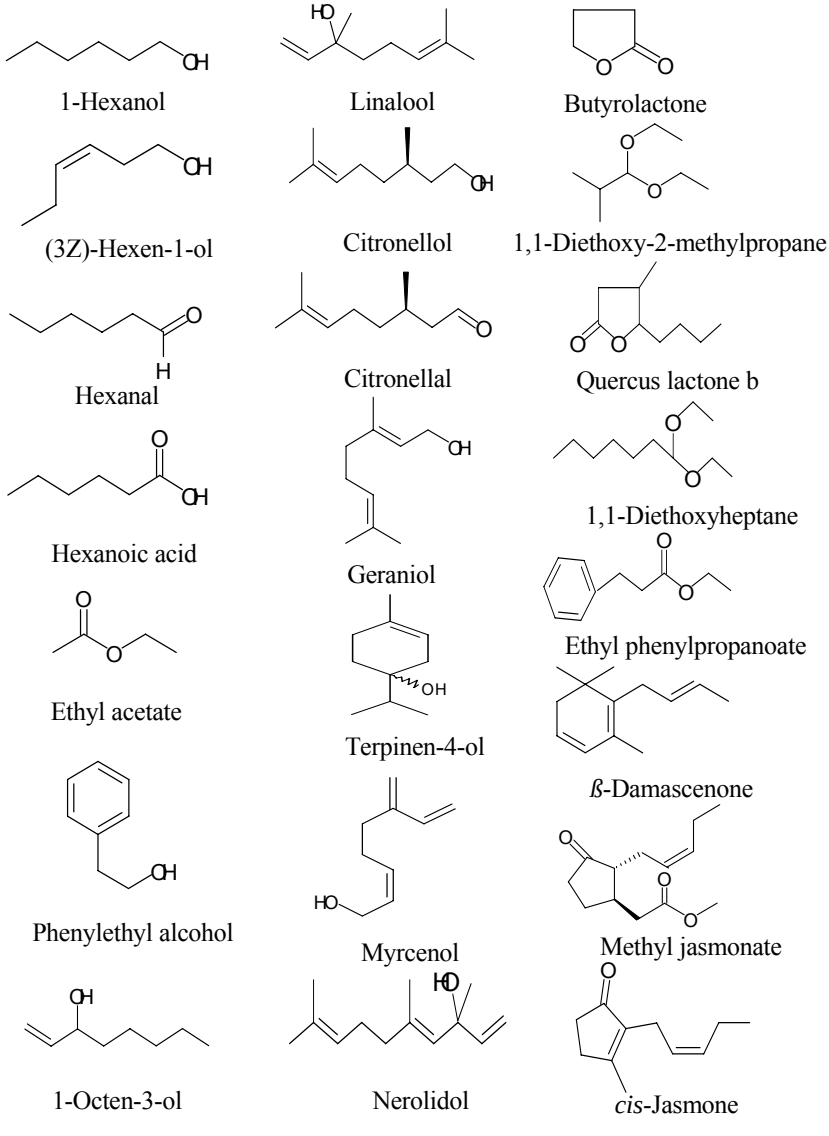

Figure 2. Molecular structures of some fragrant compounds that enhance the responses of $GABA_A$ receptors present in foods and beverages (hexanoic acid inhibits the responses).

Both fermented and non-fermented rice, bread, tea, coffee, and cacao are the major foods and beverages. Alcoholic beverages such as beer, wine, whisky, sake etc. are also integral part of western societies. Reportedly, thousands of fragrant compounds are present in foods and beverages but one or a few play a critical role for the characteristics flavor of a specific

food or beverage. For example, on the basis of the high aroma values ethanol, 2-methylpropanal, 3-methylbutanal, 2,3-butanedione, and 3-methylbutanol contribute significantly to the aroma of wheat bread (Grosch & Schieberle, 1991). Of the fragrant compounds in foods and beverages, both volatile and non-volatile compounds exist, which exert characteristics flavor and aroma. Most fragrant compounds are hydrocarbons, alcohols, aldehydes, ketones, acids, phenols or esters, which potentiate the response of the $GABA_A$ receptors in the presence of low concentrations of GABA (Aoshima & Tenpaku, 1997) (Fig. 2). Fragrant compounds themselves do not induce any response of $GABA_A$ receptors expressed in *Xenopus* oocyte. However, fragrant compounds such as 1-hexanol, hexanal, and normal alcohols potentiate the $GABA_A$ receptor-responses, which depend strongly on the concentrations of both the compound and GABA (Aoshima & Tenpaku, 1997; Aoshima et al., 2001b). Hexanol potentiates the responses of $GABA_A$ receptor more strongly than hexanal. Moreover, (3Z)-hexen-1-ol, hexanal, butyl acetate, and hexylamine potentiate the responses significantly whereas hexanoic acid inhibits it weakly and competitively (Aoshima et al., 2001b). Therefore, it is concluded that $GABA_A$ receptor-responses vary with functional groups of six-carbon hydrocarbons. Normal alcohols enhance the potentiation of the responses of $GABA_A$ receptors with the increase of their carbon chain length (Aoshima et al., 2001b). In the rat dorsal root ganglion neurons, the similar results are also found, in case of alcohols (Nakahiro et al., 1991). Phenol derivatives also potentiate the $GABA_A$ receptor mediated response strongly (Aoshima et al. 2001a; Krasowski et al., 2001). However, an increase of the hydroxyl group in the phenol compounds decreases the potentiation of the $GABA_A$ receptor's response (Aoshima et al., 2001b). Aliphatic esters play a vital role in the taste and flavor of foods and beverages. They decrease the potentiation of the $GABA_A$ receptor-response with an increase in their carbon chain length (Aoshima et al., 2006), indicating lower esters such as ethyl acetate are important in the potentiation. However, the potentiation of the $GABA_A$ receptor-responses depends on the concentration of both the esters and the GABA, similar to other fragrant compounds (Aoshima et al., 2001b).

Fragrant Compounds in Tea, Coffee and Cacao Enhance the Responses of $GABA_A$ Receptors

Tea, coffee and cacao are the major non-alcoholic beverages of the present day world. They consist of thousands of fragrant compounds, and many of them are common in these beverages. Among them, the compounds that are responsible for the characteristics flavor and aroma are probably important in the modulation of the responses of $GABA_A$ receptors. Depending on the season of harvesting and manufacturing processes, fragrant contents, taste and quality of these beverages are varied. Allantoin, benzothiazole, benzyl alcohol, catechol, citronellol, dimethylstyrene, dimethyl sulfide, geraniol, guaiacol, hexanal, 1-hexanol, hexanoic acid, (3Z)-hexen-1-ol, (E)-2-hexen-1-ol, hexylamine, *cis*-jasmone, jasmine lactone, linalool, linalool oxide, limonene, methyl jasmonate, myrcene, nerolidol, 1-octen-3-ol, penten-1-ol, 1-penten-3-ol, *cis*-2-penten-1-ol, phenylethyl alcohol, terpineol etc. play major role in flavor production in these beverages. For examples, myrcene and limonene are lemon-like; dimethylstyrine and (Z)-2-penten-1-ol are citrus-like; (E)-2-hexen-1-ol and (3Z)-hexen-1-ol (leaf alcohol) are characteristics compounds of tea, which have intense green-leafy flavor

since (E)-2-hexen-1-ol is not affected by the withering or by fermentation (Flament, 1991). All of these fragrant compounds potentiate the $GABA_A$ receptor-responses (Aoshima & Tenpaku, 1997; Aoshima et al., 2001b; Hossain et al., 2002a; Hossain et al., 2003; Hossain et al., 2004; Aoshima et al., 2006). Among these fragrants, benzothiazole, geraniol, (3Z)-hexen-1-ol (leaf alcohol), *cis*-jasmone, linalool, methyl jasmonate, 1-octen-3-ol and phenylethyl alcohol potently potentiate (more than two times of control) the $GABA_A$ receptor-responses (Table 1). The lipophilic extract of tea, dose-dependently inhibit the $GABA_A$ receptor-responses, having a non-competitive inhibition mechanism (Hossain et al., 2002a; Hossain et al., 2004). On the other hand, lipophilic extract of coffee, at very low concentrations, slightly potentiate the $GABA_A$ receptor-responses, whereas the extracts at high concentrations inhibit them (Hossain et al., 2003), possibly they contain large amount of inhibitory components than potentiators, flavor components. The extract of coffee possibly contains two types of components; components that potentiate the response with high affinity to the receptors and those that inhibit the response with low affinity to the receptor.

Table 1. Some of the fragrant compounds in teas, coffee, and/or cacao that potently enhance the response of $GABA_A$ receptors (Hossain et al., 2002a; Hossain et al., 2003; Hossain et al., 2004). The control response was obtained by perfusing the GABA solution without any compound.

Compounds	Current (%)
1 μM GABA (Control)	100
+ 1 mM Benzylalcohol	134.0 ± 8.9
+ 1 mM Dimethyl sulfide	118.1 ± 15.0
+ 1 mM Geraniol	502.0 ± 31.7
+ 1 mM Leaf alcohol	223.7 ± 12.0
+ 1 mM Linalool	348.5 ± 14.9
+ 1 mM Nerolidol	166.6 ± 19.4
+ 1 mM Phenylethyl alcohol	245.4 ± 14.1
0.25 μM GABA (Control)	100
+ 0.5 mM Benzothiazole	214.6 ± 28.5
+ 0.5 mM Catechol	122.8 ± 3.3
+ 0.5 mM 2,4-Dimethylstyrene	164.2 ± 29.3
+ 0.5 mM Guaiacol	180.0 ± 23.9
+ 0.5 mM (E)-2-Hexen-1-ol	147.7 ± 4.2
+ 0.5 mM Jasmine lactone	138.9 ± 9.1
+ 0.5 mM *cis*-Jasmone	220.3 ± 24.5
+ 0.5 mM Linalool oxide	133.6 ± 4.6
+ 0.5 mM 3-Methyl-2-buten-1-ol	121.9 ± 7.9
+ 0.5 mM 2-Methyl-3-buten-2-ol	119.0 ± 6.7
+ 0.5 mM Methyl jasmonate	211.5 ± 20.0
+ 0.5 mM 1-Octen-3-ol	404.3 ± 25.7
+ 0.5 mM 1-Penten-3-ol	135.4 ± 10.8
+ 0.5 mM *cis*-2-Penten-1-ol	124.8 ± 6.7
+ 0.5 mM Sotolone	139.4 ± 4.6

In addition, essential oils (EOs), which are prepared from various plants usually by distillation and extractions consisting of hundreds of compounds, are used in aromatherapy and for the treatment of diseases. These include alcohols, aldehydes, esters, ketones, phenols, terpenes etc. The principle compounds in EOs are fragrant compound(s), which include borneol, camphor, cineol, citral, citronellol, citronellal, eugenol, geraniol, geranyl acetate, linalool, linalyl acetate, limonene, thymol (thyme), terpene, terpinen-4-ol etc. Many of them also potently enhance the responses of $GABA_A$ receptors (Aoshima & Hamamoto, 1999; Aoshima et al., 2001b). Very small amounts (several ppm) of fragrant compounds in EOs such as cineol, citral, eugenol, citronellol, citronellal, terpinen-4-ol potently enhance the responses. Certainly, the fragrant components of EOs play a vital role in the aromatherapeutical use that induces mental tranquility and aid sleep (Hayashi, 1998). Moreover, the volatile fragrant compounds that are produced and emanated by trees and plants in the forest are called phytoncids. These generally include (3Z)-hexen-1-ol (leaf alcohol), (-)-α-pinene and hinokitiol (Kamiyama, 1983; Hatakana, 1988). These compounds also enhance the $GABA_A$ receptor-responses at the low concentration of GABA (Aoshima & Hamamoto, 1999).

Fragrant Compounds in Alcoholic Beverages Enhance the Responses of $GABA_A$ Receptors

Lipophilic components are easily incorporated into the brain. Extracts of liquors with pentane that consist of lipophilic compounds potentiate the response of $GABA_A$ receptors since they contain modulator(s) of the receptor (Hossain et al., 2002b; Aoshima et al., 2004; Aoshima et al., 2006). Lipophilic extracts of different types of liquors i. e. beer, brandy, Chinese medicinal liquor He Jiu, sake, shochyu, red wine, whisky, and white wine deferentially potentiate the $GABA_A$ receptor-responses. Among the liquors, wine and whiskey potentiate the receptor-response strongly. Liquors have many aromatic compounds (Lee et al., 2000), which determine the sensory characteristics of the drink. The major component of liquors is ethanol which affects mood and consciousness by acting on $GABA_A$ (Wafford et al., 1990), NMDA (Lovinger et al., 1989), glycine (Mihic et al., 1997; Lei et al., 2000), serotonin (Lovinger & white, 1991) receptors and K^+ channels (Lewohl et al., 1999; Kobayashi et al., 1999). Ethanol affects these targets generally at high concentrations (≥60 mM). Ethanol only at fairly high concentrations (≥50 mM) enhances GABA-currents through synaptic $GABA_A$ pentameric (2α-2β-1γ) receptors, whereas, δ-subunit containing extrasynaptic $GABA_A$ receptors such as $\alpha_4\beta_3\delta$ or $\alpha_6\beta_3\delta$ are highly sensitive to ethanol (≤3 mM) (Wallner et al., 2003). However, during the fermentation, fusel alcohols such as various butanol and pentanol derivatives are formed along with ethanol. In the later stage of fermentation, a part of these alcohols are converted into aldehydes, carboxylic acids or esters by oxidation or condensation. Various fragrant compounds in liquors such as damascenone, ethyl phenylpropanoate (EPP), hop oil, myrcenol, and the derivatives of acetate, ethoxy, lactone, and phenol potentiate the responses of $GABA_A$ receptors (Hossain et al., 2002b; Aoshima et al., 2006) (Table 2). In addition to ethanol, whiskey fragrances such as ethoxy, lactone, and phenol derivatives, and also EPP in part modulate mood and consciousness, because these compounds are several thousand times more potent than ethanol in potentiating

the $GABA_A$ receptor-elicited responses and in addition, because of their hydrophobicity, they are easily absorbed into the brain (Hossain et al., 2002b).

Table 2. Some of the major fragrant components of whiskey and/or beer that potentiate the response of $GABA_A$ receptors (Hossain et al., 2002b; Aoshima et al., 2006). The control response was obtained by perfusing the GABA solution without any compound.

Compound	Current (%)
On rat's whole brain $GABA_A$ receptors:	
10 μM GABA (Control)	100
+ 0.2 μl/ml Damascenone	128.5± 8.2
+ 0.2 μl/ml 1, 1-Diethoxyheptane	311.2±23.1
+ 0.2 μl/ml 1, 1-Diethoxy-3-methylbutane	239.3±20.9
+ 0.2 μl/ml 1, 1-Diethoxy-2-methylpropane	239.6±8.2
+ 0.2 μl/ml 1, 1-Diethoxypropane	227.6±6.4
+ 0.2 μl/ml Ethyl phenylpropanoate	330.0±29.2
+ 0.2 μl/ml Quercuslactoneb	215.7±23.9
+ 0.2 μl/ml Whiskey extract	166.3±14.7
On recombinant $_1$ and $_1$ subunits of $GABA_A$ receptors	
0.25 μM GABA (Control)	100
+ 0.02μl/ml Butyrolactone	224.6±15.0
+ 0.02μl/ml Decanolactone	168.6±18.2
+ 0.02μl/ml Dodecalactone	168.6±16.6
+ 0.02μl/ml Nonalactone	143.9±6.4
+ 1.1mM Myrcenol 29	0.9±35.5
+ 0.05 % (v/v) Hop oil Perle	119.2±12.7
+ 0.05 % (v/v) Hop oil Hallertauer	131.8±8.2
+ 0.25 % (v/v) Beer extract	143.6±6.4
1 μM GABA (Control)	100
+ 0.5mM 2-Acetylphenol	301.1±48.4
+ 0.5mM 2,6-Dimethylphenol	515.9±27.9
+ 0.5mM 4-Ethylguaiacol	421.6±9.3
+ 0.5mM 4-Ethylphenol	386.5±22.8
+ 0.5mM 2-Methoxy-4-methylphenol	248.4±18.9
+ 0.5mM 5-Methyl-2-acetylphenol	311.5±36.3
+ 1 mM Butyl acetate	153.6±8.9
+ 1 mM Ethyl acetate	260.7±19.0
+ 1 mM Ethylbutanoate	167.5±6.0
+ 1 mM Ethylheptanoate	150.0±20.0
+ 1 mM Ethylpentanoate	156.5±24.0
+ 1 mM Ethylpropanoate	249.0±6.0
+ 1 mM Pentylacetate	120.2±1.8

Whiskey itself potentiates the electrical responses of $GABA_A$ receptors generally more than ethanol at the same concentration as that in whiskey, and the potentiation also increases with the aging period of the whiskey because of the increase of fragrant compounds (Koda et

al., 2003). These mean that not only ethanol but also fragrant compounds in whiskies play an important role in the potentiation of $GABA_A$ receptor-responses, and that the concentrations and combinations of various fragrant compounds depend on the aging of whiskies. Therefore, the sedative effect of whiskey may be due to not only presence of ethanol but also presence of fragrant compounds. The addition of herbs, such as *Humulus lupulus* in beer, and *Lycium barbarum* in He Jiu, increases the quality as well as fragrant content, which in turn, have a tranquilizing effect on the brain. However, hop supercritical CO_2 extract has a pentobarbital sleeping-enhancing property and an antidepressant activity (Zanoli et al., 2005). Moreover, a fixed combination of valerian and hops acts via a central adenosine mechanism (Schellenberg et al., 2004).

Kinetic Model of Interaction and Binding Site of the Fragrant Compounds with the $GABA_A$ Receptors

Generally, fragrant compounds in foods and beverages potentiate the responses of $GABA_A$ receptors (Hossain et al. 2002 a, b; Hossain et al., 2003; Hossain et al., 2004; Aoshima et al., 2006). The potentiation of the $GABA_A$ receptor-responses increases with the concentration of each fragrant compound at the low concentrations of GABA. The addition of each of these fragrant compounds to GABA-solution shifts the GABA-dose-response curve to a lower concentration indicating binding of fragrant compound(s) to the potentiation site(s) of the $GABA_A$ receptors, enhances the GABA-binding affinity for GABA-binding sites (Aoshima & Hamamoto, 1999; Aoshima et al., 2001b; Hossain et al., 2002b). General anesthetics also act on the $GABA_A$ receptor in a similar way (Franks & Lieb, 1994). On the basis of the minimal model for ionotropic neurotransmitter receptors (Hess et al., 1983; Aoshima et al., 1987), a simple kinetic model has been proposed (Aoshima et al., 2001b) by assuming that binding of these fragrant compound(s) to the potentiation site increases the affinity of GABA for the receptor (Figure 3). This kinetic model satisfactorily explains the potentiation and the increase of desensitization-rate. Kurata et al. (1999) proposed both partial agonistic and allosteric models for the action of 1-octanol on $GABA_A$ receptors. To explain the dual modulation of $GABA_A$ receptors by alkyl-substituted γ-butyrolactones, Holland et al. (1995) proposed a simplified kinetic model of GABA-receptor-ionophore interaction, in which they assumed that one or two lactone molecules can bind to the GABA-receptor complex, either stabilizing an open state or producing a block. Table 3 shows the dissociation constant (K_p) between the major fragrant compound and the $GABA_A$ receptor, the maximum potentiation (V_m) of the receptors, and the dissociation constant of GABA (K_{1p}) when the potentiation site of the receptor is fully occupied with the compound. These values were estimated by assuming a simple equilibrium condition between the fragrant compound(s) and the receptor (Aoshima et al., 2001b). The dissociation constant (K_{1p}) of the complex between GABA and the receptor decreases from the control value in the presence of a saturating amount of each fragrant compound.

Benzodiazepines act on γ-subunit containing $GABA_A$ receptors (Gunther et al., 1995; Barnard et al., 1998) whereas alcohol or phenol derivatives enhance the responses of the

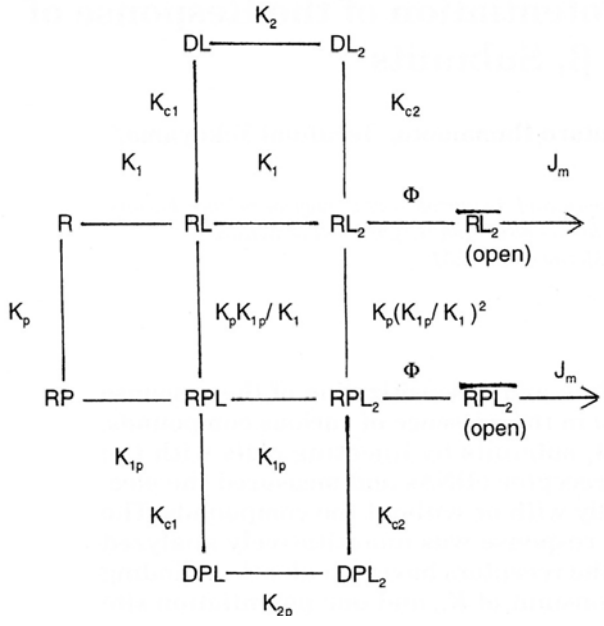

Figure 3. Minimum schematic model accounting for the potentiation of the $GABA_A$ receptor-responses (Aoshima et al., 2001b; reprinted with permission from The Japanese Biochemical Society). Active receptor (R), desensitized receptor (D), ligand (L; GABA), compound (P; potentiator), open channel ($\overline{RL}_2$, $\overline{RPL}_2$), dissociation constant (K), equilibrium constant (Φ) and maximum current (J_m) are shown in the figure.

receptors composed of only α and β-subunits. Therefore, the binding site of alcohols or phenols is different from that of benzodiazepine (Mihic et al., 1997). It has been reported that mutation of two specific amino acid residues in the α and β-subunits of the receptors abolished the potentiation by alcohols, but not the response elicited by GABA. Mascia et al. (2000) identified the specific binding site for alcohols on $GABA_A$ receptors by using an alkanethiol anesthetic to covalently label its binding site, and by mutating selected amino acids to cysteine. Thus the potentiation site for fragrant compounds is possibly located in $GABA_A$ receptors composed of only α and β subunits (Jones et al., 1995; Hossain et al., 2002b). Among the fragrant compounds in whiskey, ethyl phenylpropanoate (EPP) likely binds to the ethanol-binding site of the $GABA_A$ receptors, which is a region of 45 amino acid residues within the TM2 and TM3 domains of the subunit (Mihic et al., 1997; Mascia et al., 2000; Hossain et al., 2002b), and damascenone binds to both an inhibition and a potentiation site (Hossain et al., 2002b) similar to lipid hydroperoxide (Aoshima, 1996) or bisphenol A (Aoshima et al., 2001a). This inhibition site is probably at the interface of the receptor with membrane lipid and prevents the channel from opening (Changeux et al., 1984). Moreover, δ-subunit containing $GABA_A$ receptors are also important mediators of ethanol because δ-subunit knockout mice show multiple defects in behavioral responses to ethanol (Mihalek et al., 2001). Wallner et al. (2003) demonstrated that $GABA_A$ receptors composed by the β_3– and δ-subunit are uniquely sensitive to low ethanol concentrations (Figure 4). These receptors have a low-dose ethanol modulation site and a site activated at high alcohol doses (Wallner et al., 2006). A point mutation in β_3-subunit (β_3N265M) of the receptors abolishes the actions of high-dose alcohol (Wallner et al., 2006) as well as etomidate and propofol *in vivo* (Jurd et al.,

2003). Since δ-subunit containing $GABA_A$ receptors are producing tonic inhibitory currents in the brain and have very slow desensitization rate, and are highly sensitive to ethanol as well as mood-defining drugs, probably fragrant compounds also enhance these receptor-responses. It is also revealed that thymol, a constituent of thyme EO potently potentiates the responses of $GABA_A$ receptors through a previously unidentified binding site (Priestley et al., 2003).

Table 3. Estimated dissociation constant, K_p, maximum potentiation, V_m, and dissociation constant of GABA, K_{1p}, some of the fragrant compounds in foods and beverages (Aoshima et al., 2001b; Hossain et al., 2002a,b; Hossain et al., 2003; Hossain et al., 2004; Aoshima et al., 2006).

Compound	K_p (mM)	V_m (%)	K_{1p} (μM)
Control			59
Cineol	0.11	255	34
Citral	0.21	181	41
1,1-Diethoxyheptane	0.20	605	24
Ethanol	248	259	37
4-Ethylguaiacol	0.076	524	26
Ethyl phenylpropanoate	0.24	662	23
Eugenol	0.18	284	32
Geraniol	0.34	332	29
Hexanal	3.6	228	31
1-Hexanol	0.32	313	23
cis-Jasmone	0.45	322	33
Jsamine lactone	0.94	239	38
Leaf alcohol	0.78	258	34
Linalool	0.32	296	31
Linalool oxide	0.93	193	43
Methyl jasmonate	0.84	450	28
Myrcenol	0.35	353	31
1-Octanol	0.85	322	22
1-Octen-3-ol	0.76	688	21
1-Pentanol	2.2	279	26
Quercus lactone b	0.56	500	27
Terpinen-4-ol	0.15	635	16

The constants, K_p, V_m, and K_{1p} for geraniol, leaf alcohol, linalool and 1-octen-3-ol were estimated from the results in the presence of 5 μM GABA. The constants, K_p, V_m, and K_{1p} for others were estimated from the results in the presence of 0.25 μM GABA.

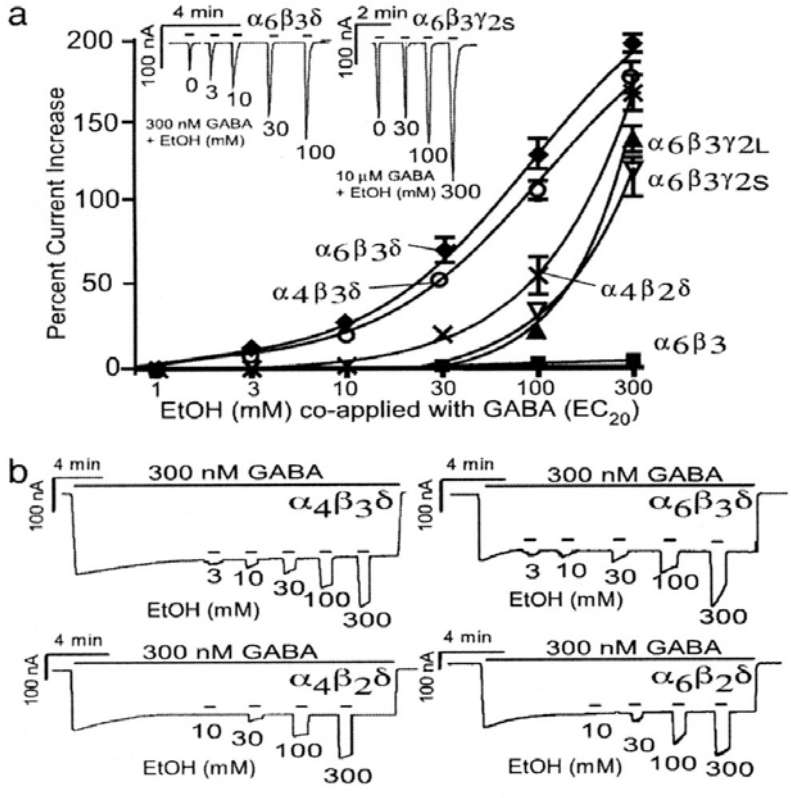

Figure 4. Both the β_3– and δ–subunits are required for high ethanol sensitivity (Wallner et al., 2003; reprinted with permission from The National Academy of Sciences of the USA). (a) EtOH current enhancement, (b) EtOH effects on tonically activated receptors.

Pharmacological and Animal Behavior Studies

Pentobarbital, which induces sleeping, has tranquilizing effects on brain through the potentiation of $GABA_A$ receptor (Matsumoto et al., 1991). From the potent activators of the $GABA_A$ receptor-elicited responses, 1,1-diethoxyheptane, ethyl phenylpropanoate, *cis*-jasmone, methyl jasmonate, myrcenol, and 1-octen-3-ol were used *in vivo* pharmacological experiments in mice. Fragrant compounds such as *cis*-jasmone, methyl jasmonate, 1-octen-3-ol, and myrcenol significantly increase the sleeping time induced by pentobarbital, suggesting that these fragrant compounds are easily absorbed into the brain, and thereby potentiate the $GABA_A$ receptor-responses, inducing anxiolytic effects on the brain (Hossain et al., 2003; Hossain et al., 2004; Aoshima et al., 2006) (Figure 5). Pentetrazole induces convulsions through the inhibition of the $GABA_A$ receptor-elicited responses in the brain (Coelho et al., 1997). Whisky fragrants-ethyl phenylpropanoate delays the convulsions induced by pentetrazole (Hossain et al., 2002b). Moreover, inhalation of Japanese blended whiskey aged for 30 years significantly prolongs the sleeping time of mice caused by pentobarbital compared to the control or ethanol whose concentration is the same as that in whisky (Koda et

al., 2003). Thus the fragrant compounds that highly potentiate the responses of $GABA_A$ receptors have tranquillizing effects on the brain *in vivo*.

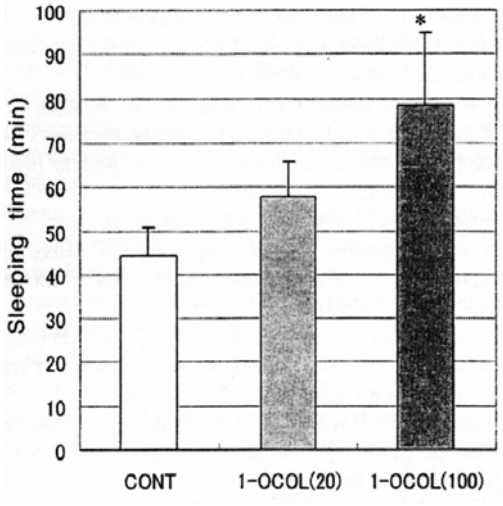

Figure 5. Effect of 1-octen-3-ol on pentobarbital-induced sleeping time in mice (Hossain et al., 2003; reprinted with permission from The American Chemical Society). CONT means control (sleeping time by the pentobarbital only); 1-OCOL (20) and 1-OCOL (100) indicate sleeping time by the pentobarbital with 20 and 100 mg/kg 1-octen-3-ol, respectively. *, $p < 0.05$ by Student's *t* test.

The biphenolic fragrant compounds honokiol and magnolol are involved in the potentiation of GABAergic neurotransmission in the rat brain (Squires et al., 1999). Administration of heliotropin in human significantly reduces anxiety (Redd et al., 1994). The inhibitory effects of the EO of SuHeXiang Wan on the CNS appear via the GABAergic system (Koo et al., 2004). Components of essential oil also accumulate in the mouse brain when they are given by means of percutaneous or vapor exposure absorption (Inoue et al., 2000; Inoue & Yamaguchi, 2000). The inhalation of chamoline, and lemon oil vapor decreases restriction-stress-induced increases in the plasma adrenocorticotropic hormone (ACTH) levels in ovariectomized rats (Figure. 6), as does diazepam, a benzodiazepine derivative (Yamada et al., 1996; Yamada et al., 2000), which acts on $GABA_A$ receptors. Essential oils and its components have anticonflict effects on mouse behavior in the Geller and Vogel conflict tests (Umezu, 1999; Umezu, 2000; Umezu et al., 2006) (Figure 7). Thus, some fragrant compounds in foods and beverages have anxiolytic effects on the brain similar to anxiolytic drugs used for psychiatric therapy.

Chlordiazepoxide, sodium pentobarbital, and ethanol increase the proportion of time spent on the open arms of a plus-maze, while anxiogenic drugs such as caffeine and picrotoxin reduce this measure in the mirrored chamber behavioral test in mice (Lister, 1987). Anxiolytic drugs such as neurosteroids and benzodiazepines significantly reduce the latency to enter a mirrored chamber in a dose-dependent manner (Toubas et al., 1990; Reddy & Kulkarni, 1997).

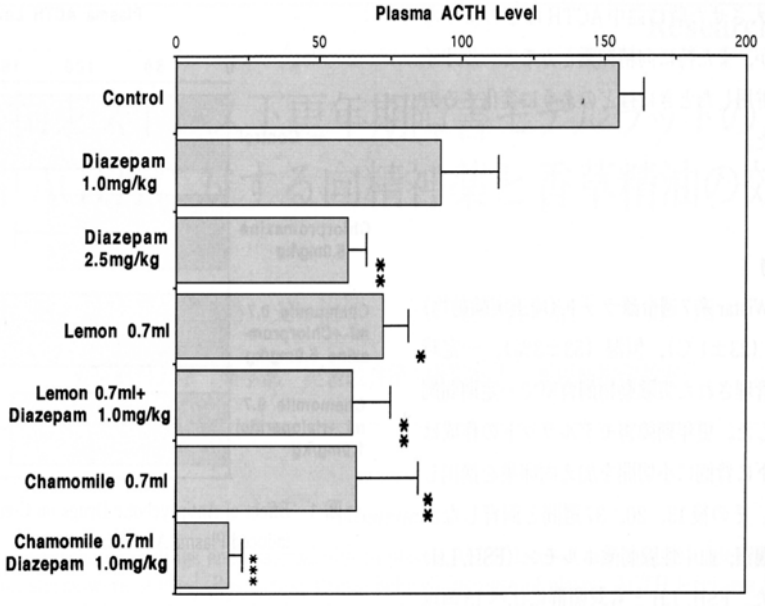

Figure 6. Effect of herb oils and diazepam on constriction stress-induced plasma ACTH level (Yamada et al., 2000; reprinted with permission from the Fragrance Journal Ltd., Japan). $*p < 0.05$ vs control, $**p < 0.01$ vs control, $***p < 0.05$ vs chamomile 0.7 ml.

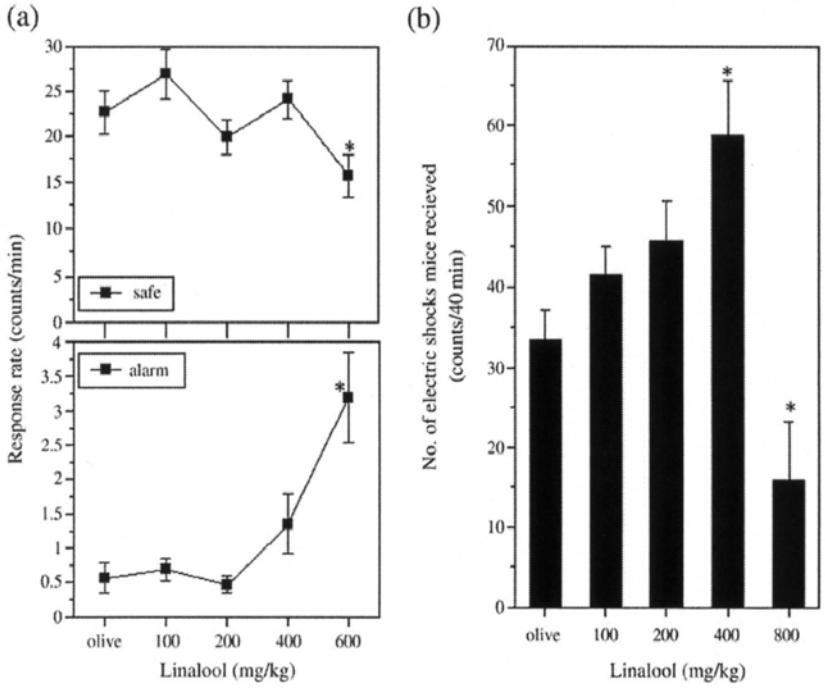

Figure 7. Effects of linalool in Geller (a) and Vogel (b) conflict tests in ICR mice (Umezu et al., 2006; reprinted with permission from Elsevier Inc.).

Conclusion

Enhancement of the inhibitory signals through $GABA_A$ receptor decreases the activity of the amygdala as a whole, resulting less excitatory signals to other areas in the brain. This in turn leads to a reduction in the physiological and psychological markers of stress and anxiety that appear as tranquility. Common foods and beverages consisting of various fragrances enhance the responses of $GABA_A$, especially the extrasynaptic receptors in the brain, modulating our consciousness or mind. In the future, it is necessary to know whether the fragrant compounds modulate gene expression patterns of $GABA_A$ receptors along with various receptors, transporters or channels expressed in the brain, which is critical control point for well being of mind.

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Chapter 10

AMPA Receptor-Mediated Neuronal Death in Motor Neuron Disease

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Abstract

AMPA receptor-mediated excitotoxicity has been proposed to play a major role in death of motor neurons in amyotrophic lateral sclerosis (ALS), the most common motor neuron disease. We demonstrated that RNA editing of GluR2 mRNA at the Q/R site was decreased in autopsy-obtained spinal motor neurons of patients with sporadic ALS. This molecular change did not occur in the other regions of central nervous system (CNS), and has been demonstrated as a molecular change inducing neuronal death in mutant mice, hence may be highly relevant to ALS pathogenesis. We found that the extent of GluR2 Q/R site-editing was reduced to various extents in motor neurons of all the sporadic ALS cases irrespective of the phenotype. On the contrary, although AMPA receptor-mediated neurotoxicity plays a role in SOD1-associated familial ALS (ALS1), the underlying molecular mechanism is an up-regulation of GluR2-lacking Ca^{2+}-permeable AMPA receptors but not GluR2 Q/R site-underediting as seen in motor neurons of sporadic ALS. The molecular mechanism increasing the GluR2-lacking AMPA receptors in ALS1 model rats may likely result from up-regulation of GluR3 mRNA as occurred in kainic acid-induced ALS model rats that we recently developed, hence over-activation of AMPA receptors might participate in death of motor neurons in ALS1. On the contrary, AMPA receptor-mediated neuronal death mechanism did not seem play a role in dying motor neurons of spinal and bulbar muscular atrophy (SBMA).

Because GluR2 Q/R site-editing is specifically catalyzed by adenosine deaminase acting on RNA type 2 (ADAR2), it is likely that regulatory mechanism of ADAR2 activity does not work well in the motor neurons of sporadic ALS. Although ADAR2-null mice die young from status epilepticus, conditional ADAR2 knockout mice that we newly developed exhibited slow progressive death of motor neurons as seen in transgenic mice for artificial Ca^{2+}-permeable GluR2 (GluR-B(N)). Therefore, it is likely that deficient ADAR2 activity induces neuronal death via underediting of GluR2 Q/R site. Indeed, ADAR2 expression level was significantly decreased in the spinal ventral gray matter of sporadic ALS as compared to

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normal control subjects. Taken together, it is likely that ADAR2 underactivity selective in motor neurons induced induces deficient GluR2 Q/R site-editing and resulting neuronal death in sporadic ALS.

Thus, there seem to be multiple different molecular mechanisms underlying death of motor neurons in various motor neuron diseases, and the molecular mechanism generating Ca^{2+}-permeable AMPA receptors that mediated neuronal death may not be uniform among death of motor neurons; an increase of GluR2-lacking Ca^{2+}-permeable AMPA receptors plays a role in ALS1, whereas an increase of Q/R site-unedited GluR2-contatining Ca^{2+}-permeable AMPA receptors in sporadic ALS.

Key words: ALS, AMPA receptor, GluR2, RNA editing, ADAR2, ALS1, SOD1, SBMA, MND

ALS Etiology: The AMPA Receptor-Mediated Neuronal Death Hypothesis

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease characterized by progressive weakness and muscle wasting leading to death within a few years after onset due to the destruction of both upper and lower motor neurons. The majority of ALS cases are sporadic, with a variety of phenotypes including limb-onset classical ALS, progressive bulbar palsy (PBP), and ALS with dementia (ALS/FTD). Cases with familial ALS accounts for less than 10% of all ALS cases, and among the causative genes so far identified, mutations in the copper-zinc superoxide dismutase 1 (SOD1) are the most common, accounting for approximately 20% of familial ALS cases.

The molecular mechanism leading motor neurons to death has not been elucidated even in SOD1-associated familial ALS (ALS1) despite the most intensively investigated ALS, and none of the causative genes so far identified in familial cases has been demonstrated to play any causal role in sporadic cases. Therefore, it is likely that there are multiple different death pathways in motor neurons and that the mechanism producing ALS-phenotype may not necessarily be the same. Several hypotheses have been proposed to explain the etiology of ALS, among which excitotoxicity mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, a subtype of ionotropic glutamate receptors, has been attracted much attention due to the fact that motor neurons are particularly vulnerable to AMPA receptor-mediated neurotoxicity in cultured spinal cord neurons. Although mechanisms of excitotoxic neuronal injury are complex and incompletely understood, intracellular Ca^{2+} overload is an important trigger and an increased influx of Ca^{2+} through activated AMPA receptor-coupled channels appears to play a key role in slow death of motor neurons in culture [1,2].

Ca^{2+} and the AMPA Receptor

Functional AMPA receptors are homo- or hetero-tetrameric assemblies that are composed of four subunits, GluR1, GluR2, GluR3 and GluR4, in various combinations. The Ca^{2+} conductance of AMPA receptors differs markedly depending on whether the receptor has the GluR2 subunit in its subunit assembly. AMPA receptors that contain at least one GluR2

subunit have low Ca^{2+} conductance (Figure 1a), whereas those lacking a GluR2 subunit are Ca^{2+} permeable [3-6]. These properties of GluR2 are generated by a single nucleotide conversion from adenosine (A) to inosine (I) by posttranscriptional RNA processing called RNA editing, during which as I is recognized as G during translation, the glutamine (Q) codon (CAG) is substituted by an arginine (R) codon (CIG; CGG) at the position called the Q/R site in the putative second membrane domain (M2) (Figure 1b) [4,5,7]. Analyses of adult rat, mouse, and human brain RNA have demonstrated that almost all GluR2 mRNA *in vivo* has R at the Q/R site, whereas Q remains at this critical position in the GluR1, GluR3 and GluR4 subunits. Thus, AMPA receptor-mediated neuronal death occurs after an increase in Ca^{2+} influx through AMPA receptor-coupled ion channels, in which either a decrease in GluR2 expression or inefficient RNA editing at the GluR2 Q/R site is the possible causative molecular change.

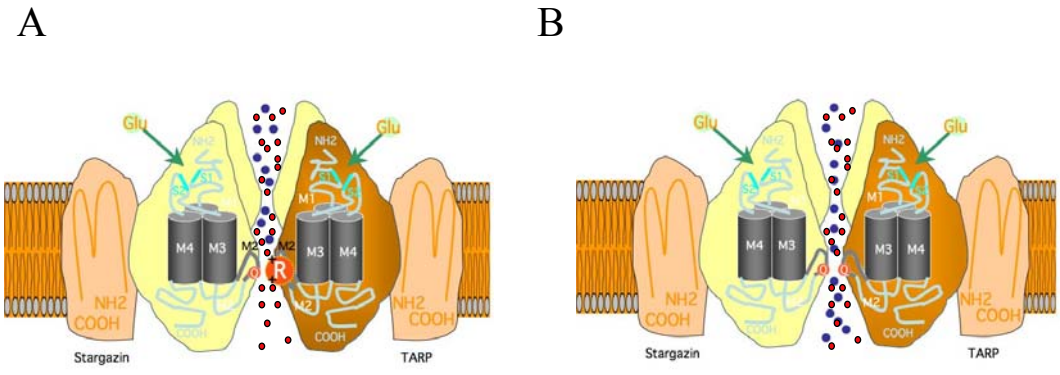

Figure 1. AMPA receptor and Ca^{2+}-permeability. A: AMPA receptor containing GluR2 subunit edited at the Q/R site (orange) in M2 (curved line in dark gray). Due to the positive charge of arginin (R) residue at the Q/R site, Ca^{2+} (blue circle) cannot pass through the channel pore, while Na^{+} (red circle) can pass through it. Majority of the AMPA receptors expressed on neurons is Q/R site-edited GluR2-containing AMPA receptors. B: Q/R site-unedited GluR2-containing or GluR2-lacking AMPA receptors. When none of the subunits have R at the Q/R position, AMPA receptors are Ca^{2+}-permeable. GluR1, 3, 4 subunits are in yellow.

In mammals, the majority of RNA editing occurs as the base conversion of A-to-I, which is most active in the central nervous system and has been found to occur predominantly in non-coding RNAs and has been considered to play a role in RNA processing including efficiency of splicing, stability, etc [8,9]. The best example of A-to-I RNA editing in non-coding RNAs is the recent finding on miRNAs that targeted different mRNAs depending on whether the miRNAs are edited or unedited [10]. On the other hand, A-to-I editing also occurs in the coding regions of mRNA encoding receptors and ion channels, including ionotropic glutamate receptor subunits [11-13], the serotonin-2C receptor ($5\text{-}HT_{2C}R$) [14], the voltage-dependent potassium channel Kv1.1 [15], and the RNA editing enzyme ADAR2 [16]. RNA editing alters the properties of these functional proteins by changing single amino acid at the specific position including the channel pore of the receptors, thereby regulating the strength of each synapse and function of neuronal networks. The regionally highly variable extent of kv1.1 I/V site-editing in the human, mouse and rat brains [15] may be a reflection of

such fine tuning of synaptic strength. In particular, the change in amino acid residue at the Q/R site of GluR2 results in marked alterations in channel properties, including Ca^{2+} permeability [3,4,12,17-22], trafficking [23], subunit assembly [24] and kinetic aspects of channel gating [13] of AMPA receptors. Furthermore, failure of GluR2 Q/R site-editing led mice to fatal status epilepticus [25], hence alterations in the AMPA receptor properties by GluR2 Q/R site-editing play pivotal role in excitation and death of neurons.

GluR2 Q/R Site-Editing and Sporadic ALS

Because several lines of evidence have indicated that molecular changes increasing Ca^{2+}-permeability of AMPA receptors may be relevant to neuronal death, particularly of motor neurons, we have investigated the molecular changes that alter the Ca^{2+}-permeability of AMPA receptors in neurons of sporadic ALS patients. In order to detect the molecular changes selective in motor neurons, we dissected motor neurons from the autopsy-obtained spinal cord of patients with a laser microdissecter (Figure 2). Although the role of GluR2-lacking AMPA receptors in neuronal death has been implicated in ALS and in delayed neuronal death after brain ischemia, we could find no evidence supporting an increase of

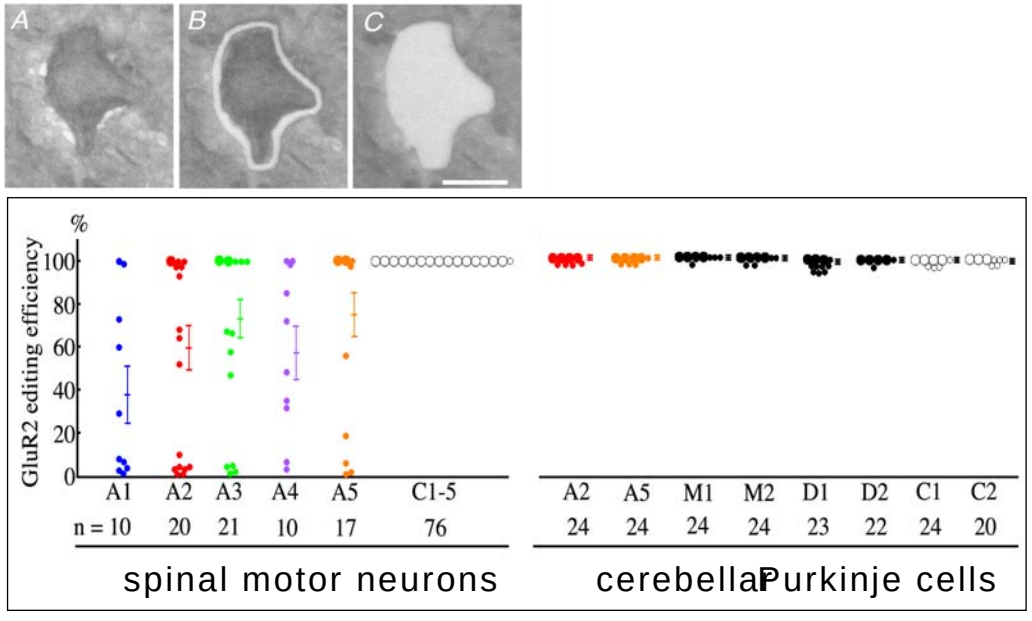

Figure 2. Upper panel: Dissection of single motor neurons with a laser microdissector. Before dissection; demargination with a narrow laser beam; after capturing the neuronal tissue. Bar = 40 μm. Lower panel: Editing efficiency at the GluR2 Q/R site in single neurons of ALS, disease control and normal control subjects (modified from Fig. 1 in [28]). The editing efficiency varied greatly, from 0% to 100% (mean: 38.1-75.3%), among the motor neurons of each individual with ALS, and was not complete in 44 of them (56%); this was in marked contrast to the control motor neurons, of which all 76 examined showed 100% editing efficiency. The editing efficiency in Purkinje cells was virtually complete (greater than 99.8%) in the ALS, disease control (DRPLA, MSA) and the normal control groups. Adopted from ref [28].

GluR2-lacking AMPA receptors in motor neurons of sporadic ALS [26]. On the contrary, GluR2 Q/R site-editing was variably decreased (from 0% to 100%) in spinal motor neurons of sporadic ALS, which was in marked contrast to the control motor neurons, of which all showed invariably 100% Q/R site-editing efficiency (Figure 2) [27,28]. Above results indicate that an increase of Ca^{2+}-permeable AMPA receptors may occur in a subset of dying motor neurons due to a reduced extent of GluR2 Q/R site-editing but not due to a reduced proportion of GluR2-containing AMPA receptors in motor neurons of sporadic ALS. Because mice deficient for GluR2 Q/R site-editing died young from status epilepticus [25], a marked reduction of GluR2 Q/R site-editing in a subset of motor neurons may likely be the death-inducing molecular change in sporadic ALS.

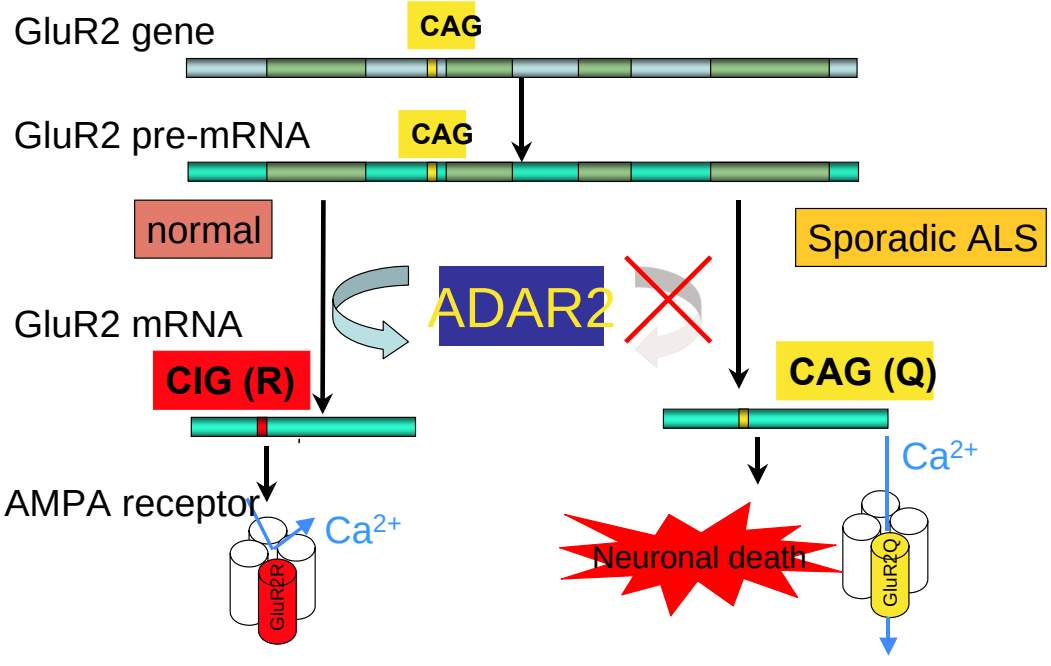

Figure 3. RNA editing and neuronal death in sporadic ALS. Codon for the Q/R site is CAG in the GluR2 gene, whereas A-to-I conversion due to ADAR2, codon for the Q/R site in mRNA is CIG, which is translated as CGG (arginine; R) in normal neurons. By contrast, due to ADAR2-underactivity, codon for the Q/R site remains CAG, which is translated as glutamin (Q) in motor neurons of sporadic ALS. An increase of Q/R site-unedited GluR2Q-containig Ca^{2+}-permeable AMPA receptors mediate neuronal death due to an increase of cytoplasmic Ca^{2+} concentration.

Progressive degeneration occurs selectively in motor neurons in ALS and other neuronal subsets undergo degeneration at a much later disease stage, if ever. We found that the reduction of GluR2 Q/R site-editing did not occur in the cerebellar Purkinje cells of the patients with sporadic ALS despite that the motor neurons expressed a high proportion of Q/R site-unedited GluR2 [28]. Additionally, we found that dying cerebellar Purkinje cells of patients with spinocerebellar degeneration (ie. dentatorubral pallidoluysian atrophy (DRPLA) and multiple system atrophy (MSA-c)) expressed only Q/R site-edited GluR2 mRNA [28]. Moreover, albeit at a tissue level, GluR2 Q/R site-editing has been reported to be preserved

even in the brain areas severely affected in several other neurodegenerative diseases including the striatum of Huntington disease, the neocortex and hippocampus of Alzheimer and Pick diseases, and the cerebellum of diseases of spinocerebellar degeneration [29-32]. Therefore, the defect in GluR2 Q/R site-editing occurs in motor neurons of sporadic ALS in a disease-specific and dying neuron-selective manner.

GluR2 Q/R Site-Editing and Neuronal Death

Mutant mice deficient for GluR2 Q/R site-editing exhibited fatal status epilepticus [25], suggesting that cortical neurons including those in the hippocampus underwent exaggerated excitation due to an increase of Ca^{2+} influx through AMPA receptors containing Q/R site-unedited GluR2. On the other hand, although GluR2-lacking AMPA receptors are also Ca^{2+}-permeable, neither GluR2 knockout mice [33] nor GluR2/GluR3 double-knockout mice [34] exhibited only minor behavioral changes but no demonstrable seizure activity or neuronal death. Therefore, it seems likely that there may be additional influence of Q/R site-unedited GluR2Q other than increasing Ca^{2+}-permeabililty on the properties of AMPA receptors.

Recently, it has been reported that the Q/R site-unedited GluR2Q subunit was more readily incorporated into functional AMPA receptors than the Q/R site-edited GluR2R subunit at the stage of tetramer (dimer of dimers) formation [24]. In addition, AMPA receptors containing the Q/R site-unedited GluR2Q subunit were more readily transported from the endoplasmic reticulum (ER) to the cell surface than those containing the Q/R site-edited GluR2R subunit during receptor trafficking [23]. Therefore, if the extent of Q/R site-RNA editing were decreased, even to a small extent, the density of Ca^{2+}-permeable AMPA receptors containing Q/R site-unedited GluR2Q may increase in the postsynaptic membrane. Indeed, neuronal death was enhanced after transfection of exogenous GluR2Q in cultured rat hippocampal neurons, while it was attenuated when the AMPA receptor trafficking was blocked, suggesting that an exaggerated increase of Ca^{2+}-permeable AMPA receptor density was required to induce neuronal death [35]. Therefore, neuronal death seems to be induced only when the density of Ca^{2+}-permeable functional AMPA receptors were significantly increased in the functioning synapses, and the difference of phenotype severity between mutant mice deficient for GluR2 Q/R site-editing [25] and GluR2-null mutant mice [33,34] may be due to the difference in the density of Ca^{2+}-permeable functional AMPA receptors at the excitatory synapse.

The role of Ca^{2+}-permeable functional AMPA receptors in neuronal death has been implicated in delayed neuronal death of pyramidal cells in the hippocampal pyramidal cells after brain ischemia. Previously, a decrease in GluR2 mRNA prior to neuronal death has been demonstrated [36], whereas a decrease in GluR2 Q/R site-editing was not demonstrated in ischemic brain [29-31] or brains of animals under conditions inducing neuronal death after cerebral hypoxia [37] or kindling [38]. However, it was recently demonstrated that dying hippocampal pyramidal cells after transient ischemia expressed increasing amount of Q/R site-unedited GluR2 mRNA and restoration of Q/R site-editing inhibited the neuronal death after ischemia [39]. Because CA1 pyramidal cells in the hippocampus undergo delayed neuronal death after ischemia [40], these observations suggest that neurons may die with a slow temporal profile when GluR2 Q/R site-editing was diminished. The time-course of slow progression of neuronal death is the characteristics in ALS as well, lending further support to

the relevance of a reduction of GluR2 Q/R site-editing to death of motor neurons in sporadic ALS. The reduction of GluR2 Q/R site-editing has been demonstrated only in the CA1 pyramidal cells after ischemia despite that ischemic insult occurred in entire forebrain area. Therefore, it is likely that a reduction of GluR2 Q/R site-editing occurs only in a subset of neurons that may have low potency keeping this site-editing and hence vulnerable to neuronal death that progresses with a slow process. It has not been elucidated, however, what factor(s) produced the motor neuron-selectivity.

When the relative expression level of each AMPA receptor subunit in various neuronal subsets was investigated at an mRNA level, the motor neurons of control subjects expressed significantly lower levels of GluR2, as compared to other neuronal subsets in humans [26]. The lower relative proportion of GluR2 mRNA among AMPA receptor subunits mRNAs in motor neurons as compared to other neuronal subsets was also found in adult rats, while GluR2 shared much lower proportion as compared to human motor neurons [41]. Above results may imply that AMPA receptors in motor neurons have lower chance to include GluR2 in their subunits than those in other neuronal subsets, resulting in an increase of the proportion of GluR2-lacking Ca^{2+}-permeable AMPA receptors. Therefore, effects of GluR2 RNA editing deficiency on neuronal excitability may be more clearly expressed in neurons expressing relatively low GluR2 [26]. According to this scheme, a small increase in Q/R site-unedited GluR2Q will greatly increase the proportion of Ca^{2+}-permeable functional AMPA receptors, thereby promoting excitotoxicity in ALS motor neurons. It is of interest that rat hippocampal type-II neurons that undergo delayed neuronal death after ischemia, expressed GluR2 mRNA at the low level less than 50% of total AMPA receptor subunits [42], as seen in rat motor neurons [41].

GluR2 Q/R site-Editing in Motor Neuron Disease

In order to rule out the possibility that deficient GluR2 Q/R site-editing is non-specifically associated with death of motor neurons in sporadic ALS, we also investigated the extent of GluR2 Q/R site-editing in dying motor neurons of motor neuron diseases other than sporadic ALS. Because we found that GluR2 Q/R site-editing was low in various types of sporadic ALS in addition to classical limb-onset type and progressive bulbar palsy, including progressive muscular atrophy type ALS with very long course, early-onset ALS with rapid progression [43], and ALS with dementia (unpublished observation), analysis of the extent of GluR2 Q/R site-editing may provide novel aspect for the classification of motor neuron diseases in terms of the death-inducing factors.

ALS1 is the most frequent familial ALS [44], and mutated human SOD1 transgenic animals have been studied extensively as a disease model of ALS1 and sometimes of all ALS cases [45], yet the molecular mechanism how mutated SOD1 induces neuronal death has not been elucidated.

In contrast with the significant GluR2 Q/R site-underediting in motor neurons of sporadic ALS [28], GluR2 mRNA in all the motor neurons of the mutated human SOD1 transgenic rats with two different mutation sites (G93A and H46R) we examined was completely edited at the Q/R site (Figure 4) [46]. We examined the motor neurons in the spinal cord segment corresponding to the hindlimbs of mutated human SOD1 transgenic rats after they manifested paralysis of their hindlimbs, indicating that the motor neurons we examined were already

pathologically affected. Because the pathogenic mechanism underlying ALS1 is considered to be the same as in mutant human SOD1 transgenic animals, motor neurons in affected ALS1 patients would be expected to have only edited GluR2 mRNA. Therefore, the death-inducing molecular mechanisms may be different between sporadic ALS and ALS1. Indeed, an association study of the SOD1 gene in a considerable number of patients with sporadic ALS reported no significant association with mutations of the SOD1 gene [47].

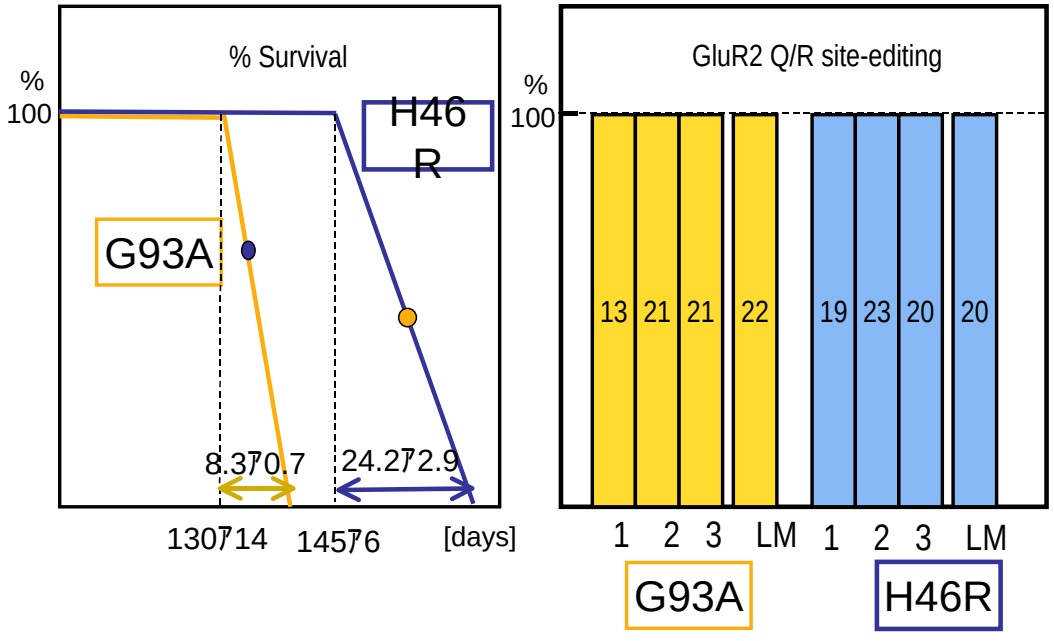

Figure 4. Extent of GluR2 Q/R site-editing was analyzed in laser-captured single motor neurons of the mutated human SOD1 transgenic rats with two different mutation sites (G93A and H46R). Analysis was conducted after transgenic rats manifested ALS signs (indicated as filled circle). More than 10 motor neurons were dissected from each rat (n=3 for each mutation) with their littermate rats. All the motor neuron examined (n=117 for transgenic rats and n=42 for littermate rats) expressed only Q/R site-edited GluR2 mRNA [46].

However, many recent studies support critical roles of Ca^{2+}-permeable AMPA receptors in motor neuron degeneration in ALS1. Transgenic animal studies have recently solidified the link between Ca^{2+}-permeable AMPA receptors and motor neuron loss in SOD1 linked familial forms of ALS. Specifically, crossing G93A SOD1 transgenic mouse models of ALS1 with either mice lacking GluR2 entirely [48] or mice with modified GluR2 encoding aspargine (N) at the Q/R site (GluR-B(N)), which is equivalent to Q/R site-unedited GluR2Q, [49] resulted in marked acceleration of the disease. Conversely, when mice with decreased numbers of Ca^{2+}-permeable AMPA receptors in their motor neurons (via targeted GluR2 overexpression) were crossed with the G93A mice, the disease was significantly delayed [50]. Because GluR2 Q/R site-editing was full in ALS1 model rats motor neurons, these lines of evidence may indicate an increase of GluR2-lacking Ca^{2+}-permeable AMPA receptors in mutated SOD1 transgenic mice. On the other hand, an increase in GluR3 mRNA has been reported in the motor neurons of SOD1 G93A mice [51]. In addition, the survival of these mice can be prolonged by the administration of GluR3 antisense protein nucleic acid [52],

which may reverse an up-regulation of GluR3 and ameliorate an increase of GluR2-lacking Ca^{2+} permeable AMPA receptors. However, as we have mentioned above, a lack of GluR2 *per se* did not induce neuronal death in GluR2 knockout mice [33]. In this regard, our recent observation in rats continuously infused with kainic acid may be worthy of note. When kainic acid was infused into the rat subarachnoid space of the caudal lumbar vertebral segments continuously for more than 1 month, Wistar and Fischer rats displayed progressive and selective motor dysfunction with neuronal death selective in the spinal motor neurons [53]. There was an up-regulation of total AMPA receptor subunit mRNAs due to a selective increase of GluR3 mRNA in motor neurons after the initiation of kainic acid infusion, suggesting that an increase in the density of GluR2-lacking Ca^{2+} permeable AMPA receptors [53]. This study implies that AMPA receptors in motor neurons underwent exaggerated activation in ALS1 model mice, leading to GluR3 up-regulation resulting in an increase in the synaptic density of GluR2-lacking Ca^{2+} permeable AMPA receptors, and ultimately in neuronal death. However, the precise mechanism underlying GluR3 up-regulation after continuous AMPA receptor activation remains elusive.

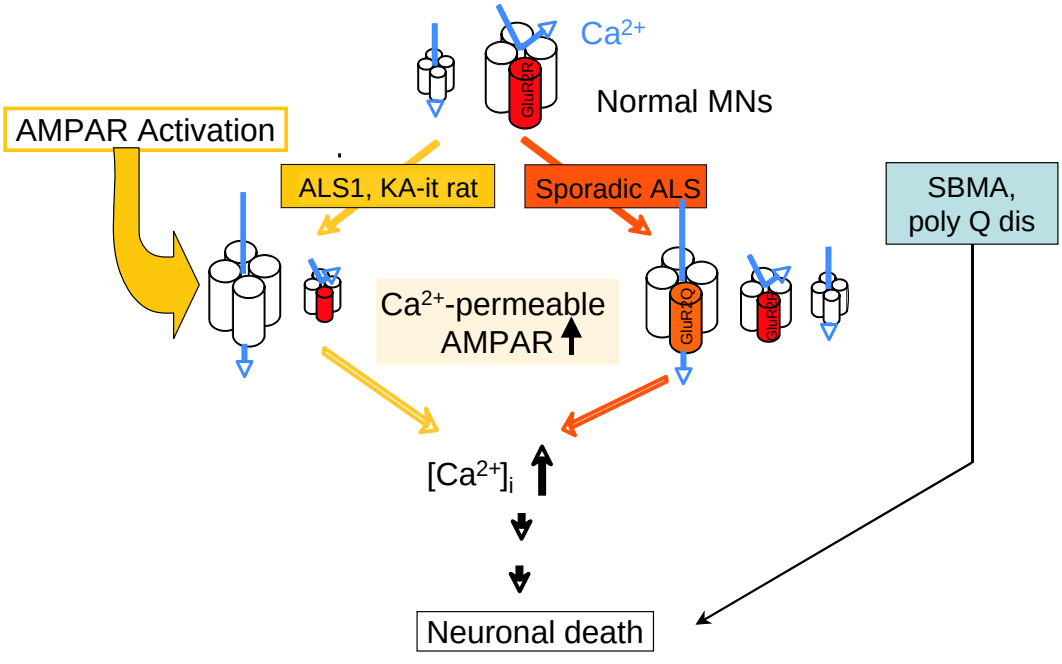

Figure 5. Under basal conditions, motor neurons possess a substantial number of Ca^{2+}-permeable as well as Ca^{2+}-impermeable AMPA receptors. Motor neurons in sporadic ALS express considerable Q/R site-unedited GluR2 mRNA, probably resulting in an increased number of Ca^{2+}-permeable AMPA channels containing unedited GluR2. By contrast, motor neurons in SOD1-associated familial ALS (ALS1) and ALS model rats by continuous intrathecal kainic acid infusion express reduced proportion of GluR2-containing AMPA receptors due to up-regulation of GluR3. Although an increase of GluR2-lacking AMPA receptors per se does not induce neuronal death, neuronal death may be resulted when AMPA receptors are continuously exaggerated. AMPA receptor-mediated neuronal death does not play a role in death of motor neurons in SBMA, a CAG-repeat expansion disease.

Therefore, AMPA receptor-mediated neuronal death plays a pivotal role in both sporadic ALS and SOD1-associated familial ALS, but the molecular mechanisms are different between

the two; deficient GluR2 Q/R site-editing increases Ca^{2+} permeable AMPA receptors in the former, whereas a relative decrease of GluR2 increased GluR2-lacking Ca^{2+} permeable AMPA receptors in the latter (Figure 5). Difference of molecular mechanisms between sporadic ALS and ALS1 were also suggested by recent findings that abnormally phospohrylated and fragmented TDP-43 was localized in ubiquitin-positive cytoplasmic inclusion bodies of motor neurons of sporadic ALS and cortical neurons of FTLD [54,55] but not in inclusion bodies of SOD1-associated familial ALS [56,57]. Investigation into the link between abnormal TDP-43 and GluR2 Q/R site underediting may promote our understanding of the ALS pathogenesis.

Another example of non-ALS motor neuron disease is spinal and bulbar spinal atrophy (SBMA), in which lower motor neurons were predominantly affected remaining upper motor neurons intact with a relatively slow clinical course. Since the CAG-repeat expansion in the androgen receptor gene has been demonstrated in SBMA [58,59], and pharmacological castration is therapeutically effective in animal models [60], the death cascade responsible for SBMA is likely different from sporadic ALS. In contrast to ALS1 model animals, it has been reported that NMDA receptor-mediated neurotoxicity but not AMPA receptor-mediated neurotoxicity plays a role in the neuronal death in model mice of Huntington disease, another CAG-repeat expansion disease [61]. In agreement with these reports, we found that GluR2 Q/R site-editing was complete in dying motor neurons in the autopsied SBMA spinal cord [46]. Therefore, it is likely that there are multiple different death pathways in motor neurons, and motor neurons in sporadic ALS, ALS1 and SBMA die by different death cascades (Figure 5).

Role of ADAR2 in Death of Motor Neurons in Sporadic ALS

The GluR2 Q/R site is almost completely edited in various brain regions, including white matter in neonatal and adult rodent brains [17,62-64]. GluR2 mRNA in human brains including white matter is, however, not always completely edited at the Q/R site [26,27,29,31,38,65-67]. We have demonstrated that neurons in various human brain nuclei express only Q/R site-edited GluR2 mRNA, but adult, but not in immature, human white matter express Q/R site-unedited GluR2 mRNA to an extent up to one-thirds of total GluR2 mRNA [26,67]. It seems likely that human glial cells physiologically express Ca^{2+}-permeable AMPA receptors: oligodendrocytes express those containing unedited GluR2 subunits, and astrocytes and Bergmann's glial cells express those lacking edited GluR2 subunits [5,18,68].

Q/R site-editing of GluR2 is an enzymatic reaction. Enzymes responsible for the A-to-I conversion have been termed adenosine deaminases acting on RNA (ADARs), and three structurally related ADARs (ADAR1 to ADAR3) have been identified in mammals [69-74]. ADAR1 and ADAR2 are expressed in most tissues [69,71,75,76] and ADAR2 recognize the adenosine residue to be edited in the Q/R site of GluR2 through the structure of the duplex that is formed between the editing site and its editing site complementary sequence (ECS), which is located in the adjacent downstream intron of the precursor (pre-) mRNA [77-79] and catalyzes conversion of A-to-I at the Q/R site of GluR2 mRNA exon 11 most efficiently. ADAR1 and ADAR2 catalyze A-to-I conversion in various RNAs in both coding and non-

coding regions. There are two isoforms generated by alternative splicing in ADAR1, with molecular weight 110 kDa and 150 kDa, respectively. There are at least 48 different variants in ADAR2 mRNA and two ADAR2 protein isoforms generated by either including or excluding Alu repeats in human brains [80]. ADAR3 is expressed exclusively in the brain, but is catalytically inactive on both extended double-stranded RNA and known pre-mRNA editing substrates [72,74].

It has been shown that the mRNA expression of ADAR2 is regulated in a cell-specific manner throughout development in rodent brain; first detected by *in situ* hybridization in the thalamic nuclei formation at embryonic day 19 (E19), with more extensive and widespread distribution by the third postnatal week when the expression is highest in the thalamic nuclei and very low in white matter [81]. RNA editing at several editing sites of GluRs was developmentally up-regulated in accordance with the increase of ADAR2 mRNA levels in rat [62,82-85] and human [67] brains, in a cultured human teratocarcinoma cell line [86], and in surgically excised hippocampus from patients affected with refractory epilepsy [87]. In addition, the extent of GluR2 Q/R site-RNA editing was determined in part by the expression level of ADAR2 mRNA in human white matter [26]. However, the ADAR2 expression level does not seem to be corresponding to the extent of RNA editing at various A-to-I positions among different tissues. Thus, it has not been precisely elucidated how ADAR2 activity is regulated in neurons.

ADAR2 knockout mice died young from status epilepticus with expressing markedly high proportion of Q/R site-unedited GluR2, but the ADAR2 knockout mice additionally expressed Q/R site-edited GluR2 without ADAR2 (GluR-B^R) exhibited normal phenotype without developing seizure activity [88]. Although whether death of motor neurons occurred in ADAR2-null mice remains unclear due to their early death, mice transgenic for an artificial Ca^{2+}-permeable GluR2 that has aspargine (N) at the Q/R position (GluR-B(N)) developed motor neuron disease late in life without developing seizure activity [49,89], suggesting that motor neurons are specifically vulnerable to neuronal death resulting from deficient GluR2 Q/R site-editing. These results suggest that ADAR2-deficiency *per se* may not be neurotoxic provided abundant Q/R site-edited GluR2 without ADAR2. Indeed, seizure-inducing phenotype of the ADAR2 knockout mice suggest that neuronal excitation was exaggerated due to an increase of functional Ca^{2+}-permeable AMPA receptors. Although histological demonstration was lacking, the neurons may have undergone seizure-related degeneration in ADAR2 knockout mice, particularly in the limbic brains including the hippocampus, as seen in epileptic rats after kainic acid injection. Therefore, we need ADAR2-deficient mice that do not exhibit seizure activity to investigate whether a lack of ADAR2 induces ALS-like slow progressive neuronal death when the mice lived long without developing status epilepticus. In order to avoid non-specific neuronal injury associated with seizure activity, we developed a mouse line in which ADAR2 was conditionally targeted in the motor neurons using Cre/LoxP system, and found that motor neurons devoid of ADAR2 underwent ALS-like slow neuronal death with expression of only Q/R site-unedited GluR2 (unpublished observation). Because we found that the expression level of ADAR2 mRNA was lower in the ventral gray matter of the autopsy-obtained spinal cord of sporadic ALS cases than in control subjects [90], the conditional ADAR2 knockout mice underwent slow neuronal death under the molecular mechanism in common with sporadic ALS.

We lend further support to our hypothesis that deficient GluR2 Q/R site-editing resulting from a reduction in ADAR2 activity is death-causing molecular change in sporadic ALS but

not in SOD1-associated familial ALS. In addition, in agreement with our results, recent reports demonstrated that abnormal TDP-43 accumulation in the inclusion bodies of sporadic ALS and FTLD but not in those of SOD1-associated familial ALS [56,57]. Therefore, due to the difference in disease-specific molecular changes, we may not properly evaluate the therapeutic efficacy on patients with ALS by using mouse model transgenic for the mutated SOD1 gene. Because GluR2 Q/R site-underediting is likely the direct cause of sporadic ALS, restoring deficient ADAR2 activity in motor neurons may be a specific therapeutic strategy for sporadic ALS, and the conditional ADAR2 knockout mouse line will provide a novel tool for developing specific therapy.

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Chapter 11

Glutamate Receptors Involved in the Interaction between Peripheral Nerve Terminals

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Abstract

Anatomical evidence has shown that glutamate receptors are localized on the peripheral terminals of primary afferent neurons. Electrical stimulation of primary afferents results in release of glutamate peripherally. This raises the question whether glutamate and its receptors enable autocrine and paracrine signaling between nerve terminals in the periphery. We found that spontaneous nerve discharge increases in a cutaneous branch from a spinal dorsal ramus in the thorax following antidromic stimulation of a cutaneous branch from an adjacent spinal segment. This enhanced activity can be blocked by local application of NMDA or non-NMDA receptor antagonists into the receptive field of the recorded nerve. It is well known that the dorsal cutaneous branches of the selected thoracic nerves run parallel to each other and no synaptic contacts at the periphery are known to exist. In addition, because each of the branches are transected distal to the spinal cord, it provides a new model by which we can abolish central interactions and investigate the role of glutamate and its receptors in non-synaptic communication between peripheral nerve terminals arising from different spinal segments. The preparation has provided evidence that glutamate released from peripheral nerves may contribute to the occurrence of secondary hyperalgesia and allodynia outside the area of initial injury. The excitatory neurotransmitters such as substance P (SP) and adenosine triphosphate (ATP) may have functions analogous to that of glutamate in peripheral tissues.

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Some inhibitory neurotransmitters such as opioid and somatostatin appear capable of antagonizing glutamate's effect in peripheral tissues. The functional balance between excitatory and inhibitory neurotransmitters may help maintain normal physiological conditions. This review will focus on the role of glutamate receptors in the peripheral nervous system. It will provide a basis for understanding that glutamate receptors in peripheral tissues could serve as a new target for drug delivery to relieve pain of peripheral origin and mechanosensitivity disorder in gastrointestinal tract.

1. Introduction

It is well known that glutamate is a major neurotransmitter in the mammalian central nervous system and it is involved in many physiologic and pathologic processes. Glutamate is the predominant synaptic transmitter in central terminations of all types of primary afferent fibers to the spinal dorsal horn (Coggeshall and Carlton, 1997). In addition, it has recently been reported that glutamate receptors are located on dorsal root ganglion cells and in peripheral nerve terminals and that they play a key role in modulating sensory input at the periphery (Carlton 2001). It is presumed that endogenous glutamate released from peripheral terminals activates the primary afferent terminals themselves and adjacent terminals. Glutamatergic ligands can cause nociception and appear to be involved in peripheral sensory input via membrane-bound receptors. In this review, we will illustrate the role of glutamate in the network of peripheral nervous system, which will suggest new therapeutic targets for treating pain and visceral mechanosensitivity disorders.

2. The Distribution and Role of Glutamate Receptors in Peripheral Nerves

Ionotropic glutamate receptors (iGluRs), which function by activating ligand-gated cation channels, include the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and kainate receptors (KA). Their names reflect the selective synthetic agonists originally used to identify each receptor. In addition, glutamate can modulate neuronal excitability and synaptic transmission by activating members of a family of G-protein-coupled receptors termed metabotropic glutamate receptors (mGluRs). On the basis of their sequence homology, pharmacology and signal transduction mechanisms, mGluRs have been classified into three main groups. Group I (mGlu1 and mGlu5) receptors are coupled to phospholipase C, activate protein kinase C and release Ca^{2+} from intracellular stores. Group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6-8) receptors inhibit adenylyl cyclase activity (Conn and Pin, 1997; Madden, 2002). In the past decade, numerous lines of evidence have shown that glutamate and its receptors play an important role in peripheral tissues (Carlton, 2001; Hinoi et al., 2004; Kirchgessner, 2001; Raab and Neuhuber, 2007).

2.1. Glutamate Receptors in Cutaneous Tissues

With the use of immunohistochemical, *in situ* hybridization and autoradiographic techniques, it has been shown that all subtypes of iGluRs are located on dorsal root ganglion cells (Sato et al., 1993; Shigemoto et al., 1992) and that iGluRs can be transported into central and peripheral terminals by vagal afferent axons (Cincotta et al., 1989). Studies at the immuno-electron microscopic level demonstrate that different subtypes of NMDA and non-NMDA receptors are localized on the axons and peripheral terminals of sensory neurons in rat and human (Carlton et al., 1995; Coggeshall and Carlton, 1998; Kinkelin et al., 2000). Approximately 20% of unmyelinated sensory axons from the glabrous skin of the rat hind paw immunostain for either NMDA, AMPA or KA receptors (Coggeshall and Carlton, 1998). Besides iGluRs, mGluRs have also been identified on peripheral primary afferent fibers and are involved in the processing of peripheral nociceptive information (Bhave et al., 2001; Carlton et al., 2001; Walker et al., 2001; Yang and Gereau, 2003; Zhou et al., 2001). In the spinal dorsal horn, glutamate receptors are mainly located in the superficial laminae, central termination sites for a substantial number of thinly myelinated (Aδ) and unmyelinated (C-fiber) primary afferents (Coggeshall and Carlton, 1997).

The presence of glutamate receptors in the receptive endings of primary afferent neurons suggests a local role for glutamate release in the periphery. Electrical stimulation of the sciatic nerve using parameters that activate either A or C fibers produces glutamate release into peripheral tissues in hindlimb (deGroot et al., 2000). Peripheral application of glutamate and its receptor agonists can induce mechanical allodynia and thermal hyperalgesia, both of which are blocked by specific glutamate receptor antagonists (Carlton et al., 1995, 1998b; Du et al., 2001, 2003, 2006; Leem et al., 2001; Zhou et al., 1996). Electrophysiological studies show that peripheral application of glutamate or glutamate receptor agonists excite cutaneous afferents *in vivo* (Cao et al., 2005; Tian et al., 2005; Zhang et al. 2006b) and *in vitro* (Du et al., 2001, 2003, 2006). The excitatory effect of glutamate can be blocked by administration of NMDA or non-NMDA receptor antagonists (Du et al., 2003, 2006).

The peripheral glutamatergic system not only plays an important role in nociceptive transduction but also in inflammatory pain (Carlton, 2001). For example, the concentration of glutamate increases in the skin after subcutaneous injection of formalin into the rat hind paw suggesting that nociceptive activity and/or the inflammation caused by formalin induces peripheral release of glutamate (Omote et al., 1998). Additional studies show that local application of glutamate receptor antagonists attenuate the nociceptive behaviors and hyperalgesia evoked by formalin (Davidson and Carlton, 1998; Davidson et al., 1997), carrageenan (Jackson et al., 1995) and bee venom (You et al., 2002) in animal models of inflammation, and also diminishes pain resulting from a burn in humans (Warncke et al., 1997). Following complete Freund's adjuvant-induced inflammation, the proportions of iGluRs expressed on both unmyelinated and myelinated nerves increase significantly (Carlton and Coggeshall, 1999).

It is not clear whether glutamate contributes to the development of inflammation. Several studies show that glutamate causes nociception but not oedema formation when injected into the rat hind paw (Zhou et al., 1996; Coggeshall et al., 1997). However, intraplantar injection of glutamate into the mouse hind paw produces nociceptive-like behaviors associated with dose-dependent paw oedema formation and it has been suggested that the increase in paw

oedema is mediated by non-NMDA iGluRs and release of nitric oxide (Beirith et al., 2002). The discrepancy between these studies indicates further investigate is warranted.

2.2. Glutamate Receptors in Deep Tissues

Accumulating evidence indicates glutamate and its receptors also contribute to peripheral sensory transduction in muscle and joint. Peripheral glutamate levels increase in arthritis patients (McNearney et al., 2000) and increase in rat synovial joints during experimental inflammation induced by kaolin and carrageenan injection (Lawand et al., 2000). Intra-articular injection of glutamate into the knee joint results in thermal hyperalgesia and mechanical allodynia, both of which are attenuated by local application of NMDA or non-NMDA receptor antagonists (Lawand et al., 1997). A very recent report shows that group I mGluR 5 plays a more prominent role than mGluR 1 in knee joint inflammatory pain including evoked and spontaneous pain (Lee et al., 2007). Injection of glutamate into the temporomandibular joint increases activity in afferent fibers of the joint and reflexly induces a prolonged increase in the electromyographic (EMG) activity of the jaw muscle through activation of peripheral NMDA and non-NMDA receptors (Cairns et al., 1998, 2001b).

A series of experiments implicate peripheral glutamate can evoke muscle pain. Injection of glutamate into masseter muscle of rats decreases the mechanical threshold and increases the activity of Aβ, Aδ and C muscle afferent fibers (Cairns et al., 2001a, 2002; Dong et al., 2006). In humans, injection of glutamate into masseter muscle induces a muscle pain response which is significantly greater in women than in men (Cairns et al., 2001a, 2003b; Castrillon et al., 2007; Svensson et al., 2003, 2005). The fact that NMDA injection into the masseter muscle potently excites muscle afferent fibers and that local application of NMDA receptor antagonists almost entirely abolishes glutamate-evoked increase in afferent discharge suggests that activation of peripheral NMDA receptors plays an important role in excitation of muscle afferent fibers (Cairns et al., 2003a; Dong et al., 2006). A very recent study shows that the sex-related differences in NMDA-evoked masseter muscle afferent discharge result from estrogen-mediated modulation of peripheral NMDA receptor activity (Dong et al., 2007). While glutamate injection into masseter muscle of rats decreases the mechanical threshold of muscle afferent fibers (Cairns et al., 2002), another experiment shows that the group I mGluR agonist, R,S-3,5-dihydroxyphenylglycol (DHPG), is capable of significantly decreasing the mechanical threshold of muscle afferent fibers to noxious stimulation (Lee and Ro, 2007). DHPG-induced mechanical hypersensitivity can be effectively prevented by pretreatment of the masseter muscle with 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), a selective mGluR 5 antagonist. Taken together, these data provide evidence that the peripherally located mGluR 5 contributes to the development of masseter hypersensitivity (Lee and Ro, 2007).

Glutamate is shuttled into synaptic vesicles by specific vesicular glutamate transporters (VGLUTs) before exocytotic release. To date, three VGLUTs, namely VGLUT1, VGLUT2 and VGLUT3, have been identified in the nervous system. VGLUTs are specific for packaging of L-glutamate; therefore, the identification of VGLUTs is considered as a more reliable marker for glutamatergic neurons than glutamate immunocytochemistry (Raab and Neuhuber, 2003). VGLUT1 has been localized in the peripheral endings of muscle spindle afferents of the triceps surae and masseter muscles (Wu et al., 2004, Pang et al. 2006). Using

electronmicroscopy immunocytochemistry, glutamate has been found enriched in muscle spindle sensory endings (Banks et al., 2002, Bewick et al., 2005). Exogenous glutamate enhances stretch-induced muscle spindle excitability. The excitability is abolished by the phospholipase D (PLD)-coupled mGluR antagonist (2R,1'-S,2'-R,3'-S)-2-(2'-carboxy-3'-phenylcyclopropyl) glycine (PCCG-13). PLD-mGluR is a newly described receptor whose transduction pathway appears different from the group I-III mGluR categories (Banks et al., 2002, Bewick et al., 2005). Resting excitability of the spindle sensory ending is, in part, maintained by this kind of mGluR in response to endogenous glutamate released from synaptic-like vesicles in the receptive ending because PCCG-13 significantly reduces stretch-induced muscle spindle excitability even in the absence of exogenous glutamate (Banks et al., 2002, Bewick et al., 2005). In summary, evidence from muscles suggests that peripheral glutamate can affect all classes of muscle primary afferent fibers through activation of glutamate receptors.

2.3. Glutamate Receptors in Sympathetic Nervous System

Many studies indicate that the sympathetic nervous system is likely involved in the generation of pain, hyperalgesia and inflammation under pathophysiological conditions (Jänig et al., 1996). However, whether neurotransmitters released by peripheral sensory fibers affect post-ganglionic sympathetic axons has not received much attention. Recently, morphological studies in normal animals have shown that in the gray rami (i.e., postganglionic sympathetic axons), 28-35% of axons express the NMDAR1 subunit, 5-10% express the GluR1 subunit of the AMPA receptor and 0-5% express the GluR5-7 subunits of the KA receptor (Carlton et al., 1998a). Almost every cell in the superior cervical ganglion of the sympathetic chain expresses the mRNA for the NMDAR1 subunit (Shigemoto et al., 1992). Two days after injection of completed Freund's adjuvant into one hind paw, the proportion of NMDA receptors increases two-fold and non-NMDA receptors increases ten-fold in the ipsilateral sympathetic gray rami. These data suggest that postganglionic sympathetic activity may be enhanced by glutamate receptor activation during inflammation (Coggeshall and Carlton, 1999). Increased activity in postganglionic fibers could lead to an increased release of norepinephrine (NE) and other substances such as prostaglandins in postganglionic efferent nerves, which in turn could enhance nociceptive activity (Coggeshall and Carlton, 1999). This peripheral sympathetic-sensory loop could modulate peripheral nociceptive transmission and also affect visceral function and local vascular changes (Carlton et al., 1998a; Carlton, 2001). For example, activation of NMDA receptor can increase NE release and induce muscle contraction in isolated pieces of the gut (Cosentino et al., 1995). Some certain neurochemicals, including glutamate, influence cardiac activity by direct interaction with sympathetic terminals in the heart (Huang et al., 1994). In addition, it has been identified that NMDA receptor and SP receptor mRNAs in pre-ganglionic sympathetic neurons of the intermedio-lateral nucleus of the spinal cord are up-regulated following injection of complete Freund's adjuvant into the front paw of the rat. The results suggest that changes in amount of receptor mRNA in these sympathetic neurons may cause sympathetically mediated pain (Ohtori et al., 2002).

2.4. Glutamate Receptors in Gastrointestinal Primary Afferents

The role of glutamate in the visceral nervous system has received attention in recent years (Hinoi et al., 2004; Kirchgessner, 2001; Raab and Neuhuber, 2007). Here we outline the glutamate's functions in the gastrointestinal tract. The enteric nervous system is the only component of the peripheral nervous system intrinsically capable of mediating reflex activity. This activity is made possible by the presence of, within the bowel wall, microcircuits comprised of primary afferent neurons, excitatory and inhibitory interneurons, and motor neurons that innervate gastrointestinal smooth muscle and glands (Liu et al., 1997). Glutamate-immunoreactive neurons are expressed in ganglia of the myenteric and submucosal plexuses in guinea pigs and rats (Liu et al., 1997). The glutamate transporter excitatory amino acid carrier 1 (EAAC1) is widely expressed on the cell bodies of enteric neurons and in the enteric ganglionic neuropil (Kirchgessner, 2001; Kirchgessner et al., 1997; Liu et al., 1997). VGLUT2 is localized in the nerve terminals of both the myenteric and submucosal plexus. The presence of VGLUT2 immunoreactivity in putative intrinsic primary afferents suggests that glutamate is an enteric sensory neurotransmitter. VGLUT2 immunoreactivity is also displayed on numerous nodose ganglion cell bodies, nerve fibers in the vagal nerve, and central terminals of vagal afferents in the nucleus tractus solitarius (Tong et al., 2001). The fact that neurons in both the nodose and dorsal root ganglia display VGLUT2 immunoreactivity and that a subset of these cells innervate the bowel, as demonstrated by retrograde tracing with FluoroGold, suggests that extrinsic primary afferent neurons have glutamatergic receptive endings in the gut (Kirchgessner, 2001; Tong et al., 2001).

NMDA, AMPA and KA receptors are all found on enteric neurons (Kirchgessner et al., 1997; Liu et al., 1997; McRoberts et al., 2001). With intracellular recordings, the depolarizing responses evoked by glutamate in myenteric neurons include both fast and slow components. The fast component is mimicked by AMPA and the slow component is mimicked by NMDA (Liu et al., 1997). With a well-characterized and clinically relevant model of visceral pain perception intravenous administration of memantine, a noncompetitive NMDA receptor antagonist, reversibly and dose dependently inhibited behavioral pain responses to transient, noxious colorectal distension (McRoberts et al., 2001). These antinociceptive effects may be mediated by NMDA receptors on small diameter afferents because single fiber recordings from decentralized pelvic nerves innervating the colon demonstrated that memantine, in a dose dependent manner, abolished activity in C-and Aδ-fibers induced by colorectal distension (McRoberts et al., 2001).

Group I (mGluR1 and mGluR5) (Hu et al., 1999; Liu and Kirchgessner, 2000), Group II (mGluR2/3) (Chen and Kirchgessner, 2002; Larzabal et al., 1999), and Group III (mGluR7 and mGluR8) (Tong and Kirchgessner, 2003) mGluRs have been detected in the enteric nervous system. Their immunoreactivities are localized in the submucosal and myenteric plexuses and the interganglionic connectives. These mGluRs appear to serve differential functions in the gut. mGluR5 agonist binding induces internalization of the receptors similar to other G-protein-coupled receptors (Liu and Kirchgessner, 2000). Patch clamp recordings reveal that activation of mGluR2/3 on enteric neurons inhibits voltage-gate Ca^{2+} currents through N-type Ca^{2+} channels suggesting a modulatory function in enteric neurotransmission (Chen and Kirchgessner, 2002). mGluR8 is expressed in nitric oxide synthase (NOS) positive neurons as well as in vasoactive intestinal peptide (VIP) positive neurons in the enteric

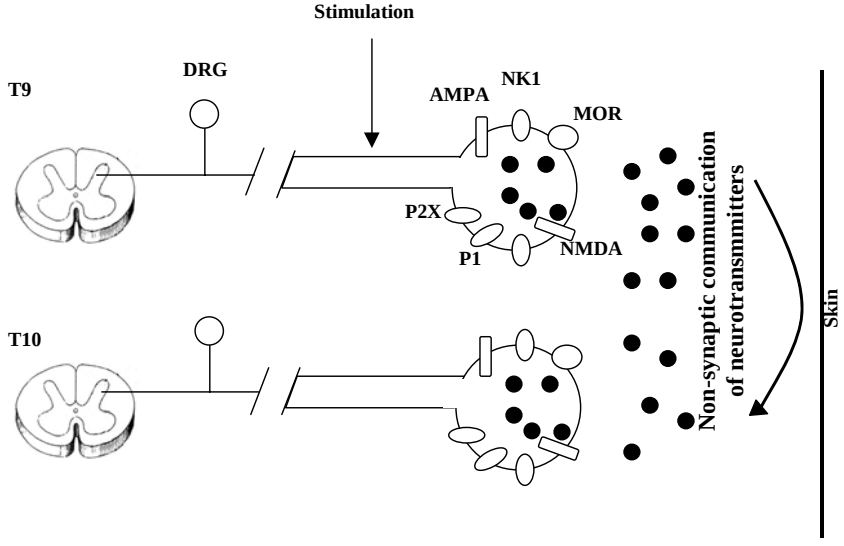
system. NOS is a marker for inhibitory motoneurons and VIP is a marker for secretomotor cells in the enteric nervous system. Therefore, activation of mGluR8 would be expected to modulate intestinal motility and secretion (Tong and Kirchgessner, 2003).

All subunits of iGluRs and mGluRs are expressed in the nodose ganglia of the vagal nerve as shown using reverse-transcription polymerase chain reaction (RT-PCR) in combination with immunohistochemistry (Page et al., 2005; Slattery et al., 2006). The excitatory modulation of gastric vagal afferent mechanosensitivity is mediated mainly via NMDA, AMPA and group I mGlu5 receptors, whereas inhibitory modulation is mediated via group II and group III mGluRs (Frisby et al., 2005; Page et al., 2005; Sengupta et al., 2004; Slattery et al., 2006; Young et al., 2007). Differential activation of these glutamate receptors would serve to maintain the relative mechanosensitivity of vagal afferent endings in the gastrointestinal tract. When the balance between effects of excitatory receptors and inhibitory receptors is tipped, for example by altered receptor expression or transducing efficacy, endogenous or dietary glutamate may cause a change in tissue function (Page et al. 2005). Taken together, all subunits of iGluRs and mGluRs have been identified in primary visceral afferents traveling in both the vagus and spinal nerves (Kirchgessner, 2001).

3. The Interaction Between Peripheral Nerve Terminals

Primary afferent nerves provide sensory information to the central nervous system, but less is known about interactions between the receptive endings of these afferents at the periphery. Recently, we have developed an electrophysiological model to investigate the mechanisms of interaction between sensory nerve terminals. The preparation was first described by Zhao et al. (1996). Briefly, two cutaneous branches to the skin of the back, each from a dorsal ramus of adjacent thoracic spinal segments (T9 and T10), are dissociated and cut close to their passage through the latissimus dorsi muscle in anesthetized rats. One branch is electrically stimulated and electrophysiological recordings obtained from the segmentally adjacent cutaneous branch (Figure 1). Small filaments from the recorded cutaneous nerve branch are repeatedly split until single unit activity is obtained. The type of afferent neuron can be identified by its conduction velocity. Antidromic stimulation of the T9 cutaneous branch activated Aβ, Aδ and C fibers in the T10 cutaneous branch (Cao et al., 2007; Sun et al., 2002, 2003; Zhang et al., 2001, 2007). Following 30s of antidromic electrical stimulation of the T9 nerve (intensity: 1 mA; pulse duration: 0.5 ms; frequency: 20 Hz) activation of the T10 nerve occurred within 1-2 minutes and lasted for 10 minutes, which was evidenced by a significant increase in discharge rate and a decrease in mechanoreceptive threshold. Spontaneous activity in Aβ, Aδ and C fibers in the T10 cutaneous branch significantly increased following stimulation of the T9 branch from 2.00 ± 0.34, 2.42 ± 0.33, and 2.19 ± 0.32 impulses/min to 4.31 ± 0.58, 5.22 ± 0.55, and 5.27 ± 0.69 impulses/min, respectively (Cao et al., 2007). These changes represented increases of 115.5 % for Aβ fibers, 115.7 % for Aδ fibers, and 140.6 % for C fibers and suggested that chemical release and diffusion from the antidromically stimulated nerve activated the adjacent cutaneous nerve. To further evaluate this, the duration before activation occurred was determined from more remote peripheral nerve terminals, three or four spinal segments apart. Discharge from the T12

cutaneous branch of the spinal dorsal ramus was recorded following antidromic stimulation of the T8 or T9 cutaneous branches (Jia et al., 2002). Subsequent to antidromic stimulation of the T9 dorsal cutaneous branch, the discharge from 59.3% (16/27) of Aδ fibers and 71.2% (37/52) of C fibers in the remote T12 nerve increased significantly by 1.5~2 min. When the T8 nerve was stimulated, the discharge from 47.8% (11/23) of Aδ fibers and 36.6% (15/41) of C fibers from the T12 nerve increased significantly by 2~2.5 min. The increased latency before remote activation as the distance increased between the stimulated segment and recorded segment is consistent with a diffusion process and indicates that interactions between sensory nerve terminals at the periphery could occur under conditions where stimulation invades a nerve terminal, e.g. during axon reflexes (see Section 4).



DRG dorsal root ganglion; AMPA: AMPA receptor; NMDA: NMDA receptor; mGluR: metabotropic glutamate receptor; P2X: P2X receptor; P1: adenosine receptor; NK1: NK1 receptor; MOR: μ opioid receptor

Figure 1. Schematic drawing of glutamate and other neurotransmitters involved in the interaction between peripheral nerve terminals. Antidromic electrical stimulation of one cutaneous branch of dorsal ramus evokes the release of some neurotransmitters (● filled circles) from peripheral terminals into the surrounding tissues. These neurotransmitters diffuse to the adjacent sensory nerves and specifically bind to different receptors located on the nerve terminals, and in turn act on the adjacent afferents.

Because the isolated nerve branches in this model are disconnected from the central nervous system, the functional link must be located in the periphery. It is well known that the cutaneous branches of adjacent thoracic spinal dorsal rami selected in the study run parallel to each but no synaptic communication has been reported between nerve terminals from adjacent segments. It could be argued that the increased activity in the recorded fiber following heteronymous antidromic stimulation results from direct stimulation due to electric current spread from the stimulating electrodes to the recorded fibers. However, this is unlikely for the following reasons: First, current spread is expected to excite the recorded units only during the stimulation and not after its termination, the latter being the case in the activity reported in this preparation. Second, since the stimulated and recorded nerves are attached to electrodes immersed in a pool of electrically isolating mineral oil, current should only spread along the stimulated nerve to the skin and not between nerves. Third, because

crushing the stimulated nerve distal to the stimulating electrodes prevented the increased activation of adjacent nerve, we conclude that intact antidromic conduction of action potentials along the stimulated nerve fibers evoked activity in adjacent nerve terminals (Zhang et al., 2007).

4. Glutamate and Its Receptors Involved in the Interaction between Peripheral Nerve Terminals

Previous studies show that some neurotransmitters may be released by antidromic stimulation of sensory nerves via the axon reflex, which contribute to the spread of neurogenic inflammation in the skin (Holzer, 1988; Lembeck, 1983). To determine whether glutamate and its receptors contribute to the interaction between peripheral nerves, iGluRs antagonists have been applied to the receptive field of recorded nerves from the preparation described in the preceding section (Cao et al., 2007). After local treatment of either NMDA receptor antagonist dizocilpine maleate (MK-801) or non-NMDA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX), the enhanced spontaneous discharge in the T10 nerve branch caused by stimulation of the T9 nerve was significantly blocked. The results indicate that peripheral iGluRs are involved in the activation of peripheral nerves following the antidromic stimulation of the adjacent nerve and we proposed that the electrical stimulation of one cutaneous branch evoked the release of glutamate from its peripheral terminals into the surrounding tissues. The released glutamate would diffuse to the adjacent sensory nerves and activate the adjacent afferents by binding to glutamate receptors located on the nerve terminals (Cao et al., 2007). In addition, our data are consistent with the idea that iGluRs contribute to peripheral nociceptor function in cutaneous nerves shown in a number of previous studies (Carlton, 2001). Fig. 1 summarizes glutamate receptors and other neurotransmitters involved in the interaction between peripheral nerve terminals.

In our experiments (Cao et al., 2007), both NMDA and non-NMDA iGluR antagonists significantly inhibited the activation of sensory nerves following the stimulation of adjacent nerves. No significant difference between the two antagonists was found for their effects on the nerve activities. This finding parallels the observation from a behavioral study that either NMDA or non-NMDA receptor antagonist can completely eliminate glutamate-induced pain (Carlton et al., 1998b). In a mouse preparation (Bhave et al., (2001), selective mGluR1 and mGluR5 antagonists independently block the second phase of mechanical sensitivity to formalin-induced inflammatory pain. Effects from blockade of the 2 receptor subtypes are not additive. In addition, the authors show that Group I mGluRs contribute to thermal hypersensitivity based upon its induction by glutamate and by the Group I mGluR agonist DHPG.. They conclude that both mGluR1 and mGluR5 contribute to the thermal hypersensitivity based upon its blockade by the selective mGluR1 antagonist LY367385 and the selective mGluR5 antagonist MPEP. It is not known if their contribution to thermal hypersensitivity is additive. Thus, activation of NMDA, non-NMDA and Group I mGluRs in the periphery can each lead to increases in mechanical pain and thermal sensitivity, and blockade of any one of these receptors can completely prevent the effects of glutamate. It is suggested that these receptors may not act completely independently in a parallel fashion

during inflammatory pain in the skin. Rather, they probably cross-talk and function serially in a complex fashion at molecular and neuronal circuit levels (Bhave et al., 2001).

Neurochemical studies indicates that neurotransmitters diffuse across the synaptic cleft (synaptic transmission) as well as diffuse through the extracellular space and affect nearby neurons (non-synaptic communication) in the central nervous system. Non-synaptic communication appears important for maintaining the physiological properties and functions of neural networks (Vizi, 2000; Vizi et al., 2004). Since synaptic connections have not been found between peripheral nerves, we have reason to believe that glutamate may contribute to interactions between peripheral nerve terminals via non-synaptic communication.

The glutamate uptake system is the primary mechanism for inactivation of released glutamate in synaptic and non-synaptic communication and for maintenance of glutamate homeostasis. Currently, five isoforms of glutamate uptake transporters have been identified: glutamate/aspartate transporter (GLAST), glutamate transporter-1 (GLT-1), excitatory amino acid carrier (EAAC) 1, excitatory amino acid transporter (EAAT) 4, and EAAT5. It has been found that spinal glutamate uptake transporters play an important role in normal sensory transmission and pathological pain states (Liaw et al., 2005; Tao et al., 2005). Accordingly, glutamate transporter modulators should be used in the future to determine the mechanism underlying glutamate involvement in the interaction between peripheral nerve terminals.

Hyperalgesia resulting from cutaneous injury can be divided into two categories: primary and secondary hyperalgesia. Primary hyperalgesia occurs at the site of injury and secondary hyperalgesia occurs outside the site of actual injury. With respect to secondary hyperalgesia, the contribution of central sensitization has been intensively investigated (Baranauskas and Nistri, 1998; Cervero et al., 2003; Klede et al., 2003). Recent studies demonstrate that secondary wide-spread cutaneous hyperalgesia is also partly maintained by peripheral impulse input from the colon/rectum in the irritable bowel syndrome patients (Price et al., 2006). Serra et al. (2004) note that the altered responsiveness in the area of secondary hyperalgesia implies that there is either an altered resting chemical environment in the skin or altered release of chemicals in response to noxious stimulation. Antidromic activation of a primary afferent nerve with high intensity electrical stimulation stimulates the induction of an axon reflex wherein action potentials generated at a receptive ending antidromically invade collateral peripheral branches of the same neuron causing neurotransmitters release from the collateral receptive endings (Holzer, 1988; Lembeck, 1983). Our studies described above (see section 3) showed that antidromic electrical stimulation of a sensory nerve evokes an enhanced response in nerves from adjacent spinal segments without involvement of the spinal cord or central sensitization. The experimental preparation provides us with a means for investigating interactions between peripheral nerve terminals. It provides the opportunity to examine the contribution neurotransmitters released from the peripheral terminals of primary afferent fibers make to the onset and development of secondary hyperalgesia and allodynia (Cao et al., 2007; Zhang et al., 2007). Further studies using this preparation could advance our understanding of peripheral sensitization in pain states and the development of therapeutic interventions for maladaptive pain without risking serious central nervous system side effects. Taken together, the model of interaction between peripheral nerve terminals may be useful for studies on the peripheral mechanism of hyperalgesia and allodynia, cross-talk in network of peripheral nerves, and information transmission along meridians in Traditional Chinese Medicine (Cao et al., 2007; Jia et al., 2002; Sun et al., 2002, 2003; Zhang et al., 2001, 2007; Zhao et al., 1996).

5. The Relationship between Glutamate and Other Neurotransmitters in the Peripheral Nerves

Several neurotransmitters, in addition to glutamate, have been demonstrated to be released from primary afferent endings. It raises the issue of whether these neurotransmitters contribute to non-synaptic communication between peripheral neurons and whether they have interaction relationships with glutamate in peripheral tissues. Some evidence has been provided to address this question. The excitatory neurotransmitters such as substance P (SP) and adenosine triphosphate (ATP) may function analogous to that of glutamate in peripheral tissues. Some inhibitory neurotransmitters such as opioid and somatostatin appear capable of antagonizing glutamate's effect in peripheral tissues. The functional balance between excitatory and inhibitory neurotransmitters may help maintain normal physiological conditions.

5.1. Glutamate and Substance P

With use of the preparation described above (see section 4), SP is reported to play a role in the interaction between peripheral nerve terminals. When the NK-1 receptor antagonist WIN 51708 was injected into the receptive field of a recorded C-fiber, its activity indirectly evoked by electrical stimulation of the adjacent nerve was blocked significantly. Furthermore, the excitation evoked by stimulation of the adjacent nerve was also diminished in adult rats that were neonatally treated with capsaicin, a treatment that destroys most SP-expressing afferent fibers (Zhang et al., 2007). These observations suggest that SP may have a function analogous to glutamate in the periphery. These findings are consistent with the fact that SP can be released from peripheral nerve terminals following antidromic electrical stimulation of sensory nerve (White and Helme, 1985) or noxious stimulation on tissues (Helme et al., 1986) and SP can excite and sensitize nociceptive C-fibers by activation of NK1 receptors in the rat sural nerve (Chen et al., 2006). An electrophysiological study showed that the glutamate-induced excitatory responses of Aδ fibers and C fibers were significantly enhanced by co-injection of SP (Zhang et al., 2006b). The mean discharge rates of Aδ fibers and C fibers with local application of glutamate alone were increased by co-injection of glutamate and SP from 5.84 ± 1.54 to 19.91 ± 4.35 impulses/min for Aδ fibers and from 5.02 ± 2.65 to 17.58 ± 5.59 impulses/min for C fibers. A behavioral study demonstrates that co-injection of glutamate and SP resulted in a significantly longer duration of nociceptive behavioral responses compared with the injection of either substance alone (Carlton et al., 1998b). A recent study on enteric neurons reveals that most of the submucosal glutamate-immunoreactive neurons in guinea pig bowel co-express SP and choline acetyltransferase immunoreactivities (Liu et al., 1997). Activation of an ionotropic glutamate receptor (NMDA) in colonic tissue causes Ca^{2+}-dependent release of SP and calcitonin gene-related peptide (CGRP) (McRoberts et al., 2001).

5.2. Glutamate and ATP, Adenosine

Adenosine triphosphate (ATP) is thought to be involved in peripheral sensory transduction. ATP is released from primary afferent fiber terminals following antidromic

stimulation of a sensory nerve (Holton, 1959). Our recent study showed that injection of α,β-methylene ATP, a P2X receptor agonist, into the receptive fields of primary afferent fibers activated and sensitized Aδ and C fibers. These excitatory effects were blocked by the P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (Zhang et al., 2006a). Other laboratories have also demonstrated that ATP activates sensory neurons in culture (Dunn et al., 2001; Grubb and Evans, 1999) and nociceptive afferent fibers in vitro (Hamilton et al., 2001). α,β-methylene ATP also activates group III and group IV muscle afferents in cats (Hanna and Kaufman, 2004). There is compelling evidence indicating that the peripheral P2X receptors are presented on nerve terminals in several tissues, including skin, bladder and tooth pulp, and that extracellular ATP contributes to sensory transduction mainly via these receptors (Cook et al., 1997; Dunn et al., 2001; North et al., 2004; Chizh and Illes, 2000).

Evidence obtained from the central terminals of primary afferents suggests that ATP, similar to substance P, also facilitates glutamate-induced nerve activity (Gu and MacDermott, 1997). In a model of a sensory synapse system made by co-culture of dorsal root ganglion (DRG) neurons and dorsal horn neurons, P2X receptors at central terminals of primary afferent fibers have been identified. Activation of these presynaptic P2X receptors has been found to facilitate quantal release of glutamate (Gu and MacDermott, 1997). However, it has not yet been investigated whether ATP released from peripheral terminals of primary afferent fibers affects glutamate's function in the peripheral tissues.

The peripheral adenosine system plays a regulatory role in both pain and inflammation (Sawynok, 1998). Adenosine A1 receptors produce peripheral mediated antinociceptive effects in various models (Liu and Sawynok, 2000; Sawynok, 1998). Adenosine A2A, A2B and A3 receptors have relatively lower affinity for adenosine compared to adenosine A1 receptors (Dunwiddie and Masino, 2001) and activation of these receptors produces peripheral pronociceptive effects (Sawynok, 1998). Therefore activation of peripheral adenosine appears to mainly play an antinociceptive role. Recently it has been demonstrated that peripheral iGluRs are involved in this process in that local injection of glutamate evokes adenosine release peripherally (Liu et al., 2002). Adenosine released by glutamate can exert an inhibitory influence on pain signaling in formalin test, and this effect is mediated by an adenosine A1 receptor (Aumeerally et al., 2004). The results suggest that glutamate-evoked peripheral release of adenosine functions as a negative feedback mechanism limiting or counteracting the glutamate-mediated peripheral sensitization (Liu et al., 2002). In summary, ATP and adenosine have complex functions in the peripheral nervous system. Continued work is necessary to investigate the interaction between adenosine, ATP and glutamate in peripheral tissues.

5.3. Glutamate and Opioids

Peripheral sensory nerves also express a number of neurotransmitter receptors that can inhibit sensory transmission. For example, μ- and δ-opioid receptors have been identified on peripheral primary afferent terminals (Coggeshall et al., 1997; Hassan et al., 1993; Ständer et al., 2002). A few studies on the interaction between glutamate and opioid peptides in the periphery have been undertaken. An electrophysiological study (Tian et al., 2005) has shown that the glutamate-induced excitatory response is significantly suppressed by co-injection of

morphine. The mean discharge rate increased by application of glutamate alone was inhibited by co-injection of glutamate and morphine from 28.96 ± 6.85 to 4.40 ± 1.76 impulses/min for C fibers and from 28.99 ± 3.79 to 2.72 ± 0.71 impulses/min for Aδ fibers. The suppressing effect of morphine was reversed by pretreatment with the non-selective opioid receptor antagonist naloxone. These findings suggest that local application of morphine can suppress the glutamate-evoked activities of the fine fibers in rat hairy skin and it provides electrophysiological evidence for peripheral antinociception of opioids. Similarly, a behavioral study has demonstrated that local cutaneous injection of DAMGO, a μ-opioid ligand, ameliorates the nociceptive behaviors caused by local injection of glutamate (Coggeshall et al., 1997).

Recently, the effects of morphine on the release of the excitatory amino acids glutamate and aspartate in the periphery have been examined (Jin et al., 2006). Antidromic stimulation of the sciatic nerve and local application of capsaicin cream to the rat plantar skin causes an increase in excitatory amino acid release. The capsaicin-induced excitatory amino acid release is suppressed by local injection of morphine. Morphine's inhibitory effect is antagonized by naloxone locally. These results indicate that activation of opioid receptors, present on the peripheral endings of small-diameter afferent fibers, can regulate noxious stimulus-induced excitatory amino acid release peripherally (Jin et al., 2006). Another interesting study shows that topical application of opioids produces analgesia in the peripheral. Repeated peripheral administration of opioids also induces tolerance, which can be reversed and prevented by NMDA receptor antagonist given peripherally (Kolesnikov and Pasternak, 1999).

In conclusion, interactions exist between membrane-bound, neuronal receptors for glutamate and other neurotransmitters such as SP, ATP, adenosine and opioids. While γ-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT), and somatostatin receptors are also observed on the peripheral primary afferent terminals, the interaction between these receptors and glutamate receptors in the periphery is not clear. Additional studies are required.

6. Potential Application of Drugs Targeting on Peripheral Glutamate Receptors

Peripheral application of glutamate receptor antagonists attenuate or block nociceptive behaviors in several animal models of inflammation and pain resulting from a burn in humans (Carlton, 2001; Warncke et al., 1997). It has been found that peripheral glutamate receptors have an important role in modulation of mechanosensitivity in gastrointestinal tract as well (Page et al., 2005; Sengupta et al., 2004; Slattery et al., 2006; Young et al., 2007). Localized application of a drug on glutamatergic system could allow for a higher local concentration of the drug at the site of symptom initiation and thereby lessen or make negligible systemic drug levels, thus the advantage of a local delivery approach would be to avoid or diminish serious side effects produced by systemic application of a glutamate receptor antagonist and other analogous drugs (Sawynok, 2003). We suggest that glutamate receptors in peripheral tissues are important new drug targets in order to relieve pain of peripheral origin and mechanosensitivity disorder in gastrointestinal tract.

Conclusion

Glutamate and its receptors are distributed in all classifications of primary afferent fibers. The distribution of glutamate receptor subtypes on peripheral sensory terminals is not homogeneous. A substantial amount of evidence indicates that glutamate and its receptors play an important role in peripheral tissues. Interactions under certain conditions exist between peripheral nerve terminals of spatially distant sensory neurons. Glutamate released peripherally implies autocrine or paracrine activation of the peripheral receptors, which regulates the functions of the afferent nerve itself and involves interactions between sensory nerve terminals respectively, via non-synaptic communication. The relationships between the receptors of glutamate and other neurotransmitters such as SP, ATP, adenosine and opioid constitute a complex network in the peripheral nervous system. Therefore, it is reasonable to believe that glutamate is a major neural signaling molecule in the peripheral nervous system as in the central nervous system. Modulation of peripheral glutamatergic system could affect many neuronal signaling mechanisms in primary afferent fibers.

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Chapter 12

Excitotoxicity and White Matter Damage

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Abstract

Glutamate is the most abundant excitatory neurotransmitter in the mammalian nervous system. It acts through ionotropic and metabotropic receptors which mediate fast excitatory and second messenger-evoked transmission, respectively. It is well known that glutamate can be neurotoxic causing neurodegeneration in many acute and chronic disorders such as ischemic stroke, epilepsy and Parkinson's disease through overactivation of glutamate receptors. In addition to neuronal death, damage to the white matter in a spectrum of clinical conditions including stroke, multiple sclerosis and other neurodegenerative disorders may be related to glutamate receptor-mediated injury. Presence of excess extracelluar glutamate resulting in excitotoxicity has also been put forward as a predominant mechanism of injury to developing cerebral white matter. Activation of glutamate receptors is thought to initiate damage in the adult and developing brain by a cascade of events such as increased calcium influx and enhanced production of cytokines which are known to cause oligodendrocyte death resulting in destruction of myelin and dysfunction of axons. Glutamate receptor antagonists have proven to be promising therapeutic agents in attenuating white matter damage in the developing and adult brain.

Introduction

Glutamate is the major mediator of excitatory signals in the central nervous system (CNS) (Tsumoto, 1990). It is involved in many of the normal brain functions such as cognition, memory and learning (Fonnum, 1984; Collingridge and Lester, 1989; Headley and Grillner, 1990). It also plays an important role in regulation of CNS development, influencing

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neuronal migration (Komuru and Rakic, 1993; Rossi and Slater, 1993), functional synapse induction (Durand et al., 1996; Hanse et al., 1997; Kurihara et al., 1997; Quinlan et al., 1999) and synapse elimination (Rabacchi et al., 1992; Danbolt, 2001).

The actions of glutamate are mediated by ionotropic [amino-methyl-propionic-acid (AMPA), *N*-methyl-d-aspartate (NMDA) and kainate glutamate receptors] and metabotropic glutamate receptors (Brandstätter et al., 1998; Gründer et al., 2001). AMPA receptors, composed of various combinations of four subunits [Glu receptor 1 - 4 (GluR1-4) or GluR-A to GluR-D; Seeburg, 1993; Hollmann and Heinemann, 1994], mediate the vast majority of excitatory neurotransmission in the CNS. The metabotropic glutamate receptors (mGluRs) have been grouped into three main classes, group I (mGluR1 and 5), Group II (mGluR2, 3) and Group III (mGluR 4,6,7,8) according to their amino acid sequence, pharmacological properties and transduction mechanisms (Conn and Pin, 1997). Group I mGlu receptors are considered to enhance cellular excitability via interactions with ionotropic receptors or ion channels (Bordi and Ugolini, 1999).

Excitotoxic cell death is a common pathologic event occurring in the CNS in a number of neurologic diseases and insults including hypoxia, ischemia, and traumatic injury (Li et al., 1999; Fern and Moller, 2000; Tekkok and Goldberg, 2001; Goldberg and Ransom, 2003). Glutamate induces and exacerbates cell death by activating ionotropic and group I mGluRs (Allen et al., 2001; Hilton et al., 2006). Accumulation of excess glutamate in the extracellular spaces leads to persistent activation of glutamate receptors resulting in excitotoxic degeneration of the neurons and glial cells (Haas and Erdo, 1991; Yoshioka et al., 1995; Matute et al., 1997; McDonald et al., 1998). Removal of excess glutamate from the extracellular space by glutamate transporters is crucial to terminate glutamate excitotoxicty.

White matter damage is an important secondary outcome of neurological disorders occuring in the developing as well as the adult brain. The main components of CNS white matter in the adult are axons, majority of which are myelinated, glial cells, and blood vessels. In the developing brain, the components are similar with the exception that majority of the axons are non-myelinated and oligodendrocytes are immature. Damage to the white matter in the developing brain contributes significantly to neonatal mortality and long-term neurodevelopmental deficits such as cerebral palsy, impaired vision, hearing impairments and mental retardation (Volpe, 2003). Multiple factors such as hypoxia-ischemia, cerebral hypoperfusion, a developmental lack of autoregulation of the cerebral blood flow, maternal intrauterine infection and prematurity are thought to be associated with the pathogenesis of periventricular white matter injury, the predominant form of brain injury during the perinatal period (Johnston, 1997; Volpe, 2001; 2003).

Impairment of cognitive function and gait disorders (Ylikoski et al., 1993; Pantoni et al., 1995; Baloh et al., 1995a, b) have been reported as a result of white matter damage in the adult brain. These lesions are associated with cardiovascular disease (Bots et al., 1993), aging and stroke (Breteler et al., 1994; Lindgren et al., 1994; Pantoni et al., 1995; Ylikoski et al., 1995), ischemia (Pantoni et al., 1996), traumatic brain injury (Onaya, 2002; Hortobagyi et al., 2007), schizophrenia (Brambilla et al., 2005; Brambilla and Tansella , 2007; Whitford et al., 2007) multiple sclerosis (Fu et al., 1998; De Stefano et al., 1999; Suhy et al., 2000; Pleasure et al., 2006) and Parkinson disease (Piccini et al., 1995).

Animal Models of Excitoxic Lesions in the White Matter

To gain insight into the pathophysiology of excitotoxic damage to the white matter, intracerebral administration of glutamate analogues that act on NMDA or AMPA/kainate receptors have been used in animals (Marret et al., 1995; Follet et al., 2000; Saliba and Marret, 2001; Tahraoui et al., 2001). Intracortical injections of ibotenate (an NMDA receptor agonist) or S-bromowillardiine (AMPA and kainate receptor agonist) in mouse brain at birth induced the formation of cysts in the white matter which mimic human periventricular white matter damage also known as periventricular leukomalacia, (Marret et al., 1995, 1999; Gressens et al., 1997, 1998; Dommergues et al., 2000; Tahraoui et al., 2001; Hennebert et al., 2004). Immature oligodendrocytes and brain macrophages/microglia have been identified to play key roles in the development of brain lesions mediated by NMDA and AMPA/kainate receptors based on results obtained in animal models of excitotoxic lesions (Tahraoui et al., 2001; Dommergues et al., 2003; Mesplès et al., 2005). *In vitro* models using oligodendroglia, isolated from mixed-glial primary cultures, have shown these cells to be highly vulnerable to glutamate-induced cell death (Oka et al., 1993).

Accumulation of Glutamate in Neurological Disorders

In the white matter, glutamate is released from axons (Kriegler and Chiu, 1993), astrocytes (Bezzi et al., 2001, 2004; Ye et al., 2003), microglial cells (Piani and Fontana, 1994), or oligodendrocytes (Domercq et al., 1999; Domercq and Matute, 1999; Li et al., 1999; Werner et al., 2001). Increased levels of cerebral glutamate have been reported in hypoxic/ischemic conditions in the perinatal brain (Silverstein et al., 1991) and elevation of extracellular glutamate in white matter in hypoxia/ischemia has been described to be possibly due to release from damaged axons (Meng et al., 1997).

Higher concentrations of extracellular glutamate have been reported in the serum or cerebrospinal fluid (CSF) of patients suffering from multiple sclerosis (Westall et al., 1980; Stover et al., 1997; Sarchielli et al., 2003) and this has been hypothesized to be caused by enhanced release from activated macrophages/microglia or alterations in the expression of glutamate transporters (Werner et al., 2001). Glutamate concentration in the CSF and brain tissue of adult patients and children suffering from traumatic brain injury (Baker et al., 1993; Palmer et al., 1994; Zauner et al., 1996; Bullock et al., 1998; Ruppel et al., 2001; Zhang et al., 2001) was also found to be higher than normal subjects. Massive increase in levels of glutamate (> 300 times the normal levels) were detected in the extracellular fluid in the brain tissue in stroke (Bullock et al., 1995). Some authors have proposed that in ischemic conditions, extracellular amino acid accumulation is restricted to the gray matter from where it diffuses slowly into the adjacent white matter and the CSF (Shimada et al., 1993).

Glutamate Excitoxicity

Glutamate excitotoxicity has been implicated in the development of white matter damage in the developing (Johnston, 2001, 2005; Volpe, 2001, Tahraoui et al., 2001; Follet et al., 2004; Jensen et al., 2003) and adult brain (Waxman et al., 1991; Li and Stys, 2000; Goldberg and Ransom, 2003; Tekkok et al., 2005). *In vivo* and *in vitro* studies have shown that oligodendrocytes and myelin can be damaged by glutamate (Li et al., 1999; Matute et al., 1997; McDonald et al., 1998; Rosenberg et al., 1999; Sanchez-Gomez and Matute, 1999; Alberdi et al., 2002). Both receptor and non-receptor mediated mechanisms (Inder and Volpe, 2000) have been described to mediate glutamate excitotoxicity. Receptor mediated glutamate toxicity occurs by activation of ionotropic (NMDA and AMPA/kainate) and mGluRs. Non-receptor mediated glutamate toxicity occurs by glutathione depletion in oligodendrocytes leading to their death by free radical mediated mechanisms (Oka et al., 1993), cytokine release and possibly disruption of blood brain barrier (BBB). In addition, a pathophysiological role for microglial cells in excitotoxic damage to the white matter has been proposed (Tanraoui et al., 2001; Dommergues et al., 2003).

Glutamate Receptor Mediated Mechanisms of Excitotoxic Cell Death

Overactivation of AMPA/ kainate receptors which damages the oligodendrocytes (Yoshioka et al., 1995; Matute et al., 1997; McDonald et al., 1998) may be a common pathway for white matter injury in the developing brain (Jensen, 2002, 2005) and also associated with several conditions such as traumatic brain injury, (Wrathall et al., 1994), multiple sclerosis (Pitt et al., 2000) and experimental allergic encephalomyelitis (Smith et al., 2000; Matute et al., 2001) in the adult brain.

Glutamate receptors are expressed on glial cells in both the gray and white matter (Verkhratsky and Steinhäuser, 2000; Belachew and Gallo, 2004; Kettenmann and Steinhäuser, 2005). Oligodendrocytes are known to express ionotropic (Patneau et al., 1994; Pende et al., 1994; Jensen, 2002, 2005; Karadottir et al., 2005) as well as mGluRs (Luyt et al., 2003; Deng et al., 2004). Recent studies have also shown the expression of functional NMDA receptors in cells of the oligodendroglial lineage and myelin (Karadottir et al., 2005; Salter and Fern, 2005; Micu et al., 2006; Matute, 2006; Verkhratsky and Kirchhoff, 2007).

Ionotropic glutamate receptors are permeable to Ca^{2+} and enhanced activation of Ca^{2+} permeable AMPA/kainate receptors has been considered as a major mechanism producing injury to the immature oligodendrocytes in the developing cerebral white matter (Fern and Moller, 2000; Follett et al., 2000; Itoh et al., 2002; Deng et al., 2003; Rosenberg et al., 2003) initiating their death (Matute et al, 2002). Support for these observations comes from studies of Follet et al (2004) who have reported that the drug topiramate [2,3:4,5-bis-*O*-(1-methyl ethylidene)-β-D-fructopyranose sulfamate] attenuates AMPA/kainate receptor-mediated cell death and calcium influx in developing oligodendrocytes and this effect is similar to the AMPA/kainate receptor antagonist 6-nitro-7-sulfamoylbenzo-(f)quinoxaline-2,3-dione (NBQX)

NMDA receptors on oligodendrocytes also contribute to white matter damage in the perinatal and adult brain (Karadottir et al., 2005; Matute et al., 2007). Activation of NMDA receptors is known to mediate Ca^{2+} increase in myelin causing loosening and vacuolation of the myelin sheath (Micu et al., 2006) rendering these sites particularly vulnerable to excitotoxic insults. These changes were attenuated by an NMDA-receptor antagonist (Micu et al., 2006).

Besides the oligodendrocytes, microglial cells also express AMPA/kainate receptors (Noda et al., 2000) and NMDA receptors (Tahraoui et al., 2001; Kaur et al., 2006). *In vitro* studies have reported microglial neurotoxicity to neurons to be mediated primarily by NMDA receptor signaling (Takeuchi et al., 2005). This observation was supported by blockade of microglia induced neurotoxicity by the NMDA receptor antagonist MK801 (Takeuchi et al., 2005). Microglia are also known to express mGluR subtypes belonging to group I (Biber et al., 1999), group II (Taylor et al., 2002), and group III (Taylor et al., 2003). Stimulation of microglial group II mGluRs induces microglial activation and neurotoxicity (Taylor et al., 2002). Future investigations are needed to ascertain whether microglial NMDA receptor signaling and stimulation of mGluRs on them are involved in damage to the oligodendrocytes in the white matter.

Although astrocytes are known to express functional glutamate receptors, they are generally believed to be resistant to excitotoxic damage (Matute et al., 2002). It is believed that over-activation of AMPA receptors can be rapidly lethal to astrocytes but desensitization normally limits this toxicity (Matute et al., 2002). In vitro studies have shown that prolonged exposure of astrocytes to AMPA/kainate receptor agonists does not affect their viability (Prieto and Alonso, 1999).

Non-Receptor Mediated Toxicity Mechanisms

Excitotoxicity and Proinflammatory Cytokines

Glutamate is known to be involved in production of inflammatory cytokines such as tumor necrosis factor–α (TNF- α) (De et al., 2005). Glutamate induced activation of AMPA receptors on adult rat microglia has been shown to enhance the production of TNFα significantly (Noda et al, 2000; Matute et al., 2001). It has been proposed that TNFα and glutamate cooperate to kill oligodendrocytes in certain conditions such as multiple sclerosis (Matute et al., 2001) and that interaction between AMPA receptor activation and TNFα may be one of the mechanisms which contributes to excitotoxic damage to the developing white matter resulting in death of oligodendrocytes (Selmaj and Raine, 1988; Louis et al., 1993), destruction of myelin and dysfunction of axons (Merrill and Benveniste, 1996). Activation of NMDA receptors on the microglial cells may also be involved in production of cytokines such as TNF- α, interleukin-1 β (IL-1β) and interleukin-6 (IL-6) as excitotoxic lesions induced by the injection of NMDA in developing rats have shown the production of these cytokines in microglial and astroglial cells (Hagan et al., 1996; Acarin et al., 2000). Besides AMPA and NMDA receptors, stimulation of microglial mGlu2 receptors can evoke the release of TNF-α (Taylor et al., 2005).

Glutamate and Blood Brain Barrier

High levels of extracellular glutamate have been reported to disrupt the BBB (Mayhan and Didion, 1996) resulting in formation of edema (Preston and Webster, 2004). Cerebral endothelial cells from many species including humans have been shown to express several forms of glutamate receptors such as NMDA (Krizbai et al., 1998; Sharp et al., 2003; Andras et al., 2007). NMDA and AMPA/kainate receptor antagonists were shown to protect against permeability increase and vascular edema formation in traumatic brain injury (Dempsey et al., 2000). Although the mechanisms of glutamate-induced disruption of the BBB integrity are not fully understood, it has been proposed that NMDA and AMPA/kainate receptors are involved in glutamate-induced alterations of the endothelial tight junction related occludin expression and phosphorylation (Andras et al., 2007).

Excitotoxicity, Glutathione Depletion and Free Radicals

Glutamate toxicity results in glutathione depletion, oxidative stress, and apoptosis (Ratan et al., 1994). Glutathione is a major cellular antioxidant which protects the cells against oxidative stress (Meister and Anderson, 1983; Mizui et al., 1992; Bobyn et al., 2002). Increase in intracellular reactive oxygen species (ROS) generation in response to glutathione depletion has been reported by several studies (Coyle and Puttfarcken, 1993; Tan et al., 1998). Glutathione depletion in oligodendrocytes leading to their death by free radical mediated mechanisms has been described to be caused by glutamate (Oka et al., 1993). Developing oligodendrocytes were reported to be vulnerable to intracellular glutathione depletion (Back et al., 1998, 2002), the mechanism for this vulnerability being intracellular accumulation of ROS (Oka et al., 1993; Yonezawa et al., 1996; Back et al., 1998).

Excitotoxicity and Glutamate Transporters

Glutamate uptake by glutamate transporters is crucial to terminate glutamate signaling and to prevent excitotoxicity. Disruption of glutamate uptake has been reported in several neurodegenerative processes (Lin et al., 1998; Maragakis and Rothstein, 2004), traumatic brain injury (Rao et al., 1998) in the adult CNS and in hypoxic/ischemic conditions in both the developing and adult brain (Torp et al., 1995; Martin et al , 1997; Raghavendra et al., 2000; Rossi et al., 2000). Although astrocytes that express glial GLT-1 and GLAST transporters are the major cell type involved in glutamate uptake in the CNS, oligodendrocytes are known to control extracellular glutamate levels by means of their own glutamate GLAST and GLT-1 transporters (Matute et al., 2001; Domercq et al., 2005). Activated microglial cells have been shown to release glutamate and also block glutamate transporters in oligodendrocytes, leading to an increase in extracellular glutamate and subsequent oligodendrocyte death (Domercq et al., 2007) by releasing inflammatory mediators such as TNF-α or IL-1 β (Fine et al., 1996; Pitt et al., 2003; Takahashi et al., 2003). Suppression of glutamate transporters in astrocytes has also been reported in hypoxic-ischemic conditions (Dallas et al., 2007).

The potential mechanisms by which excess release of glutamate can result in excitotoxic damage and death of oligodendrocytes are summed up in Fig.1.

Prevention of Excitotoxic Damage

Several studies have suggested that prevention of glutamate toxicity with AMPA/kainate antagonists is effective in diminishing white matter injury in the developing and adult CNS. In animal models, the AMPA receptor antagonist NBQX has been shown to be effective in attenuating white matter damage in the developing brain by blocking activation of non-NMDA receptors (McDonald et al., 1998; Follet et al., 2000). The drug topiramate has been reported to provide dose-dependent and long-lasting protection of developing white matter evidenced by increased survival of pre-oligodendrocytes and inhibition of microglial activation and astrogliosis in AMPA/kainate mediated excitotoxicity (Follet et al., 2004; Sfaello et al., 2005). A study, using an in situ model of coronal brain slices from adult mice containing the corpus callosum, showed that blockade of AMPA/kainate receptors with NBQX or the AMPA-selective antagonist, GYKI 52466, prevented oligodendrocyte death (Tekkok and Goldberg, 2001). Antagonists of AMPA/kainate receptors have been reported to increase oligodendrocyte survival and reduce axonal damage in experimental autoimmune encephalomyelitis which mimics multiple sclerosis (Pitt et al., 2000; Smith et al., 2000; Werner et al., 2000; Groom et al., 2003) and experimental models of transient focal ischemia (McCracken et al., 2002). In vitro studies have demonstrated that oligodendrocytes are protected from hypoxic injury by blockade of AMPA/kainate receptors thus preserving white matter function (McDonald et al., 1998; Fern and Moller, 2000; Yoshioka et al., 2000). However, although AMPA receptor antagonist, YM872 ([2,3-dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4 tetrahydroquinoxalin-1-yl] acetic acid monohydrate) may hold some promise, clinical trials of drugs that block the NMDA receptors in acute ischemic stroke have been disappointing (Akins and Atkinson, 2002).

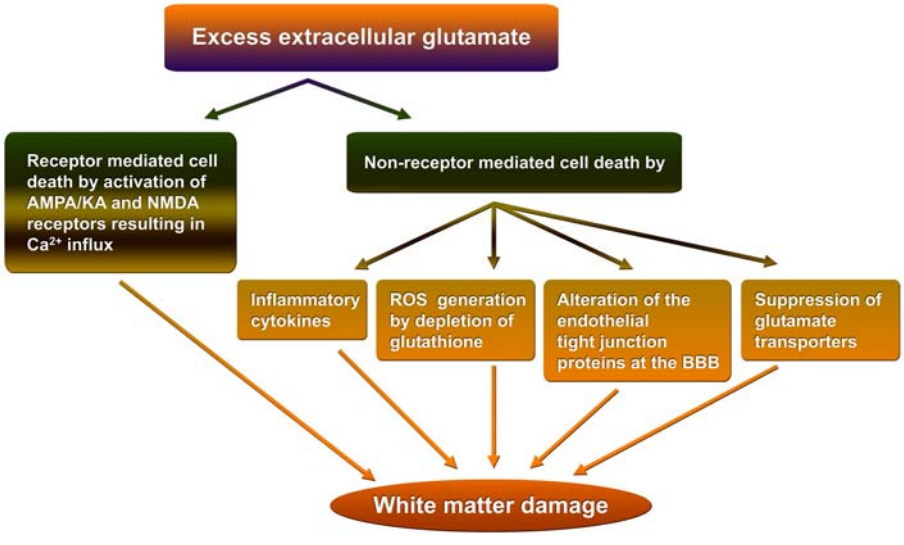

Figure 1. Potential mechanisms of oligodendrocyte damage in the white matter by excess release of glutamate. AMPA/KA, amino-methyl-propionic-acid/kainite; ROS, reactive oxygen species; BBB, blood-brain barrier.

Recent studies have suggested therapeutic potential of melatonin, an antioxidant and a free radical scavenger (Reiter, 1997; 2003), in reducing excitotoxic damage to the developing periventricular white matter (Husson et al., 2002). Intraperitoneally administered melatonin in mice has been reported to result in an 82% reduction in size of ibotenate-induced white matter cysts in comparison to the controls; this effect was believed to be mediated by specific melatonin receptors and not its antioxidant properties (Husson et al., 2002). Our recent investigations have shown that melatonin reduces the glutamate concentration following hypoxic injury in the periventricular white matter of neonatal rats (Kaur et al., unpublished observations). This supports the beneficial effect of melatonin in diminishing glutamate excitotoxicty. Vasoactive intestinal peptide (Gressens et al., 1997) and nociceptin antagonists (Laudenbach et al., 2001) were shown to reduce white matter damage and protect it against ibotenate induced excitoxicity. Protection against excitotoxic damage to the white matter in the neonatal period by drugs such as minocycline which target microglia, suppressing their activation, has been investigated in animal models (Dommergues et al., 2003).

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Abbreviations Used

AMPA : amino-methyl-propionic-acid
BBB : blood brain barrier
CNS : central nervous system
CSF : cerebrospinal fluid
GluR : glutamate receptor
IL-1β : interleukin -1 β
mGluRs : metabotropic glutamate receptors
NBQX : nitro-7-sulfamoylbenzo-(f) quinoxaline-2, 3,-dione
NMDA : N-methyl-D-aspartate
ROS : reactive oxygen species
TNFα: tumor necrosis factor -α

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Chapter 13

Regulation of Synaptic Transmission and Brain Function by Ambient Extracellular Glutamate

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Abstract

The concentration of glutamate in brain extracellular fluid is presumed to be biologically negligible under nonpathological conditions. But this is unlikely to be true. In fact, measurements suggest that ambient extracellular glutamate concentrations are high enough to cause significant steady-state desensitization of glutamate receptors, implying that ambient extracellular glutamate may be a critical regulator of synaptic transmission and brain function. Unfortunately, in vivo measurements of ambient extracellular glutamate have serious caveats, and new less invasive techniques for measuring extracellular composition have yet be developed. It's also unclear what controls ambient extracellular glutamate; virtually nothing is known about the molecular regulation of this glutamate pool. Finally, there are significant challenges posed by the idea that brain function can only really be understood in the context of a three-dimensionally-intact and volumetrically-perfused structure. Nevertheless, tackling these difficulties promises significant reward.

Introduction

Nervous systems are perfused by complex and generally poorly understood ‘stews’ of extracellular fluid. One component of this extracellular fluid is dissolved glutamate. The concentration of dissolved ambient extracellular glutamate perfusing the nervous system, if high enough, could have profound effects on nervous system function. Most neurotransmission in the mammalian central nervous system is mediated by glutamate receptors. Consequently, neural circuit function depends heavily on glutamate receptor

function, and changes in glutamate receptor abundance and function underlie learning and memory [1-4]. Here, I briefly review how ambient extracellular glutamate can regulate synapse strength by activation or deactivation of glutamate receptors.

Ambient Extracellular Glutamate and Regulation of Glutamatergic Synapse Strength

Many neurophysiologists incorrectly assume that the concentration of ambient extracellular glutamate perfusing the nervous system in vivo is biologically negligible. This is untrue. Cerebrospinal fluid (CSF) is most easily extracted, and therefore has been most extensively analyzed. Most studies report that healthy subjects have CSF glutamate in the range of 3-10 μM [5-7]. However, glutamate levels in CSF might misrepresent perineuronal glutamate levels in the brain. Glutamate in CSF could be quite low compared to perisynaptic glutamate because of frequent synaptic release, which transiently raises synaptic glutamate to 1000-3000 μM [8, 9]. On the other hand, glutamate in CSF might be quite high compared to perisynaptic glutamate due to localized glutamate uptake.

A number of techniques have been used to measure extracellular glutamate in brain tissue. Microdialysis is the most common sampling approach, and yields estimates for extracellular glutamate that are typically in the range of 1-5 μM after correction for recovery [10-12]. High recovery push-pull perfusion and direct sampling methods both sample from a more limited volume than microdialysis, which is useful for assays of localized synaptic activity. Push-pull perfusion measurements of extracellular glutamate are similar to those from microdialysis: < 2 μM [13]. Direct sampling typically yields lower numbers: ~0.5 μM [14]. Electrochemical sensors capable of measuring glutamate in situ often report values much higher than other methods [15, 16]. For example, Kulagina et al (1999) and Oldenziel et al (2006) measured extracellular glutamate concentrations in vivo from rat striatum using electrochemical microsensors, and obtained values that averaged 29 μM and 18 μM, respectively. Some of the discrepancy between these electrochemical measurements and other techniques may be due to low electrochemical glutamate selectivity. Consistent with this, electrochemical microarray measurements combined with a background subtraction approach estimated rat striatal and frontal cortex extracellular glutamate at approximately 2 μM, within the range obtained by other techniques [17]. Thus, numerous studies and techniques suggest that ambient extracellular glutamate concentration in the resting/anesthetized mammalian brain is in the range of 0.5 to 5 μM. As discussed below, this concentration is sufficient to have profound effects on brain function.

However, a major criticism of analytical extracellular glutamate measurements is that sampling probes likely disrupt brain tissue, leading to spuriously high extracellular glutamate measurements. In an attempt to circumvent this problem, Herman and Jahr (2007) measured ambient extracellular glutamate using patch clamp recordings of standing NMDA receptor ‘standing currents’, which are presumably proportional to ambient extracellular glutamate concentration [18]. Their measurements suggested that ambient extracellular glutamate in rat brain slices is only 25 nM, kept very low by strong glutamate uptake. 25 nM is remarkably low – approximately the theoretical lower limit based on glutamate transporter properties [19, 20]. Is this realistic? All cells import and concentrate glutamate. For example, plasma

glutamate is 30-100 μM, while red blood cell cytoplasmic glutamate is approximately 500 μM, and muscle cell cytoplasmic glutamate is approximately 5000 μM [21-23]. This represents a 10-100 fold gradient. Synaptic vesicle glutamate transporters in glutamatergic neurons produce a gradient that is similar in magnitude. For example, the concentration of glutamate in the cytoplasm of glutamatergic neurons is approximately 10,000 μM, and synaptic vesicles within these neurons have glutamate concentrations near 100,000 μM [6, 8, 24-27]. If the glutamate gradient between neuronal cytoplasm and extracellular fluid were similar to the gradients achieved by other cell types or synaptic vesicles, then ambient extracellular glutamate would be approximately 100 μM. However, 100 μM ambient extracellular glutamate would completely preclude intercellular glutamatergic transmission and trigger massive neurodegeneration. Therefore, the concentration of ambient extracellular glutamate in the nervous system must be lower, consistent with very high efficiency glutamate uptake in the nervous system and the conclusions of Herman and Jahr [6, 28-30]. Unfortunately, careful examination of the report by Herman and Jahr reveals that their convoluted standing current technique grossly underestimated experimentally-controlled ambient extracellular glutamate in the presence of glutamate uptake blockers, implying that their estimate of 25 nM for ambient extracellular glutamate might be far too low.

Another approach to measuring ambient extracellular glutamate measurements in vivo are genetically-encoded optical probes for glutamate – essentially, glutamate receptors whose fluorescence changes after agonist binding. Such a sensor was recently described by Namiki et al (2007). This sensor, termed 'EOS' (for Glutamate (E) Optical Sensor) improves upon previous enzyme-linked and GFP-based probes [31, 32], which could not be used in vivo and/or had low sensitivity. EOS, generated from the glutamate-binding region of a GluR2 AMPA receptor, reliably shows relatively fast fluorescence changes in response to nanomolar concentrations of glutamate [33]. Unfortunately, EOS fluorescence is generated by a fluorescent dye that must be bath applied and which then conjugates to the protein via introduced cystines. Thus, while a promising tool for cell culture and perhaps brain slice studies, EOS does not allow in vivo extracellular glutamate measurements. Recent insights into ionotropic glutamate receptor binding region structure and channel gating [34, 35] will hopefully allow engineering of better optical glutamate biosensors.

Why does the exact concentration of ambient extracellular glutamate matter so much? There is evidence that extracellular glutamate controls several important neurological processes, including neuronal and glial cell differentiation and migration during development [36-40]. Here, we will focus on regulation of glutamatergic synapse strength. The easiest way to imagine ambient extracellular glutamate affecting any biological process, including synaptic transmission, is via glutamate receptors. A typical initial thought is that ambient extracellular glutamate, if it does anything at all, might perpetually activate glutamate receptors. However, another possibility is that ambient extracellular glutamate might lead to constitutive desensitization of receptors and therefore suppression of glutamatergic signaling. Which actually occurs depends on the glutamate sensitivity of receptor activation and desensitization relative to the concentration of ambient extracellular glutamate. The exact glutamate sensitivity of activation and desensitization depends on glutamate receptor type.

There are two main types of glutamate receptor in the nervous system: ionotropic (pore-forming) glutamate receptors and metabotropic (G-protein coupled) glutamate receptors. Mammalian ionotropic glutamate receptors are functionally and molecularly differentiated into three subgroups based on agonist pharmacology and subunit composition: 1) N-methyl-

D-aspartate (NMDA) receptors, 2) amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, and 3) kainate receptors. As their names imply, NMDA receptors display particular sensitivity to NMDA, AMPA receptors display particular sensitivity to AMPA, and kainate receptors display particular sensitivity to kainate [41]. Differences between the three subtypes are attributed to the fact that although all ionotropic glutamate receptors are thought to be tetrameric, each receptor subtype is assembled from a different set of subunit proteins. For example, NMDA receptors are assembled from NR1, NR2, or NR3 subunits, AMPA receptors are composed of various combinations of GluR1, GluR2, GluR3, and GluR4 subunits, and kainate receptors are composed of GluR5, GluR6, GluR7, KA1, and KA2 subunits [41-43]. (It should be noted that glutamate receptors are also present in many non-neuronal cell types, where the subunit composition and pharmacology is likely to differ [44-46]). There are several different types of NR1, NR2, and NR3 subunit, each encoded by different genes, and alternative forms of each of the subunits mentioned above can be created by alternative splicing or post-translational modification [47, 48]. Thus, there is significant diversity in the subunits that assemble to form receptors, and therefore significant functional and cell biological diversity within each of the three ionotropic glutamate receptor subtype families. Presumably, an organism's ability to 'mix and match' subunits in different cell types and in different stages of development allows it to 'fine-tune' receptor properties.

Mammalian metabotropic glutamate receptors are also divided into three subfamilies, or 'groups', based on molecular and pharmacological differences. Group I metabotropic glutamate receptors are formed from mGluRI or mGluR5 subunits and often activate phospholipase C pathways. Group II and Group III metabotropic receptors are comprised of mGluR2/mGluR3 or mGluR4/mGluR6/mGluR7/mGluR8 subunits respectively, and generally suppress adenylate cyclase activity [43, 49, 50]. As with ionotropic glutamate receptors, further diversity is generated through alternative splicing and poststranslational modifications [50].

Which glutamate receptors are likely to be activated by ambient extracellular glutamate? AMPA and kainate receptors mediate most of the fast excitatory neurotransmission in the CNS and require the highest concentrations of glutamate to activate. For example, native AMPA receptors in rat neurons are activated by glutamate concentrations between approximately 100 μM and 10,000 μM, with an EC50 about 700-1000 μM [51-53]. Kainate receptors are activated at slightly lower glutamate concentrations, with EC50s around 300-800 μM [54-56]. Thus, neither AMPA nor kainate receptors are likely to be activated by ambient extracellular glutamate. AMPA receptors heterologously expressed in oocytes can be activated by substantially lower glutamate concentrations: 20-2000 μM [57, 58], but this is still rather high relative to ambient extracellular glutamate, and may represent anomalous pharmacology and/or gating due to lack of modulatory accessory proteins or perfusion, since AMPA receptors heterologously expressed in HEK cells show glutamate dose-response curves similar to those from native receptors [59].

Compared to that of AMPA and kainate receptors, NMDA receptor activation is much more sensitive to glutamate. In the presence of the co-agonist glycine, both native and recombinant NMDA receptors are activated by 0.5-50 μM of glutamate, with most EC50s around 2-4 μM [60-62], although single channel measurements from heterologously-expressed receptors have yielded EC50 values that are substantially lower: 0.45 μM [63]. These EC50s, compared to estimates of ambient extracellular glutamate concentration in vivo, suggest that a substantial fraction of NMDA receptors could be constitutively activated

by ambient extracellular glutamate. Consistent with this, Sah et al (1989) showed that NMDA receptors in hippocampal CA1 pyramidal cells were constitutively activated [64], and attributed this tonic activation to ambient extracellular glutamate. Tonic NMDA receptor activation appeared as membrane current 'noise' in hippocampal slice recordings that was blockable with the NMDA receptor blocker APV [64]. This observation has been replicated [65], and the source of ambient glutamate probed in more detail (see below). Tonic activation of NMDA receptors has also been noted in dentate gyrus granule cells and salamander retinal ganglion cells [66]. Tonic activation of NMDA receptors is thought to primarily affect cell excitability. Depolarization increases the size of tonic NMDA receptor currents due to release of voltage-dependent Mg2+ block, thereby increasing the tendency toward regenerative depolarization [67].

Metabotropic glutamate receptors are also activated by relatively low concentrations of glutamate. The exact EC50 for glutamate activation of mGluRs depends on receptor subtype. Measured EC50s range from 0.02 μM (for mGluR8) to 1000 μM (for mGluR7), though most EC50 values for most mGluR subtypes are around 10 μM (for review see [68]). In principle, tonic activation of mGluRs could be either excitatory or inhibitory depending on downstream effectors. Indeed, both excitatory and inhibitory effects have been noted and attributed to tonic activation of mGluRs. For example, activation of excitatory sodium/calcium exchange in baroreceptors has been attributed to tonic activation of group I mGluRs [69]. Tonic activation of group III mGluRs causes suppression of supraoptic nucleus spontaneous activity and also inhibition of hypocretin/orexin neurons [70, 71]. At the level of network function, tonic activation of mGluRs triggers 40 Hz ('gamma') oscillations in hippocampal and neocortical slices [72, 73], and these oscillations are thought to underlie 'binding' of sensory stimuli [74]. Thus, although it is difficult to predict how tonic activation of mGluRs might affect brain function, it is likely that such effects are computationally important.

Perhaps more important than the possibility of tonic activation of glutamate receptors is the high likelihood that ambient extracellular glutamate causes constitutive receptor desensitization. Glutamate triggers both activation and desensitization of glutamate receptors. Activation is faster than desensitization, and thus typically precedes desensitization when glutamate concentrations rise quickly. Thus, receptors are transiently activated in response to fast application of agonist. However, activation will not necessarily happen at all during slow increases in extracellular glutamate or in response to ambient extracellular glutamate. In ionotropic glutamate receptors, activation is not required for desensitization, which can occur from unopened channel states, and desensitization is much more sensitive to glutamate than activation. For example, the EC50 for NMDA receptor desensitization in cultured hippocampal neurons is only 1-2 μM [62, 75]. In one study, the EC50 for glutamate 'predesensitization' of NMDA receptors in excised outside-out nucleated patches from cultured embryonic mouse neurons was only 0.3 μM [76]. In that study, 80% of NMDA receptors were desensitized by 1 μM glutamate [76]. Native AMPA and kainate receptors have desensitization EC50 values approximately 4 μM and 3-13 μM, respectively [54, 55, 75, 77], making at least some constitutive desensitization of these receptor subtypes by ambient extracellular glutamate also likely. Thus, the most likely effect of ambient extracellular glutamate on glutamate receptors will be constitutive desensitization, rather than activation.

Metabotropic glutamate receptor desensitization involves phosphorylation, altered intracellular protein interactions, and receptor internalization [78, 79]. In the case of ionotropic receptors, desensitization represents a shift to a very stable nonconducting

(synaptically silent) protein conformation [34, 35, 80]. Recent evidence suggests that mobility and/or trafficking of ionotropic glutamate receptor proteins may also be altered by desensitization [81-83]. Consistent with the idea that steady-state glutamate receptor desensitization by ambient extracellular glutamate suppresses synaptic transmission in vivo, Zorumski et al (1996) showed that synaptic currents recorded from cultured rat hippocampal neurons were significantly decreased by relatively low concentrations of bath-applied glutamate [75]. Specifically, the EC50 of suppression for AMPA receptor-mediated synaptic currents was 3.8 μM glutamate, and 1.3 μM glutamate for NMDA receptor-mediated synaptic currents [75]. Similarly, excitatory postsynaptic currents in cultured embryonic chick neurons can be reduced by about one-half after bath application of 10 μM glutamate [84]. More recently, the number of functional synaptic glutamate receptors in *Drosophila* synapses was shown to be regulated by glutamate concentrations in a normal physiological range [83]. Many other studies have shown that synaptic current sizes in glutamatergic synapses are increased after application of desensitization inhibitors, further strengthening the idea that glutamate receptor desensitization significantly limits synaptic transmission in vivo. However, AMPA receptor desensitization is fast enough to limit peak synaptic responses, making it difficult to determine how much synaptic augmentation after desensitization inhibitors is due to steady-state desensitization. Nevertheless, if ambient extracellular glutamate is 0.5 to 5 μM, as suggested by analytical chemistry measurements, the EC50 values for glutamate receptor desensitization strongly suggest that glutamatergic synaptic transmission strength in vivo might be less than one-half what it might otherwise be without steady-state desensitization.

Why would the brain constitutively cripple synaptic transmission? One possibility is that constitutive receptor desensitization provides a means for regulating synaptic strength. In basic principle, modulation of glutamatergic synapse strength by regulation of glutamate receptor desensitization is not different from other nervous system phenomena. For example, steady state receptor desensitization by ambient extracellular glutamate is analogous to steady state inactivation of voltage-gated channels by resting membrane potential. Steady state inactivation of voltage-gated channels is an important regulator of membrane excitability and synaptic transmission in many different tissues. For example, approximately two thirds of rat skeletal muscle voltage gated sodium channels are inactivated at a resting potential of -90 mV [85, 86]. Consequently, only one-third of muscle sodium channels are normally available for action potential generation. As a result, the number of functionally available sodium channels can be modified rapidly and reversibly by phosphorylation or interactions with modulators that shift steady-state inactivation. In presynaptic terminals, the functional availability of voltage gated calcium channels is a strong determinant of neurotransmitter release. It was recently shown that the synaptic protein rim1 interacts with presynaptic voltage gated calcium channels to reduce voltage gated calcium channel steady-state inactivation, therefore facilitating presynaptic calcium influx and increasing subsequent synaptic transmission [87].

If glutamatergic synapse strength were limited in vivo by steady-state receptor desensitization, then glutamatergic synapse strength would be highly regulated by anything that chances the EC50 of desensitization. The concentration dependence of steady-state receptor desensitization is likely modified by mechanisms known to regulate glutamate binding and desensitization kinetics, such as phosphorylation, or interactions with allosteric regulatory proteins such as TARPs (Transmembrane AMPA Receptor Regulatory Proteins) [88-96]. Glutamatergic synapse strength will also be modified by anything that changes levels

of ambient extracellular glutamate. Glutamate secretion under nonpathological conditions is usually attributed only to fusion of synaptic vesicles in neurons – e.g. synaptic transmission. But glia also secrete numerous transmitters, including glutamate [97-99], suggesting that glia may be an important point source for ambient extracellular glutamate. Glutamate secretion in astrocytes in particular has been relatively well studied, and involves calcium-dependent glutamate secretion mechanisms similar to those used by neurons [99]. However, ambient extracellular glutamate levels in brain are largely calcium independent and insensitive to TTX [12, 100-103]. In *Drosophila*, complete block of synaptic vesicle fusion does not affect suppression of ionotropic glutamate receptor function by ambient extracellular glutamate [104]. Therefore, ambient extracellular glutamate is likely mainly a product of nonvesicular glutamate release. Unfortunately, nonvesicular neurotransmitter release is still somewhat controversial, primarily because the molecular mechanisms remain poorly understood.

There are numerous recognized ways that glutamate can be released nonvesicularly in the nervous system, including swelling-activated anion channels, gap junction hemi-channels, purinergic (P2X) receptors, and cystine-glutamate exchangers [12, 105, 106]. Although still understudied, there is excellent evidence that cystine glutamate exchange, in particular, is a strong regulator of ambient extracellular glutamate and behavior.

Cystine-glutamate exchangers mediate 1:1 exchange between extracellular cystine and intracellular glutamate [107-112]. Functional xc- system transporters are heterodimeric transmembrane proteins composed of 'heavy' 4F2hc subunits, and 'light' xCT subunits [107, 113]. 4F2hc subunits are thought to regulate protein trafficking, and are utilized by several different types of amino acid transporter. xCT subunits, in contrast, are required for amino acid selectivity and transport, and are found only in xc- system transporters [107, 114-117].

Cystine-glutamate exchangers appear to be present in many types of cultured mammalian cell lines, and were originally assumed to function purely as a cystine-uptake mechanism for glutathione synthesis during oxidative stress [118-120]. However, in neurons and glia, the xc- transport system is dispensable for glutathione synthesis but rate-limiting for nonvesicular export of glutamate [12, 121]. (Neurons and glia do not need to rely on system xc- for cystine uptake because they contain large numbers of excitatory amino acid transport (EAAT) family proteins. EAATs are best known as sodium-dependent transporters for glutamate uptake, but EAATs also efficiently import cysteine -- the reduced form of cystine used in glutathione synthesis [6, 121-125]). Furthermore, mouse brain xCT is primarily expressed in meninges and circumventricular organs [112, 126], where it would largely contribute to the free glutamate content of cerebrospinal fluid (CSF) -- but not general cystine homeostasis in brain cells. Consistent with the idea that cystine-glutamate transporters play a role in glutamate homeostasis, subsequent pharmacological studies suggest that cystine-glutamate transporters are an important source of extracellular glutamate in rat brains [12, 127]. Specifically, Baker et al (2002) showed a 60% drop in extrasynaptic glutamate levels in rat striatum after application of cystine-glutamate exchange antagonists, while blockade of voltage-dependent sodium and calcium channels had little or no effect. Baker et al (2002) also showed that cystine-glutamate exchange activity in the striatum was negatively regulated by metabotropic glutamate receptor activation via camp-dependent protein kinase, consistent with the idea that ambient extracellular glutamate levels are actively maintained by the brain [12]. To verify these results genetically and pursue the possibility that cystine-glutamate exchange regulates ionotropic glutamate receptor function, Augustin et al (2007) identified and genetically deleted a *Drosophila* xCT gene [83]. Ambient extracellular glutamate levels in these

Drosophila xCT mutants dropped to approximately one-half normal [83], in agreement with the results from rats published by Baker et al. Consistent with the idea that ambient extracellular glutamate suppresses glutamate receptor function, the decrease in *Drosophila* ambient extracellular glutamate was also associated with a doubling of functional synaptic glutamate receptors [83].

If cystine-glutamate exchange regulates ambient extracellular glutamate, and ambient extracellular glutamate modulates glutamatergic transmission as argued above, then cystine-glutamate exchange function should also control behavior. It does. Baker et al (2003) showed that rats undergoing cocaine withdrawal had drops in ambient extracellular glutamate. Intracranial perfusion of cystine or systemic administration of N-acetylcysteine, which should increase cystine-glutamate exchange, normalized ambient extracellular glutamate levels and ameliorated cocaine-seeking behavior [127]. Modulation of xCT transporters also affected phencyclidine (PCP)-induced drug behavior [128]. In the *Drosophila* xCT mutants described by Augustin et al (2007), the mutated xCT gene was named 'genderblind' because mutants showed equal sexual preference for both male and female partners. Subsequent work verified that this genderblind phenotype was due to abnormally strong glutamatergic transmission, such that normally aversive or neutral male pheremones became attractive to other males (Grosjean et al, submitted). Indeed, this *Drosophila* homosexual behavior could be induced or reversed by altering glutamatergic synapse strength genetically or pharmacologically independent of xCT transporters. Thus, both mammalian and invertebrate studies are consistent with the idea that ambient extracellular glutamate, regulated prominently by cystine-glutamate exchangers, controls glutamatergic synapse strength and thus spatially modulates glutamatergic circuit function in vivo.

The idea that ambient extracellular glutamate might be stronger than electrical activity (e.g. LTP or LTD) or other relatively well-studied phenomena (like modulation of receptor trafficking) for control of glutamatergic synapse strength is somewhat of a neuroscientific heresy. It suggests that an understanding of brain computation is unlikely to be obtained by experiments performed in cell culture or in slice preparations outside any native chemical milieu. However, it's long been clear that the brain is a neurosecretory organ suffused with dozens or hundreds of signaling compounds that probably should not be ignored. The addition of glutamate to the list of neuromodulatory compounds circulating throughout the brain does not pose a fundamentally new challenge. Nevertheless, it is a challenge that must be faced if we're ever to truly understand brain function. We need to stop thinking of the brain as a wiring diagram, and more like the autoendocrine organ that it truly is. A good first step is understanding the regulation and role of ambient extracellular glutamate.

Conclusion

The exact concentration of ambient extracellular glutamate in the intact brain is somewhat controversial. Nevertheless, most measurements suggest that the concentration of ambient extracellular glutamate perfusing the intact brain is sufficient to cause constitutive desensitization of most glutamate receptors. Glutamate receptor desensitization renders receptors functionally silent and causes significant suppression of glutamatergic synapse strength. xCT-based transporters nonvesicularly secrete glutamate, and may be strong regulators of ambient extracellular glutamate in the brain. Consistent with this,

pharmacological or genetic manipulation of xCT function causes large changes in ambient extracellular glutamate, glutamatergic synapse function, and complex animal behavior. Despite these intriguing findings, ambient extracellular glutamate in the brain is still often assumed to be biologically insignificant and is therefore typically overlooked and understudied. This needs to change.

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Chapter 14

Glutamate and Ethanol: The Role of the Ionotropic NMDA Glutamate Receptors in Alcoholism

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Abstract

Glutamate is the major excitatory neurotransmitter of the brain, which acts at specific receptors located on the post-synaptic membrane of most CNS neurons. These receptors then exert their effect either *via* a G-protein-linked signalling cascade (metabotropic) or though the opening of an intrinsic ion channel (ionotropic).

The ionotropic glutamate receptors can be sub-divided into classes based on their affinity for selective agonists. The *N*-methyl-D-aspartate (NMDA) class of receptors ($GluR_{NMDA}$) are heteromeric receptors that are both ligand- and voltage-gated. The opening of the $GluR_{NMDA}$ ion channel is dependent on the binding of both glutamate and the co-agonist glycine, together with attenuation of the plasma-membrane potential to remove the Mg^{2+} block. Activation of $GluR_{NMDA}$ receptors leads to an influx of Na^{+} and Ca^{2+} ions into the cell, altering its membrane potential further toward the threshold for the generation of an action potential. The increase in intracellular Ca^{2+} also affects several Ca^{2+}-dependent signalling pathways.

Amino-acid transmission and the action of ethanol in the human brain are closely linked. Ethanol acts at the $GluR_{NMDA}$ to specifically alter its activity, especially by reducing Ca^{2+} influx. This interaction is influenced by the subunit composition of the receptor. Ethanol is thought to bind to the NR1 subunit, but this binding is altered by the identity of the NR2 subunits that make up the complex.

Under conditions of persistent ethanol exposure, such as in the alcoholic brain, $GluR_{NMDA}$ undergoes a process of adaptation. This adaptation is brought about by the regulation of the expression of the various subunits, leading to an alteration in the number and type of subunits that comprise the receptor complex. This process is notably influenced by the genotype and co-morbid pathology of the individual.

Here we review the effects of acute ethanol administration and long-term ethanol exposure on $GluR_{NMDA}$ sites. We also present recent research on the quantification of expression of $GluR_{NMDA}$ subunits.

Introduction

The amino acid glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). Glutamate is released into the synaptic cleft when the arrival of an action potential triggers pre-synaptic vesicular exocytosis. The increase in local glutamate concentration is detected by glutamate receptors present on the membrane of the postsynaptic neuron.

These receptors can be divided into two general groups, the metabotropic (mGluR) and the ionotropic (iGluR) glutamate receptors. The mGluR are G-protein-linked receptors that induce changes in the functionality of the postsynaptic neuron through activation of second messenger pathways. Dependent on sub-type, this leads to the modulation of the activity of phospholipase C or adenylyl cyclase. The mGluR are responsible for longer-term effects such as regulating neuronal excitability, synaptic transmission and plasticity [1].

The iGluRs, in contrast, respond to glutamate by opening an intrinsic ion channel that allows the influx of cations into the cell. This results in an increase in the membrane potential of the postsynaptic neuron, and subsequently to the generation of an action potential. The iGluRs can be further divided dependent on their response to the artificial agonists α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA), 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) or *N*-methyl-D-aspartate (NMDA).

The AMPA and kainate (non-NMDA) receptors mediate fast, short-lived responses to glutamate and are permeable to Na^+, K^+ and, dependent on the presence of key subunits, Ca^{2+}. These receptors are characterised by smaller conductance amplitudes, shorter open times, and a greater number of conducting states than the NMDA receptors.

The NMDA Glutamate Receptors

The response of NMDA glutamate receptors ($GluR_{NMDA}$) to glutamate is quite different from that of non-NMDA receptors. It is characterised by a voltage-dependent block mediated by Mg^{2+} binding within the ion channel. This binding is strongly voltage-dependent and is relieved by depolarisation of the neuron, such as through the activation of non-NMDA receptors. Removal of the block allows Na^+ and Ca^{2+} to flux into the cell, which further depolarizes the plasma membrane and also affects numerous Ca^{2+}-dependent signalling cascades.

$GluR_{NMDA}$ Receptor Subunits

Seven subunits for the $GluR_{NMDA}$ have been described; NR1, NR2A-D, NR3A and NR3B. The NR1 subunit is absolutely required for the formation of a functional receptor and is found in combination with NR2 and/or NR3 subunits. The stoichiometry of the complex has been the subject of some debate, but the consensus of opinion is that the $GluR_{NMDA}$ is a hetero-tetramer consisting of 2 NR1 subunits and 2 NR2 and/or NR3 subunits [2, 3].

The NR2 and NR3 subunits are encoded by distinct genes (GRIN2A-D, GRIN3A-B) where as the NR1 subunit is encoded by a single gene (GRIN1). However, NR1 undergoes

differential RNA splicing involving exons 5, 21 and 22, giving rise to eight different splice variants (exon numbering based on rat mRNA; see Figure 1).

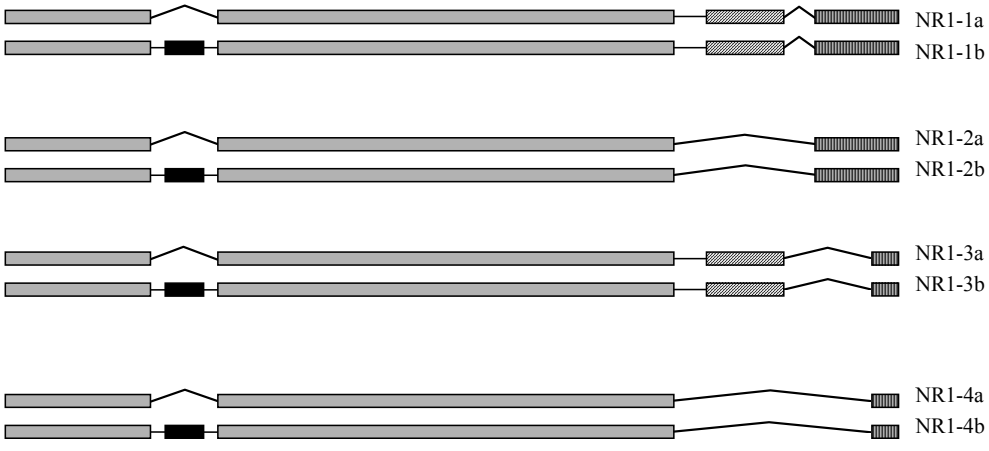

Figure 1. A graphical representation of the eight splice variants of GRIN1 highlighting the differentially spliced exons. Exon 5 – black; Exon 21 – diagonal stripes; Exon 22 – vertical stripes. Exon numbering is that of the rat mRNA. Adapted from [7].

The differing splice variants of NR1 demonstrate different pharmacokinetics and are further modulated by the influence of the NR2 or NR3 subunits present in the complex. Thus it can be seen that the combinatorial nature of $GluR_{NMDA}$ leads to a diversity in agonist and antagonist responses [4-7].

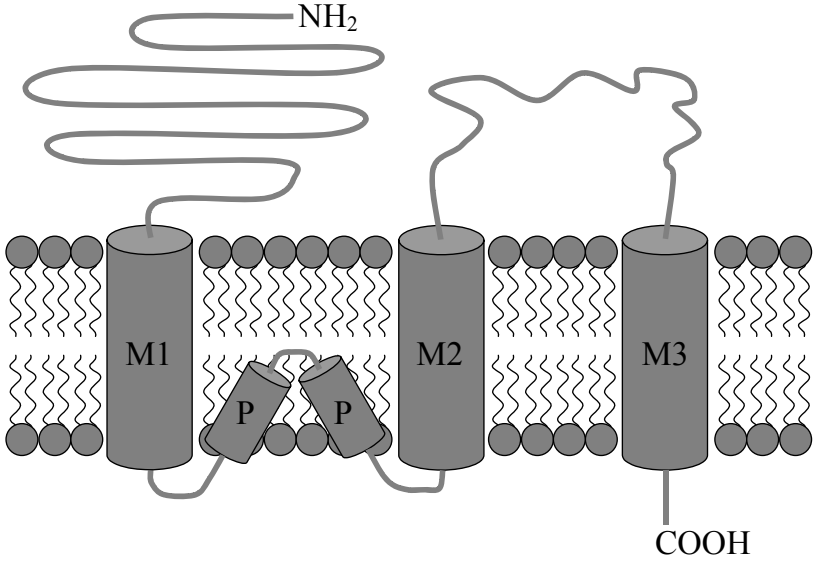

Figure 2. A graphical representation of the topology of an archetypal $GluR_{NMDA}$ subunit.

All of the subunits of $GluR_{NMDA}$ are thought to adopt a similar tertiary structure, with 3 trans-membrane helices (M1, 2 and 3) and a fourth hydrophobic domain that is thought to partially penetrate the membrane (P; see Figure 2). The amino-terminal domain and the agonist binding domain project into the extracellular milieu [2]. Initial predictions of the membrane topology of $GluR_{NMDA}$ suggested the presence of 4 trans-membrane helices, M1 refers to the same helix; however, some nomenclatures identify the second hydrophobic region as M2 (equivalent to P) and the third and fourth as M3 and M4 (equivalent to M2 and M3 respectively) [8]. For the purposes of this article the former nomenclature will be used.

Properties of $GluR_{NMDA}$

The $GluR_{NMDA}$ complex can bind a number of agonists, antagonists and other compounds that modulate its function. The binding parameters of these substances are dependent on the identity of the component subunits of the receptor complex. The intrinsic ion channel within the receptor is the site at which Mg^{2+} binds, giving the receptor its voltage-dependent properties. The compounds ketamine, phencyclidine and dizocilpine (MK-801) bind within the ion channel and thus block the influx of ions. The complex also has distinct, specific binding sites for its natural co-agonists glutamate and glycine: these sites are found on the NR2 and NR1 (and NR3) subunits respectively. The glutamate site binds NMDA. The receptor also contains a polyamine binding site, where binding of the polyamines spermine or spermidine can potentiate its function [1].

NR1/NR3-containing receptors act as excitatory glycine receptors: as this chapter concerns glutamate-mediated transmission they will not be discussed further. However, NR1/NR2-containing receptors bind both glutamate and glycine. This allows $GluR_{NMDA}$ to act as a coincidence detector, not only of glycine and glutamate, but also of an increase in plasma-membrane potential through the voltage-dependent Mg^{2+} block.

Physiological Role of $GluR_{NMDA}$

Many synapses have multi-component excitatory post-synaptic currents (EPSCs). The faster component is mediated by non-NMDA receptors and the slower component by $GluR_{NMDA}$, which has a slower rise time and a decay time some 100 times greater [9]. $GluR_{NMDA}$ are thought to play a significant part in long-term potentiation (LTP), plasticity, and development within the brain [10]. In addition, the large influx of Ca^{2+} *via* $GluR_{NMDA}$ affects many Ca^{2+}-dependent signalling pathways, leading to alterations in the levels of components such as protein kinase C (PKC), Ca^{2+}/calmodulin-dependent kinase, phosphatases, and phospholipases, to name a few [11].

The Action of Ethanol

The effects of ethanol in the brain are mediated by its action at $GluR_{NMDA}$ and GABA receptors. The effects at $GluR_{NMDA}$ can be divided into the acute, immediate effects of alcohol administration or consumption and the long-term effects of repeated alcohol intake.

Acute Effects

Ethanol, at mM concentrations, acts at $GluR_{NMDA}$ to attenuate LTP and inhibit hyper-excitability [12]. The effects on LTP have been proposed to be a mechanism by which alcohol-induced memory loss may occur [13]. The sensitivity of $GluR_{NMDA}$ to ethanol is regionally specific. NMDA-stimulated neuronal activity is inhibited in the inferior colliculus and hippocampus but not in the medial septum [14]. This variation in response is also found within neuronal cell-types. *In vitro* measurements on locus ceruleus neurons showed that NMDA-induced depolarization was only poorly inhibited by ethanol [15, 16], whereas ethanol was a potent inhibitor of NMDA-dependent norepinephrine release [17]. These data imply that there is a cell-level variation in the response to ethanol.

Despite a large body of research, the precise nature of the interaction of ethanol with $GluR_{NMDA}$ remains enigmatic. Whole-cell patch-clamp measurements demonstrated that NMDA-induced currents could be inhibited by a number of aliphatic *n*-alcohols with increasing numbers of C atoms from methanol to octanol [18]. Increasing the number of C atoms further (nonanol and decanol) no longer inhibited $GluR_{NMDA}$. These experiments also showed that the IC_{50} was inversely proportional to the number of C atoms, with heptanol having the smallest IC_{50} of 3.4 mM [18]. Further whole-cell patch-clamp measurements showed that the inhibition of $GluR_{NMDA}$ by ethanol was independent of voltage, pH, and the binding of NMDA, glycine, spermine, Mg^{2+}, Zn^{2+}, and ketamine [19].

As may be inferred from the combinatorial and diverse nature of $GluR_{NMDA}$, the response to ethanol is varied and dependent on subunit composition. Recently, molecular biological techniques and recombinant expression studies have been used to investigate the action of ethanol at $GluR_{NMDA}$. Subunit composition dramatically alters the extent to which ethanol is able to inhibit the generation of a glutamate-induced currents [20]. For NR1-2a subunits, the identity of the NR2 subunit had little effect on sensitivity to ethanol. However, for NR1-1a, -3a and -4a, similar levels of inhibition were only seen when they were in combination with NR2B, with all other combinations being less sensitive to ethanol. For NR1-1b and -2b, co-expression with NR2C showed the greatest sensitivity to ethanol, with the NR1-2b/NR2C combination showing the most sensitivity to ethanol of all NR1/NR2 combinations. The relative inhibition of $GluR_{NMDA}$ varied from 15% (NR1-1b/NR2C) to 55% (NR1-2b/NR2C), depending on combination. This study demonstrated that the ethanol sensitivity of $GluR_{NMDA}$ is not associated with the identity of single subunit [20]. Taken together these data indicate that ethanol acts at a distinct, separate site within $GluR_{NMDA}$.

Site-directed mutagenesis in combination with recombinant expression has been used in an attempt to identify the position of the postulated ethanol-binding site on $GluR_{NMDA}$. These studies have been based on single amino acid changes in the membrane-associated domains of the $GluR_{NMDA}$ subunits, and have indicated that, whilst the M2 domain of NR2A is involved in the response to ethanol, this domain does not directly bind ethanol [21]. However, the M3 domain of NR2A and the M2 and M3 domains of NR1 are thought to contribute to ethanol binding [22-25].

It can be seen that the effect of ethanol on $GluR_{NMDA}$ is determined by the intimate interactions of the subunits. This is particularly evident in the modulation of the response to ethanol and the apparent inter-subunit nature of the site of action of ethanol.

Chronic Effects

The macroscopic effects of long-term alcohol abuse are well documented. These can manifest in the living alcoholic from mild cognitive deficits to the Wernicke-Korsakoff syndrome, and other neurological defects. Further pathology can be observed in autopsy brain, where significant indices of alcoholic misuse can be found. These include a loss of white matter and a localized neuron loss, with neuronal density reduced by 22% in the frontal cortex but unchanged in the motor, temporal or cingulate cortex [26]. Neuronal death in alcoholics might be mediated by excitotoxicity, which can be brought about by dysregulation of the $GluR_{NMDA}$-mediated excitatory response.

Much effort has been made to elucidate the effects induced by long-term alcohol abuse. Many studies have used animal or cell-culture models to identify the molecular changes that occur. These approaches may be divided into several main paradigms: the constant drinking paradigm, where alcohol exposure is constant and maintained until end-point; the withdrawal paradigm, where alcohol exposure is constant but is terminated prior to end-point; and the repeated deprivation paradigm, where alcohol exposure is periodic and interspersed with periods of cessation. These models can be further divided according to differences in the amount (concentration) of ethanol available and to the timing of the end-point (during active drinking vs. during withdrawal period).

With repeated exposure to alcohol the functionality of $GluR_{NMDA}$ is increased, apparently as a mechanism to overcome the inhibitory effect of ethanol. This alteration can occur in a number of ways. The response to glutamate can be altered through post-translational mechanisms such as phosphorylation or subunit switching, or through more global mechanisms that lead to an increase in the number of $GluR_{NMDA}$.

The levels of expression of $GluR_{NMDA}$ subunits, and the phosphorylation state of those subunits, vary markedly with the alcohol-exposure paradigm used and the timing of sampling: before withdrawal, shortly after withdrawal, and after prolonged withdrawal.

Exposure of naïve dorsal striatum to ethanol causes an inhibition of LTP [27, 28], even at relatively low concentrations (10 mM) [28]. Higher concentrations of ethanol lead to the development of LTD [28] and can also lead to long-term facilitation (LTF) of $GluR_{NMDA}$, where the post-exposure EPSC increases to a level greater than the pre-exposure value [27]. The formation of LTF may be due to altered phosphorylation of the NR2B subunit: it correlates well with an increase in Fyn kinase, which can phosphorylate NR2B [27]. This observation has particular significance for the development of alcoholism, because transient exposure to ethanol may require further ethanol consumption to overcome the hyper-excitation caused by LTF [27]. The role of phosphorylation in the acute adaptation of $GluR_{NMDA}$ to ethanol was supported by the observation that there is a significant increase in the phosphorylation of Serine 897 of NR1 just 40 min post-infusion [29]. This phosphorylation correlates with the development of ethanol-induced tolerance to NMDA-dependent increases in blood pressure, and is eliminated by a PKA inhibitor and cAMP analogues, implying a role for cAMP-dependent protein kinases [29]. In contrast, there is a decrease in NR2A subunit phosphorylation in rat cortex in response to ethanol, even though NR2A subunit expression is increased. This was hypothesized to be due an alteration in the C terminus of the subunit [30]. Additionally, the experimental paradigm used strongly influences the degree of these changes. A constant drinking paradigm led to a large increase

in the NR1 subunits, in particular the NR1-1, -2 and -3 splice variants. However, a repeated deprivation paradigm, with end-point within an alcohol consumption period, showed little alteration in the levels of any NR1 variants [31].

During withdrawal, significant changes in the level and identity of the $GluR_{NMDA}$ and its subunits can be seen [32-35]. Animals subjected to a constant consumption paradigm followed by 12 or 36 hours of withdrawal showed no difference in [^{3}H]MK-801 binding immediately after withdrawal. However, a large increase in [^{3}H]MK-801 binding was seen 12 hours after withdrawal; this binding decreased by 36 hours, implying a transient increase in the overall number of $GluR_{NMDA}$ [32]. Quantification of $GluR_{NMDA}$ subunit mRNA and protein in cultured mouse cortical neurons also revealed significant changes in expression levels. Following both chronic and chronic-intermittent ethanol exposure, the levels of NR2B mRNA and protein rose significantly. After withdrawal, however, these levels decreased in the chronic exposure model but were maintained in the chronic-intermittent exposure model. In contrast, the NR1 subunit was not significantly affected at either the mRNA or protein level [33]. These observations are supported by immunological quantification of $GluR_{NMDA}$ subunits in rat hippocampal slices. There, a repeated deprivation paradigm showed little change in NR1, NR2A or NR2B levels 1 day post-withdrawal, but significantly higher levels of NR2A and NR2B subunits 7 days post-withdrawal with no change in NR1 [34]. Such alterations in $GluR_{NMDA}$ subunit levels may not only be paradigm-specific but also limited to subsets of neurons. In a constant drinking paradigm followed by 24 hours of withdrawal, *in situ* hybridization histochemistry showed little or no change in NR1 levels and no significant difference in the overall expression of any of the NR2 subunits. However, when GABAergic neurons were analysed separately, a significant increase in NR2A was observed [35].

It can be seen that ethanol exposure can dramatically alter the expression of the subunits of $GluR_{NMDA}$. An alteration in the levels of these subunits is associated with alteration in the morphology of neurons, where a constant drinking paradigm not only caused an increase in specific NR1 subunits (see above), but also led to an alteration in the dendritic structure of medium spiny neurons of the rat nucleus accumbens (NAcc) [31]. Low doses of alcohol can improve memory in a rat model; this is mediated by an over-expression of the NR1 subunit, particularly in the hippocampus [36]. These studies are starting to unravel the possible molecular basis for alcohol dependence, by showing that ethanol can cause long-term effects in the NAcc, an area of the brain associated with addiction. Ethanol may also alter memory, which could lead to stronger positive reinforcers for alcohol drinking behaviour.

The Human Alcoholic

Animal and cell-culture models have provided a wealth of information about the effects of ethanol exposure on $GluR_{NMDA}$. The mode of action of acutely administered ethanol seen in these studies is likely to be largely applicable to the human brain. The chronic effects of alcohol are more enigmatic. The pattern of ethanol exposure is a major determinant of the changes in $GluR_{NMDA}$ seen and these changes can be region-, cell-type-, and subunit-specific. It is therefore imperative that studies of the effect of long-term alcohol abuse on the $GluR_{NMDA}$ in the human brain are carried out.

The monitoring of $GluR_{NMDA}$ levels and subunit composition in the living brain would be a highly desirable paradigm to study the effects of ethanol. Imaging techniques such a

positron emission tomography (PET) can measure the relative levels of target proteins in the brain and have been used with some success in the study of addictive substances [37]. However, study of $GluR_{NMDA}$ has been hampered by the lack of suitable imaging agents. Some progress has been made recently with the development of a PET tracer specific for the glycine binding site of $GluR_{NMDA}$ [38] and a potential PET tracer specific for NR2B [39].

Assessing changes in $GluR_{NMDA}$ in autopsy brain tissue has several advantages over whole-brain imaging methods. The changes in multiple subunits can be assessed with relative ease in each subject, and can be related to pathology, brain area and genotype. For example, *in situ* hybridization histochemistry has been used to ascertain the spatial distribution and relative levels of the NR1 and NR2 subunits in post-mortem human brain [40].

A more accurate method for mRNA quantification is to use quantitative RT-PCR to determine the levels of the different $GluR_{NMDA}$ transcripts. Quantitative competitive RT-PCR has been used to quantify the relative levels of two isoforms of the $GABA_A$ receptor β subunit in human alcoholic autopsy brain tissue [41]. This study demonstrated the potential of such an approach for elucidating changes in expression due to alcoholism.

Recent work in our laboratory has used RT-PCR in combination with real-time PCR to quantify NR1, NR2A and NR2B subunit transcripts in superior frontal cortex and motor cortex from control, alcoholic and cirrhotic-alcoholic cases. We found a general reduction in all transcripts in both areas of the cirrhotic-alcoholic group compared with controls, although this reduction was not significant for NR2A (both frontal and motor) or NR2B (frontal). For alcoholics without comorbid disease NR1 and NR2A transcript levels did not change significantly, although there was a slight increase in NR2A levels in both areas. The NR2B subunit showed the most difference, where the level found in the frontal region was demonstrably higher than in the homotropic area of controls. However, there was no effect in motor cortex, where only a small increase was seen. Thus, the NR2B levels in the two regions differed significantly [42].

Conclusion

The $GluR_{NMDA}$ mediates many of the acute effects of ethanol. The interaction of $GluR_{NMDA}$ with ethanol is dependent on both the NR1 and NR2 subunits.

Long-term ethanol exposure has a marked effect on the response and expression of $GluR_{NMDA}$, through a number of mechanisms. These can be rapid, such as changes in phosphorylation, or slow, by altered expression.

In animal experiments the alcohol exposure paradigm has a strong influence on longer-term effects, such as changes in subunit levels. Therefore, while animal models provide an avenue for identifying changes that may occur, and the biochemical and physiological consequences of these, it is difficult to determine which paradigm, if any, best models the effects of alcohol consumption in alcoholics. Hence, animal models must be complemented with human studies.

The study of human autopsy brain tissue continues to yield critical data on the long-term changes that occur in response to alcohol misuse. These data will be enhanced by advances in our ability to determine the identity and abundance of $GluR_{NMDA}$ and its various subunits.

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Chapter 15

Chemoreception of Umami in Caterpillars

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Abstract

Umami is a human taste quality, in most cases produced by some amino acids, such as glutamic acid and aspartic acid. This chapter briefly introduces to the concept of umami taste, presents current status of the research on umami in mammals and in larvae of Lepidoptera (caterpillars), speculates whether or not umami is a separate taste modality in caterpillars as it is in humans or rodents, and (using the case of the codling moth, *Cydia pomonella* L., as an example) delineates the directions for using comparative taste physiology in rational plant protection.

Introduction

Taste begins with chemical stimuli acting on specialized cells of chemoreceptive anatomical structures, for instance taste buds in humans or contact chemoreceptors in insects. There is a body of evidence that, in mammals, different chemical compounds influence membranes of these cells in different manners and signals from different chemicals are often transduced via separate cellular mechanisms. This fact supports ancient ideas of Democritus, Aristotle and Plato that taste can be "divided" into relatively small number of discrete sensations that today are called primary (or cardinal) tastes. In twentieth century thought on taste was dominated by a concept of Hans Henning, who proposed that, in humans, every taste was composed of mixture of four primary tastes: sweet, sour, bitter and salty, and visualized these primary taste stimuli as the apices of a hollow tetrahedron - Henning's tetrahedron.

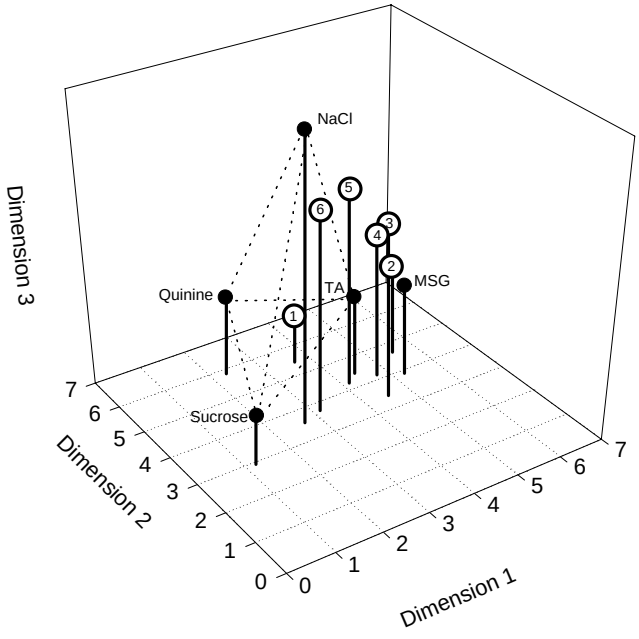

Figure 1. Three-dimensional geometry of human taste. Four primary tastes (sweet, sour, salty and bitter) constitute apices of Henning's tetrahedron (solid points, dotted lines). The taste of grapefruit juice (1) is represented as a combination of the four primary tastes, and – consequently- falls into the *inside* of the tetrahedron. MSG is represented by a solid point *outside* of Henning's tetrahedron. Stimuli produced by other foods classified as umami, such as skipjack tuna (2), pork (3), chicken (4), beef (5) or mushrooms (6), also fall outside of Henning's tetrahedron. Collated and modified from [39] and [52]. Analogical information for a caterpillar of any species is lacking.

A basic idea associated with Henning's tetrahedron was that every taste sensation could be represented by a single point inside or on the surface of this tetrahedron. This idea could be experimentally verified by presenting a human subject first with a sample containing a substance of a primary taste, next with a sample containing tested substance, and requesting the subject to judge how much these two samples are similar in quality. This procedure is repeated for each primary taste. Similarity ratings are subsequently subjected to multidimentional scaling which yields a spatial configuration reflecting the similarities among the taste of tested substance and each of four primary tastes represented by the apices of Henning's tetrahedron (Figure 1). Indeed, the taste of many foods (e.g. grapefruit juice) can be represented by points in Henning's tetrahedron if examined that way. However, experiments of Yamaguchi [52] who used the same methodology to study taste of certain meats, fish, mushrooms, and some vegetables, showed that there are natural foods which may be only represented by points *outside* of Henning tetrahedron (Figure 1). Amino acid salts, such as monosodium glutamate (MSG) and foods rich in free amino acids, such as pork, beef, chicken, skipjack tuna, and mushrooms fall into this category (Figure 1). Yamaguchi [52] therefore proposed that a fifth primary taste, represented by MSG, exists. The idea that glutamate and its salts elicit a unique taste was not new at that time. Almost eighty years earlier, Ikeda [18] extracted glutamate from dried kelp and named its taste "umami", which means "delicious" in Japanese.

Umami Chemoreception in Mammals

Several classes of compounds are described by humans as umami; (i) amino acids and their salts such as L-Glutamate (L-Glu), monosodium glutamate (MSG), and L-Aspartate (L-Asp); (ii) inosine and guanosine derivatives, such as inosine 5'-monophosphate (IMP); and guanosine 5'-monophosphate (GMP); (iii) certain short peptides; (iv) glutamate receptor agonists (e.g. L(+)-2-Amino-4-phosphonobutyric acid, L-AP4); and (v) some organic acids [40, 41]. How umami substances are perceived by mammalian chemoreceptors is a subject of debate. Although it is generally accepted that umami substances excite G-protein- coupled membrane receptors (GPCRs) of mammalian taste cells, different receptors and different signal transduction mechanisms are proposed by researchers.

A member of the metabotropic glutamate receptor family, taste-mGluR4, was shown to function as a taste receptor for umami [8]. Taste-mGluR4, which was cloned from rat taste buds, is a truncated variant of brain-mGluR4. (In taste-mGluR4 about 50% of extracellular domain is lacking, which – according to the authors- explains why much higher concentrations of glutamate are needed to excite taste-mGluR4 in comparison to brain-mGluR4). Chaudhari et al. [8] also showed that taste-mGluR4 decreases cytosolic cAMP levels upon excitation by L-Glutamate. It is not clear how taste-mGluR4 is linked to decrease in cAMP concentration, but there is behavioral evidence that a taste-specific G-protein, Gα gustducin, plays a role here since Gα gustducin- knockout mice do not perceive umami [42]. Terminal steps of cell response to umami via taste-mGluR4 remain speculative. It is proposed that stimulation of protein kinase A and inhibition of potassium channels lead to cell depolarization [40]. One the other hand, Nelson et al. [27] and Zhao et al. [54] argue that two rodent GPCRs (T1R1 and T1R3) combine to form a broadly tuned L-amino-acid receptor, the excitation of which elicits umami taste. The T1R1 + T1R3 heterodimer, upon binding MSG, is believed to stimulate phospholipase C (PLC). Stimulation of PLC, in turn, leads to calcium release from internal calcium stores via stereotypic 1,4,5-triphosphate (IP_3)- dependent pathway which gates a taste-transduction membrane channel allowing sodium influx to the cell and its depolarization [6, 53]. It is not clear how PLC is stimulated, but – again - a role for Gα gustducin is postulated here. Human T1R1 and T1R3 were cloned and functionally expressed showing response to substances reported to elicit umami taste in humans: L-Glu, L-Asp and L-AP4 [21]. I am not aware of any report of cloning or functional expression of human taste-mGluR4.

Umami Chemoreception in Caterpillars

Although chemoreception of amino acids in insects has attracted attention of some researchers, reports on umami chemoreception in caterpillars are scarce. Only two umami substances, L-Glu and L-Asp, were systematically studied, using two different experimental approaches.

In one approach, a modification of standard electrophysiological equipment described by Miller [25] was used. A glass or plastic capillary suction microelectrode (active electrode) was filled with tested umami substance plus an electrolyte and placed over the tip of insect chemosensory organ (sensillum). The low-resistance reference electrode was placed directly

in caterpillar's body (sometimes the caterpillar's head was cut off and mounted directly on the reference electrode), and entire preparation was clamped to an amplifier and a recording device. Details and variants of this system are described elsewhere [13]. This standard and uniform methodology allows verification whether or not tested umami substance is sensed at all, identification of umami- sensitive chemosensory organs, and comparison of the results among various species that may have different feeding habits. However, it does not provide any information about functional significance of the neural impulses produced by the umami stimulus.

In another approach, larvae were exposed to residues of umami substances on a feeding substrate, and their behavior was recorded and quantified. Here, most commonly, feeding intensity was estimated by measuring the amount of feeding substrate consumed. In one case mass of fecal pellets was used as feeding intensity estimator. In some studies, preference of treated feeding substrate over control food was measured (Table 1).

The behavioral approach allows evaluation of whether or not tested umami substance has phagostimulatory or phagoinhibitory properties, but the results obtained with such methods are difficult to compare among various species. The caterpillars of different species may have different feeding habits, different dynamics of habituation or sensitization to particular stimuli, or different circadian patterns of feeding and sensory physiology. Moreover, there was no uniformity among the researchers as far as the carrier for tested umami substances is concerned. Some researchers used neutral feeding substrate, such as foamed polystyrene [2], others used artificial diets spiked with tested substances [20], and in some research natural feeding substrates [17] were used. All these factors must be accounted for in inter-specific comparisons of the previous studies on umami in caterpillars. In most cases such a comparison is either very difficult or simply impossible.

Additionally, to identify the taste stimuli, the researcher must discriminate between pre- and post-ingestive effects of tested chemicals on feeding behavior: the chemicals which modify probing of the feeding substrate or induce feeding are likely to be taste stimuli, whereas the chemicals which simply increase food intake or fecal pellet production by the larvae may be not taste stimuli, but the factors which, upon ingestion, are sensed by the gut or central nervous system and alter food intake mechanisms of the caterpillar by direct or indirect actions on hunger controlling centers. The latter precautionary measure is particularly important in studies on umami substances, since these are known to be not only caterpillar taste stimuli [34, 44], but also insect neurotransmitters [50].

Table 1 shows an inventory of caterpillar species which exhibit gustatory responses to umami substances. Responses to L-Glu or L-Asp were mostly investigated. Caterpillars of at least fourteen species do sense L-Glu or L-Asp or both (Table 1). One species, codling moth, *Cydia pomonella* (L.) responds to L-AP4, additionally. Eight species were studied using electrophysiological methods. In the remaining six species, modification of feeding behavior in response to umami was observed and quantified. Lack of a robust and rigorous physiological analysis of umami taste in caterpillars is striking. In most of the studies, the effects of umami substances were tested at one concentration only and no dose-response curves were shown, which does not allow a speculation as to the threshold values for gustatory stimulation among the species. Information about possible inhibitory effects of extremely high concentrations of tested substances is also missing, for the same reason.

Table 1. Caterpillars in which umami substances produce gustatory responses.

Species	Umami substance tested			Method	Refs.
	Glutamate	Aspartate	L-AP4		
Estigmena acrea (Drury)	+ (saturated)	O (saturated)	n.t.	E	12
Malacosoma americana (Fabr.)	+ (saturated)	+ (saturated)	n.t.	E	12
Danaus plexippus (L.)	+ (saturated)	+ (saturated)	n.t.	E	12
Papilio polyxenes (L.)	+ (saturated)	+ (saturated)	n.t.	E	12
Calpodes ethlius (Stoll)	+ (saturated)	+ (saturated)	n.t.	E	12
Grammia geneura (Strecker)	+ (5 mM)	O (5 mM)	n.t.	E	5
Pieris brassicae (L.)	+ (10 mM)	+ (10 mM)	n.t.	E	22
Pieris rapae (L.)	+ (10 mM)	+ (10 mM)	n.t.	E	22
Pyrausta nubilalis (Hubn.)	+ (20 mM)	O (20 mM)	n.t.	Ba	4
Spodoptera littoralis Boisd.	+ (12.5 or 125 mM)	O (12.5 or 125 mM)	n.t.	Bb	2
Choristneura fumiferana (Clem.)	+ (2 mM)	+ (20 mM)	n.t.	E	28
Choristneura fumiferana (Clem.)	+ (2 or 20mM)	+ (20 mM)	n.t.	Bb,Bc	1, 17
Choristoneura rosacea (Harris)	+ (6 to 60 μM)	n.t.	n.t.	Bc	36
Uraba lugens (Walker)	+ (50 mM)	+ (50 mM)	n.t.	Bd,Be	9
Cydia pomonella (L)	+ (0.06 to 0.6mM)	+ (0.6 to 6mM)	+ (0.5 to 5 mM)	Bc,Bf	24, 34, 37 38

Concentrations of tested umami substances are given in parentheses
+ response occurred
O no response found
n.t. not tested
Methods used: (E) electrophysiological study of chemoreceptors, (Ba) feeding preference in a choice assay on artificial carrier of tested substance, (Bb) feeding intensity on artificial carrier of tested substance, (Bc) feeding intensity on natural feeding substrate, (Bd) feeding induction on artificial feeding substrate, (Be) fecal pellet counts, (Bf) feeding induction on natural feeding substrate

Electrophysiological analysis of gustatory response to umami was accompanied by behavioral assays only in one case [1, 28], thus, although we know that larval chemosensillae of eight species do respond to umami, we still do not know if and how this response may modify feeding behavior in seven out of eight investigated species. Also, in four studies using behavioral methods [1,2,17,36], gustatory response to umami was measured in a way which did not discriminate between pre- and postingestive effects of tested substances, which raises additional questions as to the nature of the response observed. In some cases, ingested umami substances could have had modulatory effects on hunger controlling centers rather than have produced a typical chemosensory response, via taste cells at the input.

Perhaps the most extensive bioassay- based studies on umami chemoreception were done in neonate larvae of the codling moth. In this subject, employing the electrophysiological methods was impossible: the neonate is approximately two millimeters long, and its small, soft body makes it impossible to use currently available electrodes due to rapid desiccation of the larva upon puncture. (Procuring a non-destructive reference electrode is a problem that must be solved). However, properly established behavioral assays are informative enough.

Although codling moth larvae are internal fruit feeders, the females of this species lay a vast majority of eggs on foliage, not fruit [19], and it is the newly hatched neonate larva that ultimately decides which fruit is to be infested. Moreover, neonate larvae of the codling moth exhibit high potential for dispersal in their pursuit for host fruit [32]. Because gravid females of the codling moth have remarkable and inheritable potential for migration [45, 46] and are not discriminative in their selection of oviposition substrate [3, 10, 15], between-generation predictability of oviposition and food resources is low in this species. Once having infested the fruit, however, the codling moth larva never leaves it until pupation [16, 49], thus codling moth environment has high degree of within-generation predictability. In such a situation learning of host plant choice is especially beneficial to an animal with discrete generations [48], and indeed, codling moth neonates learn how to choose host fruit through collecting gustatory stimuli just before the choice of the fruit is made. Gustatory stimuli are collected by feeding on the foliage and used either for inducing food preference or for conditioning food aversion. A combination of these two forms of single-experience learning provides a basis for ultimately making the decision which fruit is to be infested [32]. Codling moth neonates probe foliage and collect gustatory stimuli, then disperse in search of fruit, and – finally – infest the fruit which produces gustatory stimuli similar to those collected on foliage [32]. Because L-Glu and L-Asp constitute up to 80% of total free amino acids in apple peel [23] one may expect that ability to perceive umami on foliage or fruit, or both, is essential to the neonate in host fruit acceptation or rejection.

Feeding on foliage by codling moth neonates has two distinct phases, regardless whether the larvae are exposed to feeding stimulants or not. During the first phase, the larva explores the foliage surface and starts feeding after some time. During the second phase, feeding continues with variable dynamics, depending on the time of the day [34]. Both the time of feeding commencement and the amount of leaf tissue consumed after a fixed number of hours could be measured, and serve as parameters of feeding behavior [30, 31, 33, 34]. Monosodium glutamate (MSG) was the most extensively studied umami substance in terms of its effects on feeding of codling moth neonates [30, 34, 37]. Exposure to MSG affects both phases of feeding in the neonates, accelerating feeding commencement by approximately one hour and increasing foliage consumption almost twofold [30, 34, 37]. Interestingly, these two

phases of feeding behavior appear to be mediated by two different sets of glutamate receptors (GluRs), which likely share their pharmacological properties with vertebrate GluRs.

In vertebrates, GluRs divide into two distinct families; ionotropic glutamate receptors (iGluR) directly coupled to calcium channels in the plasma membrane, and metabotropic glutamate receptors (mGluRs) activating G-proteins and related second messenger systems, a process which leads to opening calcium channels on internal calcium stores (e.g. endoplasmatic reticulum). Exposure of the neonates to the residues of a broad-spectrum agonist of vertebrate mGluRs accelerates feeding commencement, but does not affect the amounts of consumed foliage. On the other hand, a specific agonist of vertebrate iGluRs, *N*-methyl-D-Aspartate, NMDA, or calcium chloride residues have no effect on the time of feeding commencement, but both increase amounts of consumed foliage [35]. Moreover, stimulatory effects of NMDA are abolished at presence of calcium chelator, EDTA [35]. These results suggested that MSG induces feeding via metabotropic, rather than ionotropic GluRs, and that codling moth taste receptors which perceive umami substances while the larva is probing the feeding substrate share their pharmacology with vertebrate GluRs. This assumption was supported by a later study, where codling moth feeding on foliage was induced with selective agonists of vertebrate mGluR subtypes [37]. Three vertebrate mGluR agonists were used; (1S,3R)-1-Aminocyclopentane-1,3-dicarboxylic acid, (1S,3R)-ACPD, which stimulates group I mGluRs; (2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate (2R,4R)-APDC, which stimulates group II mGluRs; and L-AP4, which stimulates some group III mGluRs. L-AP4 is also known to elicit umami taste in humans [21, 40]. All these agonists induced precocious feeding in codling moth neonates, but in each case, a different signaling pathway modulator reversed their stimulatory effects [37]. Feeding stimulatory effects of (1S,3R)-ACPD were reversed by phospholipase C inhibitor, U 73122, but remained unaffected by adenylate cyclase activator, NKH 477, or phosphodiesterase E inhibitor, Rolipram. (For systematic IUPAC names of these specific signal transduction modulators please see the footnote of Table 2). (2R,4R)-APDC induced feeding in presence of U 73122 or Rolipram, but lost its feeding stimulatory effects in presence of NKH 477. L-AP4, in turn, did not induce feeding in presence of Rolipram, but maintained its feeding stimulatory effects in presence of U 73122 or NKH 477.

There are three findings worth of emphasizing here. First, these results suggest that the receptors used for probing the feeding substrate by codling moth neonates in presence of umami substances are GPCRs that share pharmacological characteristics with their counterpartners found in vertebrates (group III mGluRs). Second, mGluR agonists that stimulate groups I and II of vertebrate mGluRs also initiate leaf consumption in the caterpillar. mGluRs belonging to group I or II have not been reported previously either for vertebrates or for invertebrates in the context of taste. It seems that a unique set of GluRs is involved in taste perception by codling moth larvae. Third, the experiments on the effects of specific mGluR agonists combined with drugs specifically affecting signal transduction suggest that each tested mGluR agonist (and – likely- each subtype of mGluRs involved in perceiving umami taste) uses a different signaling pathway as the insect probes the leaf surface.

Table 2. Pharmacological analysis of umami taste in codling moth caterpillars

Umami substance tested	Signal transduction modulator			Reference
	U 73122 [a]	NKH 477 [b]	Rolipram [c]	
MSG	o	---	--	39
L-Asp	o	--	--	25
L-AP4	o	o	---	39

[a.] 1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)- 1H-pyrrole-2,5-dione, a phospholipase C inhibitor

[b.] N,N-Dimethyl-(3R,4aR,5S,6aS,10S,10aR,10bS)-5-(acetyloxy)-3-ethenyldodecahydro-10,10b-dihydroxy-3,4a,7,7,10a-pe ntamethyl-1-oxo-1H-naphtho[2,1-b]pyran-6-yl ester b-alanine hydrochloride, a potent adenylate cyclase activator

[c.] 4-(3-cyclopentyloxy-4-methoxy-phenyl)pyrrolidin-2-one, a phopsphodiesterase E inhibitor

Effects: (o) no effect, (--) moderate inhibitory effect, (---) strong inhibitory effect

However, not all three signaling pathways are used in perceiving umami. NKH 477, or Rolipram abolished MSG- caused acceleration of feeding commencement, but U 73122 did not affect MSG- stimulated feeding [37]. In codling moth caterpillars, MSG probably induces feeding via group II and III mGluRs, using two pathways which differ at their initial steps; one likely depends on adenylate cyclase, whereas the other likely uses phosphodiesterase E. Both pathways likely converge downstream, and ultimately respond to MSG by decrease in cAMP concentrations [37]. MSG does not appear to signal through PLC in codling moth neonates. Therefore, it seems that codling moth neonates respond to MSG using GPCR coupled to a cAMP- based signaling system. Codling moth neonates likely respond to MSG in a manner similar to that postulated by Chaudhari et al., [8] for vertebrates. Codling moth neonates probably do not use a PLC- coupled homologue of T1R1 + T1R3 heterodimer to perceive umami, as vertebrates seem to do in some experiments of Nelson et al. [27] and Zhao et al. [54].

Preliminary experiments on feeding induction in codling moth caterpillars by L-Asp yielded quite similar results. L-Asp accelerated feeding by approximately 45 minutes, and these stimulatory effects were abolished by NKH 477, and Rolipram, but not by U 73122 [24]. Pharmacological analysis of gustatory response to umami substances in codling moth caterpillars is summarized in Table 2.

Is Umami a Separate Taste Modality in Caterpillars?

Codling moth neonates do perceive umami substances, and they likely use specific receptors to do so. Moreover, these receptors share their pharmacological properties with vertebrate taste-mGluRs. The question should be posed, whether or not umami is a separate taste modality in caterpillars, as it is in humans and rodents. Originally, such a hypothesis was studied in humans using psychophysiological tests and multidimentional scaling [52] explained in the Introduction. For obvious reasons, psychophysiological tests for similarity requiring verbal reports on perceived similarity or dissimilarity of two subsequently probed substances can not be employed in animals. However, in those animals which can learn to prefer or avoid certain tastes, one can verify, by behavioral tests, whether or not one taste

modality resembles another. In one procedure (conditioned taste aversion), rats are presented with a taste stimulus produced by a substance 1 and injection of lithium chloride to induce gastric distress. This procedure produces a conditioned aversion to the substance 1 [7, 14]. Next (after recovery from any ill effects of the conditioning procedure) the rats are presented with choice between the substance 1 and a substance 2, and preference of one substance to another is recorded (licking ratios were used by Stapleton et al., [47] as an estimator of the reaction to the substance 2). If, the rats conditioned to avoid the taste of the substance 1 also avoid the substance 2 (the conditioned aversion to the taste 1 generalizes to the taste 2), these two substances taste similar to the animals. If the rats prefer the substance 2 to the substance 1, the opposite is true. Using this methodology Stapleton et al., [47] demonstrated that in the rat the taste of MSG generalized to L-Asp and vice versa. In another study, with the same method, Chaudhari et al., [7] demonstrated that in rats taste aversion to MSG generalizes to L-AP4, and Nakashima et al., [26] showed the same for mice.

Interestingly, in the rat, conditioned aversion to MSG or L-Asp did not generalize to NMDA, and vice versa, showing that in this rodent, as in codling moth neonates, pre-ingestive effects of umami are mediated by metabotropic rather than ionotropic GluRs [47]. These results were further supported by another behavioral analysis of umami taste in the rat, where the animals were subjected to taste discrimination operant conditioning with either positive reinforcement or positive punishment. In brief, taste stimulus 1 was administered and followed by delivery of small quantity of drinking water (positive reinforcement) and this procedure was followed by delivery of taste stimulus 2 and subsequently – a weak electric shock (positive punishment) to the animal's tongue (see [11] for more details). Again, the rats conditioned this way did not distinguish between the taste of MSG and that of L-Asp, or between the taste of MSG and that of L-AP4 [11].

One can train codling moth neonates to avoid or prefer certain tastes. Either of two training paradigms could be used here: conditioned taste aversion or food preference induction [32]. The former paradigm does not differ between rats and codling moth neonates, only deleterious foliage of *Ginko biloba* is used to produce gastric distress instead of lithium chloride. Next, the neonates are allowed to feed on untreated foliage for 24 hours (to assure recovery from exposure to Ginkgo) and presented a choice between two apples or two pieces of foliage, one treated with saccharin and the other treated with solvent. Almost all larvae conditioned to avoid saccharin by pairing its taste with Ginkgo – avoid saccharin- treated food [32]. Food preference induction is specific to insects, and probably is a kind of operant learning with positive reinforcement. Here, the neonate is presented with a taste stimulus which likely is a reward itself. Next, the larvae are allowed to spend a few hours feeding on untreated foliage, and then are presented a choice of two pieces of food, one treated with saccharin and one treated with solvent. Almost 100% larvae conditioned by food preference induction choose saccharin- treated food [32]. Either of these two training paradigms in combination with a choice assay may be used for assessing dissimilarities between saccharin and other taste modalities in codling moth neonates.

Although the research here is still in its initial stage, preliminary results suggest that the neonates distinguish the taste of saccharin and that of MSG. Twenty larvae were conditioned to avoid the taste of saccharin (as described above), and presented a choice in between two sections of apple leaf, one section treated with saccharin and the other treated with MSG, in a small circular arena. Nineteen larvae still avoided saccharin- treated foliage, suggesting that the taste of MSG is not confused with that of saccharin. In another experiment, 20 larvae were

conditioned to prefer saccharin by induction of food preference (as described above), and presented a choice in between saccharin- and MSG- treated food in aforementioned choice test. Fifteen larvae preferred saccharin- treated food, suggesting that despite different conditioning paradigms had been used, the tastes of MSG and saccharin were not confused one with each other. Thus, it is likely that saccharin and MSG represent two different taste modalities in codling moth neonates. More experiments are needed to confirm this hypothesis. Whether or not codling moth neonates discriminate between the taste of MSG and that of L-Asp or L-AP4 has not been studied.

How to Utilize the Knowledge of Umami Taste in Caterpillars? The Case of the Codling Moth

Codling moth is a cosmopolitan pest which primarily attacks apples, a commodity which, worldwide, is worth more than 15 billion US dollars annually. In most locations there are two or three full generations of this pest each growing season, causing problems every year. Growers can seldom tolerate losses exceeding 0.4%. In unmanaged orchards fruit infestation by codling moth exceeds 80%, which potentially translates to global losses exceeding 10 billion US dollars annually. (For comparison, annual gross domestic product of more than 70 countries in the world, including a member of European Union, Malta, is lower than 10 billion US dollars).

Because codling moth larvae infests apples within 12 hours after hatching from eggs, and stay inside of the fruit until their development is complete, growers have limited measures to control this insect. Codling moth is currently controlled by excessive use of broad-spectrum insecticides such as azinphosmethyl (which has to be used at rates exceeding one pound per acre due to increasing resistance of the pest) or neonicotinoids (which may pose a danger to pollinators, such as bees). There are attempts to employ more environmentally friendly insecticides such as *Bacillus thuringiensis*, but efficacy is too low to provide satisfactory control. The problems associated with use of broad- spectrum insecticides to control this pest, including environmental pollution and danger to human health, will likely escalate.

So far, only mating disruption and attract-and-kill technology serve as alternatives to broad-spectrum insecticide use for codling moth control in apple orchards. Mating disruption, however, is effective at low population density and does not solve problems caused by gravid codling moth females migrating to the orchard from neighboring areas [43, 51] which is typical of small apple orchards in patchy landscape common for apple production worldwide. Attract-and-kill technology is inefficient against pesticide-resistant populations of codling moth [29].

There are no well working control measures against codling moth Other than conventional spraying with broad-spectrum insecticides, there are no approaches that work. Alternate approaches that provide a supplementary strategy of control are greatly needed.

One possible strategy would be development of such apple varieties, which produce fruit unattractive to codling moth larvae, but still attractive and appealing to the consumer (i.e. to the human). This could be achieved by genetically modifying the apple, rationally breeding a new variety, or finding an existing variety among many ones being cultivated in apple repositories worldwide. In either case, before such an effort is made, a question should be

posed whether codling moth larvae discriminate basic tastes (sour, sweet, salty and bitter) and their combinations in the same way as humans do. If the answer is "yes" production of the apple which is not tasty to codling moth and tasty to humans is impossible. But if there are dissimilarities between cardinal taste representations in humans and those of codling moth neonate larvae, such an effort could be rationally proposed and directed. Answering the question whether or not codling moth neonates distinguish umami as a separate taste modality would be the first step toward this goal.

Conclusion

Despite general and practical significance, the taste of umami in caterpillars is, unfortunately, a heavily underinvestigated subject. However, there are caterpillars (some of them belonging to economically important species) which exhibit gustatory responses to the substances described by humans as umami. Further studies on these species will require standardization of the bioassays used for examination of gustatory responses to umami before any comparative analysis could be made. The study on the codling moth shows that the pharmacology of cellular events underlying chemoreception of umami in humans and caterpillars may be similar, thus the receptors and signal transduction pathways involved in perception of umami may share basic properties between insects and the man. The question arises whether or not umami is a separate taste modality in caterpillars. Answering this question will delineate the directions for rational crop protection with minimum or no chemistry involved.

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Chapter 16

Glutamate Synapses in Olfactory Neural Circuits

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Abstract

The amino acid glutamate is the major excitatory neurotransmitter in the brain. Neuronal interactions and signaling in the olfactory system are mediated by a variety of neurotransmitters, principally including glutamate. In the main olfactory bulb (MOB) of the brain, glutamate acts at multiple levels of synaptic processing. At the level of sensory input to the MOB glutamate is released from olfactory nerve terminals into olfactory glomeruli. Glomeruli are structural and functional odorant receptor-specific units that serve as the initial sites of synaptic processing in the olfactory system. They house the dendrites of projection neurons and local interneurons, juxtaglomerular cells. Projection neurons of the MOB, mitral and tufted cells, are glutamatergic themselves, and glutamate released from their dendrites acts to mutually excite and couple the activity of output cells within the same glomerular unit. Glutamate released from these neurons also mediates dendrodendritic transmission at synapses with juxtaglomerular cells and with GABAergic granule cells deeper in the MOB, controlling MOB output to higher centers of the brain. At this level of processing, axons of centrifugal fibers originating in olfactory cortical areas in turn provide spatially segregated glutamatergic input to the MOB.

New experiments in the MOB have used tract tracing, recordings of field potentials, patch-clamp recordings, microdissection, flash photolysis, two-photon-guided local stimulation, and optical-imaging techniques. These experiments describe novel cellular and network olfactory mechanisms that process odor-specific activity patterns elicited by activation of glutamatergic inputs to MOB neurons. Neuronal activity in the MOB is controlled by glutamate acting at ionotropic α-amino-3-hydroxy-5-methyl-4-

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isoxazolepropionate (AMPA) receptors, kainate receptors, and N-methyl-D-aspartate (NMDA) receptors, as well as metabotropic glutamate receptors coupled to intracellular second messengers. Glutamate activates serial excitatory and inhibitory neural circuits in the MOB such that olfactory processing is regulated through interplay of responses to excitatory glutamate receptors and inhibitory neurotransmitter receptors. Olfactory coding is both spatially and temporally dynamic: glutamate receptors are a key element generating the rhythmic, correlated activity within the MOB that underlies the neural encoding of odors.

Introduction

The focus of this review is on the amino acid glutamate as the principal excitatory neurotransmitter in the MOB of the brain, the first central relay station for olfactory processing. Glutamate binds to receptors that are broadly classified as ionotropic or metabotropic. Ionotropic glutamate receptors (iGluRs) act through ligand-gated ion channels, and are divided into AMPA/kainate receptors and NMDA receptors. NMDA receptor activation also requires that D-serine or glycine of glial origin binds to a specific extracellular site on the receptor complex (Martineau *et al,* 2006), and that the post-synaptic membrane is depolarized to relieve Mg^{2+} block of the ion channel (Mayer *et al,* 1984; Nowak *et al,* 1984; Moriyoshi *et al,* 1991). Metabotropic glutamate receptors (mGluRs) act through G-protein-coupled second messenger pathways, and are divided into eight subtypes in three groups (reviewed below). These receptors may be postsynaptic, presynaptic, or extrasynaptic. The functions of iGluRs in the MOB have been extensively studied, and emerging evidence now also suggests major roles for mGluRs at all levels of olfactory processing.

The relatively recent development of the rodent MOB slice preparation has greatly facilitated the functional exploration and understanding of the mammalian olfactory system. MOB slice preparations provide functionally preserved intrinsic MOB circuitry with access to olfactory receptor neuron axons in the olfactory nerve layer (ONL) of the MOB, allowing stimulation of afferent inputs, visual identification of functional layers and cell types, as well as access to axons of output neurons, mitral/tufted cells, leaving the MOB through the lateral olfactory tract (LOT) (Nickell *et al*, 1994; Liu *et al*, 1994; Aroniadou-Anderjaska *et al*, 1997; Keller *et al*, 1998; Aroniadou-Anderjaska *et al*, 1999a). More recently, MOB slices with microdissection cuts in them to isolate specific synaptic pathways or containing only selected layers of the tissue have been developed (Aungst *et al,* 2003) as well as slices preserving axon pathways projecting back into the MOB from olfactory cortex (Laaris *et al,* 2007; Balu *et al,* 2007). These preparations have allowed specific glutamatergic pathways at different levels of processing to be studied separately, using electrophysiological and imaging techniques.

Glutamate is the major excitatory neurotransmitter in the MOB. Most synaptic processing in the MOB involves serial or reciprocal glutamatergic/GABAergic circuits, principally between the dendrites of MOB output neurons and local interneurons, with specific stages of processing occurring in discrete layers of the tissue. We will begin by reviewing the structural and functional organization of the MOB, before describing the roles of glutamate synapses in olfactory processing.

Structural Organization of the MOBob

Afferent Projections to the MOB

The nasal epithelium houses bipolar olfactory receptor neurons. Each of these projects a sensory dendrite into the thin layer of nasal mucus covering the nasal epithelium, and an axon to the ipsilateral MOB. Most Oolfactory receptor neurons express one odorant receptor protein of a large gene family (Buck and Axel, 1991; Young *et al*, 2002). However, aA small subset of olfactory sensory neurons seems to lacks odorant receptors, and, instead, expressinges genes for trace amine-associated receptors (TAARs). These may represent a dedicated class of pheromone receptors. (Liberles and Buck, 2006).. Olfactory receptor neurons bind odorant (or pheromone) molecules dissolved in nasal mucus, and thereby transduce a chemosensory sensory signal into an action potential that is propagated to the MOB (Zufall *et al,* 1994).

The essential cytoarchitecture of the MOB is shown schematically in Figure 1. The MOB is an allocortical structure with distinctly laminar organization (Shipley and Ennis, 1996; Ennis *et al,* 2007). The axon of each olfactory receptor neuron traverses the most superficial layer of the MOB, the ONL, and forms synapses with MOB neurons in one of many spheroidal neuropil structures, the olfactory glomeruli (1800 in the mouse MOB [Klenoff & Greer 1998], 8000 in humans [Meisami et al 1998]) . Olfactory receptor neurons that express the same odorant receptor protein send their axon to the same two (or very few) topographically fixed glomeruli arranged bilaterally in each bulb, and thus four glomeruli in total (Vassar *et al*, 1994; Mombaerts, 1999; Mombaerts *et al*, 1996; Mombaerts, 2006). Recent evidence suggests that animals, including humans, process inputs to contralateral glomeruli to derive information about the relative strength of odours present at each nostril, thus smelling in stereo (Rajan *et al,* 2006; Porter *et al,* 2007). In addition to determining the response profile of olfactory receptor neurons, rodent odorant receptors help to guide axonal projections through the ONL to specific glomeruli and generate precise synaptic conncectivity in the MOB even in the absence of odorant-evoked neural activity (Wang *et al,* 1998; Lin *et al,* 2000). This topographic map is established independently of cues provided by, or synapse formation with, the major neuronal cell types in the MOB (Bulfone *et al,* 1998). Hence, glomeruli in the MOB form a spatially organized map reflecting the odorant specificity of olfactory receptor neurons that are distributed widely in the nasal epithelium. The integrity of this map is maintained during the ongoing turnover of olfactory receptor neurons, as they die and are replaced from a population of stem cells in the nose (Gogos *et al,* 2000).

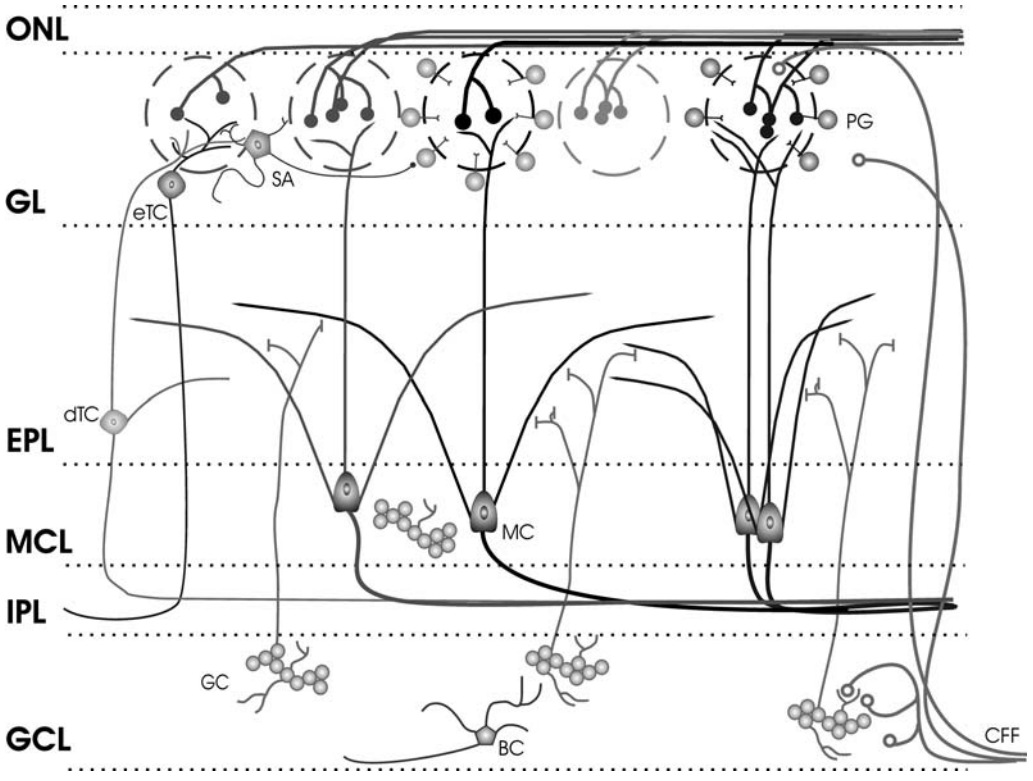

Figure 1. Olfactory bulb circuitry. Olfactory receptor neuron axons enter the main olfactory bulb through the olfactory nerve laver (ONL), to synapse with periglomerular cells (PG), mitral cells (MC) and tufted cells (of which external, eTC, and deep, dTC, are shown) within the glomerular layer (GL). Olfactory receptor projections to glomeruli are odorant receptor-specific, and mitral cell dendrites within a glomerulus are coupled electrically and through glutamate spillover. Short Axon (SA) cell axons receive synaptic input from eTCs and form extensive interconnections between glomeruli, making glutamate synapses with PG cells, while mitral cell apical dendrites convey sensory information to deeper layers of the bulb. In the external plexiform layer (EPL), mitral and (deep) tufted cells extend lateral dendrites which release glutamate onto the dendrites of granule cells (GC). Mitral cell bodies are located in the mitral cell layer (MCL), which is also densely packed with granule cells. Mitral and tufted cell axons project through the internal plexiform layer (IPL) to olfactory cortex (their axon collaterals branching into the GCL are not shown). A subpopulation of external tufted cells projects axons through the IPL to synapse on granule cell proximal dendrites on the opposite side of the main olfactory bulb, but whether or not these are the same cells that release glutamate onto SA cells is not known. The granule cell layer (GCL) contains the major population of inhibitory granule cells. Blane's cells within the GCL make inhibitory contact with granule cells. Centrifugal fibers (CFF) shown projecting to the GL and GCL include glutamate-releasing axons of olfactory cortex pyramidal cells receiving mitral cell output. These contact the basal dendrites of granule cells, while mitral cell dendrites contact their apical dendrites. (Several inconsistently described interneuron types of unknown function are not shown (see Pressler *et al,* 2006)). (Original drawing by Cristina Shirley).

Glomerular Layer (GL)

Subjacent to the ONL, the GL houses an array of olfactory glomeruli. Each of these is 80 to 160 μm in diameter and surrounded by neurons and glia (Shipley and Ennis, 1996). Odorant receptor axons converging into the MOB drive the formation of glomeruli, and the maturation of olfactory bulb output cells (Kobayakawa *et al,* 2007, online supplementary material). Glomeruli house a diverse array of neuronal elements. In addition to axon terminals of olfactory receptor neurons, several types of local interneurons, collectively referred to as juxtaglomerular cells, send dendrites into the glomerular neuropil. Numerous morphological juxtaglomerular cell types are distinguished, including external tufted cells, 'short axon' cells (which actually have long axons extending through the GL (Aungst *et al,* 2003)), and at least two forms of periglomerular cell. In addition, the single apical dendrite of each MOB output neuron, mitral/tufted cells, arborizes in one glomerulus. Olfactory receptor neurons make direct synaptic contact with mitral/tufted cells and with at least some juxtaglomerular cells (Pinching and Powell, 1971). Juxtaglomerular cells in turn intercommunicate extensively (reviewed in Wachowiak and Shipley 2006). A large subset of periglomerular cells contains dopamine (Halasz *et al*, 1981; Davis and Macrides, 1983; McLean and Shipley, 1988) or GABA (Ribak *et al*, 1977). Periglomerular cells presynaptically inhibit glutamate release from olfactory receptor neurons through metabotropic GABAergic and dopaminergic transmission (Hsia *et al*, 1999; Berkowicz and Trombley, 2000; Ennis *et al*, 2001). Periglomerular cells form reciprocal dendrodendritic synapses with mitral/tufted cells (Pinching and Powell, 1971; Shipley and Ennis, 1996). In addition, centrifugal axons originating in higher olfactory centers arborize in glomeruli and may provide modulatory feedback to the GL (reviewed in Shipley and Ennis, 1996; Laaris *et al,* 2007).

External Plexiform Layer (EPL)

Subjacent to the GL lies the EPL. This layer is sparsely populated with cell bodies, including Van Gehuchten cells and tufted cells. The latter are generally classified as external, middle, and deep tufted cells according to their location relative to the surface of the MOB. The EPL is characterized by a rich neuropil, primarily consisting of the extensive lateral dendrites of mitral/tufted cells, and granule cell dendrites sent from deeper in the MOB.

Mitral Cell Layer (MCL)

The cell bodies of mitral cells, 25 to 35 μm in diamater (Shipley and Ennis, 1996) form a thin distinct band delineating the MCL, immediately below the EPL. Mitral/tufted cells are the principal output neurons of the MOB, receiving olfactory receptor neuron input at their dendrites within glomeruli, and projecting glutamatergic axons through the lateral olfactory tract to olfactory cortex. Each mitral/tufted cell sends a single apical dendrite towards the surface of the MOB to form a tuft in one glomerulus. Several lateral dendrites (6-8) originate at the cell body and extend up to thousands of micrometers through the EPL, where they form reciprocal dendrodendritic synapses with granule cell dendrites. The lateral dendrites of mitral cells may also receive input from centrifugal fibers and Van Gehuchten cells (Jackowski *et al*, 1978; Rall *et al*, 1966). Additionally, a large population (~100,000) of granule cells populates

the MCL (Frazier and Brunjes, 1988), outnumbering mitral cells (~40,000) (Meisami, 1989). Like those of granule cells located deeper in the bulb, the apical dendrites of these superficial granule cells extend into the EPL to form dendrodendritic synapses with mitral/tufted cells.

Internal Plexiform Layer (IPL)

Subjacent to the MCL is the IPL. Like the EPL, this layer contains few cell bodies but is densely packed with neuropil, including mitral/tufted cell axons and granule cell dendrites. Centrifugal axons emanating from several brain regions also pass through the IPL, including serotoninergic fibers from the Raphe nuclei (McLean and Shipley, 1987), noradrenergic fibers from the locus coeruleus (McLean *et al*, 1989) and cholinergic fibers from the nucleus of the diagonal band (Shipley *et al*, 1996). Axons of cholecystokinin-containing tufted cells also pass through the IPL to terminate on the dendrites of granule cells on the opposite side of the MOB (Liu and Shipley, 1994).

Granule Cell Layer (GCL)

The GCL contains several cell types, the most prominent of which is the eponymous granule cell. These GABAergic neurons are densely packed into groups (or islands), have no axon, but send apical dendrites to the EPL where they interact with mitral/tufted cells, and shorter basal dendrites receiving glutamate synapses from axon terminals (reviewed below). Other less well characterized cell types in this layer include GABAergic short-axon interneurons, Golgi cells and Blane's cells. Little is known about the function of these cell populations (Shipley and Ennis, 1996), although Blane's cells have recently been found to make inhibitory synaptic contact with granule cells (Pressler and Strowbridge, 2006). Immediately below the GCL, in the core of the MOB, lies an ependymal layer continuous with the ventricular lining. Throughout adult life, neuronal precursors migrate to the MOB through the rostral migratory stream, enter the GCL through the ependymal layer and differentiate to become functional granule cells, or continue to the GL to differentiate into periglomerular neurons (Lledo et al, 2006; Whitman and Greer, 2007).

Functional Organization of the MOBob

Patterned Inputs from the Nose to the MOB

Recent technological advances in *in vitro* and *in vivo* recording have allowed detailed exploration of the neural interactions that govern olfactory processing in the MOB. Stimulation of olfactory receptor neurons with an odor activates a distinct pattern of glomeruli (Astic and Saucier, 1983; Coppersmith *et al*, 1986; Kauer and Cinelli, 1993; Johnson *et al*, 1998; Mori *et al*, 1999; Wachowiak and Cohen, 2001; Wachowiak and Shipley, 2006; Wachowiak *et al*, 2004). An odorant activates a particular set of olfactory receptor neurons, and their axons terminate in the MOB such that each glomerulus serves as the address for particular odorant features (Shepherd *et al*, 2004). Functional imaging and 2-DG data (reviewed in Wachowiak and Shipley, 2006) confirm that different odorants

differentially activate sets of glomeruli. In order to discriminate and recognize odors (often consisting of multiple odorants at different concentrations) the olfactory system must process and decipher these complex patterns of glomerular activation.

Processing of Patterned Inputs within the MOB

The MOB processes odor-specific patterns of input that are distributed across glomeruli, sending the resulting output to higher brain centers as patterns of action potentials distributed across mitral/tufted cell axons. Olfactory receptor neurons expressing the same odorant receptor on their surface, the MOB glomeruli to which their axons project, and the mitral/tufted cells with their apical dendritic tufts within those glomeruli constitute functional odorant receptor-specific neuron groups. Activation of one neuron group influences activity in others with different odorant receptor specificity through lateral inhibitory interactions mediated by local inhibitory interneurons within the MOB. These lateral interactions may enhance contrast within and discrimination between patterns of MOB neural activity representing odors, and they occur at two distinct stages of synaptic processing.

At the first stage of synaptic processing in the MOB, at the level of sensory synaptic input from olfactory receptor neurons, an extensive glutamate-releasing lateral network exists between glomeruli (Aungst *et al,* 2003). This network is formed by the axons of cells called 'sShort aAxon' cells before the full extent of their arborization was recognized. Glomerular layer short axon cells do not receive direct glutamatergic input from olfactory receptor neurons, but are activated by glutamate released by a population of external tufted cells that do. Activation of the short axon cell network then results in widespread depolarization within the glomerular layer, mediated by ionotropic glutamate receptors. Figure 2 shows the result of directly activating this network while imaging membrane potentials with voltage-sensitive dye.

These experiments shown in Figure 2 were done in tangential MOB slices containing the glomerular layer but not deeper layers, and prepared from animals in which the ONL had been ablated. The widespread depolarization seen propagating throughout the glomerular layer is mediated by iGluRs, resulting in depolarization of periglomerular cells (Aungst *et al,* 2003). These cells then release GABA to inhibit mitral cell responses to olfactory receptor neuron input. That this circuit results in lateral inhibition mediated entirely within the glomerular layer can be clearly demonstrated in olfactory bulb slices in which all lateral circuits other than the glomerular layer have been cut, as depicted in Figure 3. Glutamate release from short-axon cells results in robust lateral inhibition; a single stimulus effectively terminates mitral cell responses to olfactory receptor neuron input in surrounding regions of the glomerular layer (Aungst *et al,* 2003). Activation of one odorant receptor-specific neuron group can thus inhibit responses of surrounding neuron groups with different odorant specificities, through glutamatergic/GABAergic circuits intrinsic to the MOB. This may represent a form of center-surround processing analogous to that seen in the retina (Aungst *et al,* 2003).

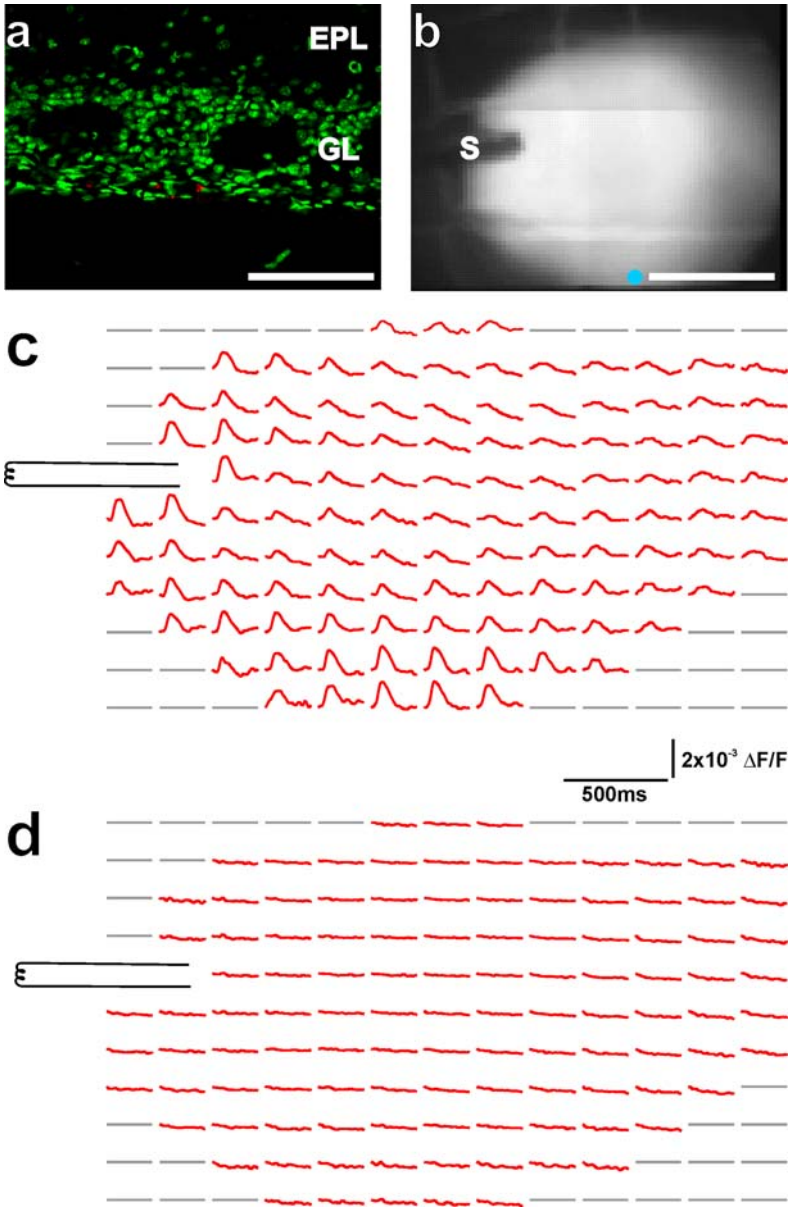

Figure 2. Excitation mediated by glutamate release propagates throughout the glomerular layer. Optical recordings of membrane voltage were made in brain slices cut tangential to the olfactory bulb surface, containing the glomerular layer. Slices were prepared from mice in which the olfactory nerve had previously been ablated by $ZnSO_4$ lavage of the nasal epithelium. (a) The membrane tracer DiD (red) confirmed that the olfactory nerve could not contribute to optical responses. (b) Image taken through the recording camera showing the olfactory bulb surface slice, stained with the voltage-sensitive dye RH414. The stimulating electrode is visible to the left (s). (c) Stimulation of the glomerular layer resulted in widespread transient depolarization (upward deflections, 100 μm x 100 μm spatial signal averages). (d) This depolarization was abolished by blockade of ionotropic glutamate receptors. (Scale bars are 100 μm in a, 500 μm in b. The cyan dot in b represents the size of a single glomerulus). From Aungst *et al*, (2003), , *with permission of AAASNature* 426: 623-9.

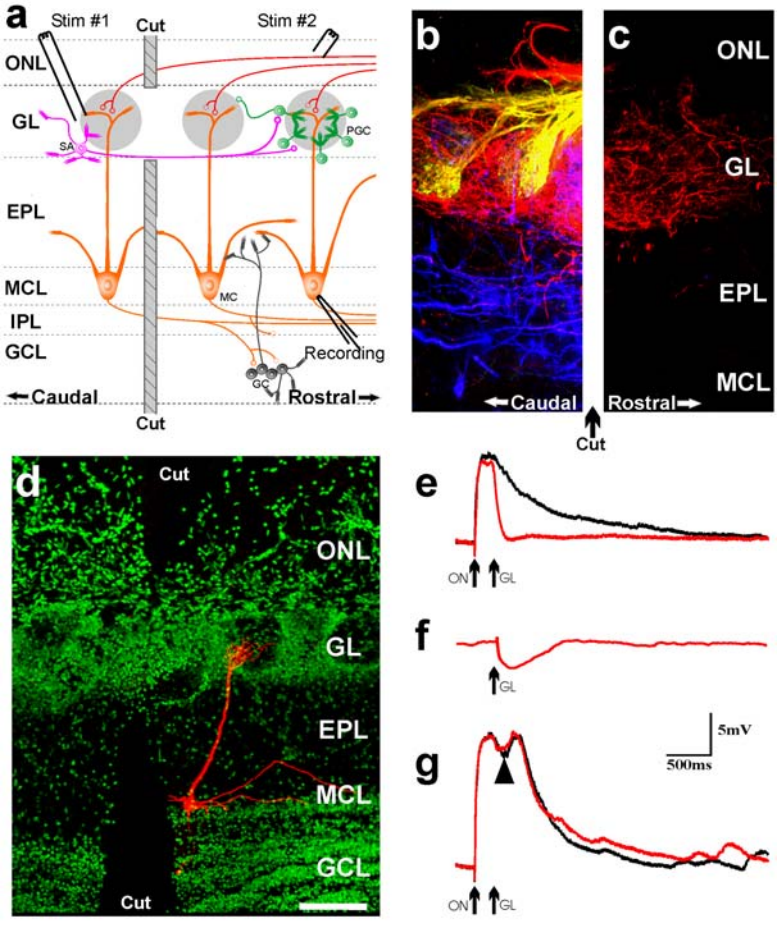

Figure 3. Glutamate-releasing connections between glomeruli control mitral cell responses to olfactory nerve input. (a) Experimental design. Cuts were made in olfactory bulb brain slices, sparing only lateral connections between glomeruli. Mitral cell responses to olfactory nerve stimulation (Stim # 1) were recorded on one side of the cuts, and the glomerular layer stimulated on the other (Stim #2). (b) The membrane-soluble tracers DiA, DiI and DiD microinjected into the olfactory nerve layer, glomerular layer and EPL respectively to the left (caudal) of the cuts labeled all layers of the slice. (c) To the right (rostral) of the cuts only the glomerular layer was labeled with tracer (DiI). (d) A mitral cell labeled during recording with biocytin (red), and nuclear counterstain (green). The cuts are clearly visible in the slice. (e) Long lasting mitral cell depolarization (black trace), initiated by glutamate release from olfactory nerve terminals (ON, arrow) and sustained by mutual glutamate excitation among the apical dendrites of multiple mitral cells. When the glomerular layer circuit was stimulated (GL, arrow) this response was terminated (red trace). These data were recorded from the cell shown in panel (d). (f) Inhibitory post-synaptic potential recorded from a mitral cell in response to stimulation of the glomerular layer (GL, arrow). (g) When GABA receptors were blocked (gabazine, 5 μM) stimulation of the glomerular layer no longer inhibited mitral cell responses (black trace, olfactory nerve stimulation alone; red trace, olfactory nerve plus glomerular layer stimulation). With inhibitory transmission blocked, mitral cell responses to olfactory nerve stimulation were larger and contained multiple peaks (arrowhead). The scale bar in d represents 100 μm in b,c,d. From Aungst *et al,* (2003), *Nature* 426: 623-9From Aungst *et al,* 2003 with permission of AAAS.

At the second major stage of synaptic processing, lateral inhibition is mediated by interactions within the EPL between the dendrites of mitral/tufted cells and granule cells (Jahr and Nicoll, 1982). Functionally, this occurs at the level of action potential output from the MOB, as action potentials are propagated away from the mitral cell body along lateral dendrites in EPL as well as axons to olfactory cortex (Lowe, 2002). Glutamate released by mitral cell dendrites activates GABA release from the dendrites of granule cells (reviewed in Schoppa and Urban, 2003), and may thereby inhibit the output of surrounding mitral cells belonging to different functional groups. Mitral cell inhibition mediated by granule cells, however, is relatively weak (Schoppa and Urban, 2003; Cang and Isaacson, 2003), even when studied *in vitro* in bathing media containing additional glycine or no Mg^{2+} to enhance activation or conductance at NMDA receptors respectively (see Schoppa and Urban, 2003). In contrast to lateral inhibition activated by the extensive glutamatergic interconnections in the glomerular layer, lateral inhibition activated by glutamate release within the EPL is effectively disabled *in vitro* (reviewed in Halabisky and Strowbridge 2003), and has not been shown to inhibit mitral cell responses to olfactory receptor neuron input. Although the relative ineffectiveness of lateral inhibition within the EPL *in vitro* could be related to truncation of mitral cell lateral dendrites, and therefore the extent of their interactions with granule cells, it now seems likely that the EPL level of processing requires input from higher brain centers absent in brain slices (Balu *et al,* 2007, see below). Recent viral tracing studies, of synaptic pathways from the olfactory nerve and from olfactory cortex, suggest that unlike retina where center-surround inhibition is graded with distance, lateral inhibition in the EPL is highly selective, involving widely separated odorant-specific neuron groups (Willhite *et al,* 2006).

The MOB may thus operate as a serial, two stage, lateral inhibitory network, the first stage at the level of input to mitral cells, intrinsic to the MOB, the second at the level of output from mitral cells, possibly mediating odorant-specific feedback from olfactory cortex (Aungst *et al,* 2003; Balu *et al,* 2007; Willhite *et al,* 2006). These two stages of processing are summarized in Figure 4. Each stage of processing may reduce noise while enhancing contrast between the activities of different odorant receptor-specific neuron groups, thus sharpening the patterns of neural activity representing odors. In addition, periglomerular cells and granule cells may act locally, within odorant receptor-specific neuron groups of the MOB, providing feedback inhibition through dendrodenditic interactions with mitral/tufted cells (Shepherd *et al,* 2004). Glutamate neurotransmission drives all these aspects of olfactory processing.

Ionotropic Glutamate Receptors in the MOB

AMPA, kainate, and NMDA ionotropic glutamate receptors are tetramers assembled from different combinations of receptor subunits, endowing them with distinct functional properties (reviewed in Dingledine *et al,* 1999). Receptor subunits specifically assembling into AMPA and kainate receptors are differentially distributed within the MOB (Montague and Greer, 1999; Davila *et al,* 2007). AMPA receptors of variable subunit composition are found on glia within the ONL, mitral/tufted cell bodies and apical and lateral dendrites, periglomerular cell bodies and dendrites, short axon cells within the EPL and GCL, and granule cell bodies and proximal dendrites in GCL. In these studies (Montague and Greer, 1999) AMPA receptors were notably absent from granule cell dendrites within the EPL. Other studies (Sassoe-Pognetto and Ottersen, 2000) have, however, described clustered

localization of AMPA receptors at post-synaptic sites on granule cell dendrites in EPL. Kainate receptor subunits have been described on cell bodies and apical and lateral dendrites of mitral/tufted cells, and on granule cell bodies in the GCL (Montague and Greer 1999; Davila *et al,* 2007), with a differential distribution of kainate receptor subtypes on mitral cells and interneurons (Davila *et al,* 2007). NMDA receptors are extensively co-localized with AMPA receptors in granule cells, clustered at post-synaptic sites of interaction with mitral cell dendrites in EPL. In contrast, AMPA and NMDA receptors are not clustered in mitral cell lateral dendrites, but are a sparse population of extrasynaptic receptors (Sassoe-Pognetto and Ottersen, 2000), consistent with a role in mitral cell dendritic autoexcitation (see below). NMDA receptor expression varies widely among different regions of the MOB, being greatest in GCL and EPL, least in GL, with that in GL less than 30% of that in GCL (Sassoe-Pognetto *et al,* 2003). In addition, a form of AMPA receptors permeable to Ca^{2+} may be expressed throughout the MOB, in subsets of most neuron types (Blakemore *et al,* 2006).

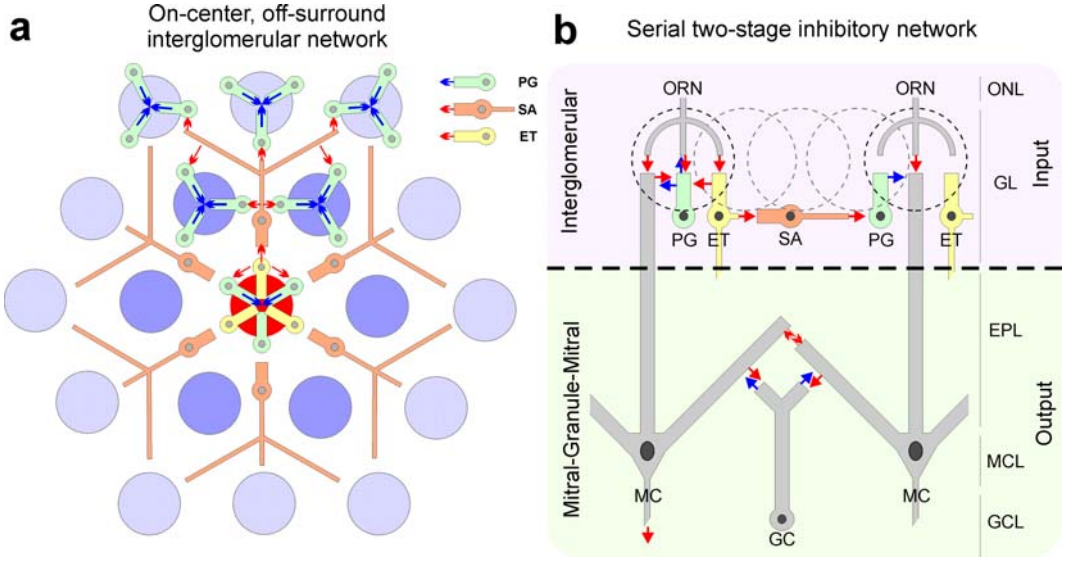

Figure 4. Lateral glutamate/GABA networks in the olfactory bulb. (a) The on-center/off-surround circuit in the glomerular layer, depicted in plan view. Activation of the central (red) glomerulus results in excitation (glutamate release, red arrows) of periglomerular (PG) cells which release GABA (blue arrows) in surrounding glomeruli to inhibit mitral cell responses to olfactory nerve. (b) The two proposed levels of center-surround inhibition in the olfactory bulb. At the level of olfactory nerve input, the intrinsic interglomerular circuit is activated by external tufted (ET) cells which release glutamate onto short axon (SA) cells, which in turn release glutamate to activate inhibitory periglomerular (PG) cells. ET cells and PG cells also participate in local dendrodendritic interactions within individual glomeruli. At the level of mitral cell output, the mitral-granule-mitral circuit is formed by mitral cell lateral dendrites which release glutamate to excite GABA release from granule cell dendrites. Recent findings suggest that activation of GABA release within this circuit requires synaptic glutamate input to granule cell bodies, from the axons of olfactory cortical pyramidal cells receiving mitral cell axonal output (see text). From Aungst *et al,* (2003), *Nature* 426: 623-9From Aungst *et al,* 2003 with permission of AAAS.

Ionotropic Responses to Olfactory Receptor Neuron Input

Numerous studies confirm that excitatory synaptic transmission in the MOB is mediated by glutamate. Olfactory receptor neurons release glutamate at their axon terminals in the olfactory glomeruli to activate iGluRs on the apical dendrites of mitral/tufted cells (Berkowicz *et al,* 1994; Bardoni *et al,* 1996; Ennis *et al,* 1996, 2001; Aroniadou-Anderjaska *et al,* 1997; Chen and Shepherd, 1997; Keller *et al,* 1998). Intrinsic membrane properties of mitral cells determine whether olfactory receptor neuron synaptic input to the mitral cell apical dendrite generates somatic spikes that are relayed to higher order olfactory structures, and are propagated along mitral cell dendrites to release glutamate onto granule cells (Chen *et al*, 1997; Bischofberger and Jonas, 1997; Heyward *et al*, 2001). The complex synaptic connectivity within glomeruli and local circuits between them has recently been comprehensively reviewed (Wachowiak and Shipley, 2006). Glutamate released by olfactory receptor neurons activates iGluRs on juxtaglomerular cells, which in turn activate or inhibit multiple glomerular elements. For example, dopamine and GABA released by juxtaglomerular cells presynaptically modulate glutamate release from olfactory receptor neurons, through D2 and $GABA_B$ receptors respectively, reducing subsequent post-synaptic responses in mitral cells (Hsia *et al*, 1999; Berkowicz and Trombley, 2000; Ennis *et al*, 2001). Imaging studies show that this action of GABA results from inhibition of voltage-dependent Ca^{2+} entry into olfactory receptor neuron terminals (Wachowiak *et al,* 2005). Furthermore, in response to olfactory nerve input, mitral and tufted cell apical dendrites release glutamate to activate not only juxtaglomerular interneurons, but also generate autoexcitation and mutual excitation among output neurons (Schoppa and Westbrook, 2002).

Glutamate released from olfactory nerve terminals may synchronize activity in intrinsically bursting external tufted cells, which act to amplify responses to sensory input by synchronous dendritic release of glutamate onto multiple elements within glomeruli, including short axon cells, periglomerular cells, and mitral cells (Hayar *et al,* 2004a, 2004b, 2005). These tufted cells may express two functionally distinct populations of Ca^{2+}-permeable AMPA receptors, one acting as postsynaptic receptors for olfactory nerve input, the other as autoreceptors for dendritic glutamate release (Ma and Lowe, 2007). Ca^{2+}-permeable AMPA receptors may serve to amplify synaptic output of glutamate from tufted cell dendrites, through positive feedback at the level of intracellular Ca^{2+} (ibid). A major function of external tufted cells may be to relay olfactory receptor neuron excitation to short axon cells, via iGluRs, which in turn release glutamate at ionotropic synapses with periglomerular cells in surrounding glomeruli (Aungst *et al,* 2003; Wachowiak and Shipley, 2006).

Mitral cells respond to olfactory nerve input with long-lasting epochs of action potential generation. The long lasting post-synaptic depolarization underlying this response consists of a fast AMPA receptor mediated component and a slow, longer lasting NMDA receptor mediated component (Aroniadou-Anderjaska *et al,* 1997; Carlson *et al,* 2000; Ennis *et al,* 2001). These events are essentially all or none, not graded with stimulus intensity (Carlson *et al,* 2000). They are initiated by brief synchronous release of glutamate from olfactory nerve terminals, and sustained by asynchronous release of glutamate from mitral and tufted cell dendrites, and consequent activation of dendritic NMDA receptors (Carlson *et al,* 2000; Hayar *et al,* 2004b; De Saint Jan and Westbrook, 2007).

Ionotropic Coupling among Mitral Cells

In response to olfactory receptor neuron synaptic input, mitral/tufted cell dendrites release glutamate which activates iGluRs (and mGluRs) on other cell types in the glomeruli, and granule cells in the EPL (Trombley and Westbrook, 1990; Bardoni *et al,* 1996; Isaacson and Strowbridge, 1998; Schoppa *et al,* 1998; Aroniadou-Anderjaska *et al,* 1999a). Glutamate released by mitral cell dendrites also acts at extrasynaptic (or perisynaptic) iGluRs distributed on mitral cell dendrites (Sassoe-Pognetto and Ottersen, 2000, Davila *et al,* 2007). Such glutamate 'spillover' mediates recurrent excitatory interactions among apical and lateral dendrites of mitral/tufted cells, producing mutual excitation, and autoexcitation mediated by AMPA, kainate, and NMDA receptors, (Isaacson, 1999; Aroniadou-Anderjaska *et al,* 1999b; Carlson *et al,* 2000; Salin *et al,* 2001; Schoppa and Westbrook, 2001). Recent studies suggest a specific role for presynaptic kainate receptors on mitral/tufted cell dendrites, in enhancing glutamate release and spillover within glomeruli (Davila *et al,* 2007). These interactions contribute, along with electrical coupling between mitral cell apical dendritic tufts (Christie and Westbrook, 2006; Christie *et al,* 2005), to the coordinated depolarization of odorant receptor-specific groups of mitral cells, those with their dendrites receiving olfactory receptor neuron input within the same glomerulus.

Extrasynaptic spillover of glutamate, released from and acting on mitral cell dendrites, may serve to amplify, coordinate, and prolong mitral cell responses to olfactory receptor neuron input (reviewed in Schoppa and Urban, 2003). It has been suggested, however, that glutamate released by granule cell dendrites, not mitral cell dendrites, generates apparent autoexcitation in mitral cells within the EPL (Didier *et al,* 2001). Glutamate released by mitral cells was proposed to activate NMDA receptors on granule cells, which in turn release glutamate to activate AMPA receptors and pharmacologically novel extrasynaptic NMDA receptors on mitral cell dendrites. These findings, based on iontophoretic drug applications, have been refuted in studies using flash photolysis of caged glutamate, and explained as an artifact resulting from the high glutamate concentrations achieved by iontophoresis (Lowe, 2003). There is thus no functional evidence for release of glutamate from granule cells (or for co-release with GABA, but see Seal and Edwards, 2006). The NMDA receptor-dependence of transmitter release from granule cells has itself also recently been questioned (Schoppa, 2006; see below).

Ionotropic Activation of Granule Cells

Granule cells are the major, continuously replenished population of MOB inhibitory interneurons, releasing dendritic GABA to regulate the output of mitral cells. Interactions between the dendrites of mitral cells and granule cells in EPL have been extensively studied, but only recently has their role in olfactory processing been clarified. Glutamate released from lateral dendrites of mitral and tufted cells depolarizes granule cell dendrites in EPL, which can in turn generate GABAergic feedback and feedforward inhibition of mitral/tufted cells (Aroniadou-Anderjaska *et al,* 1999b; Chen *et al,* 2000; Isaacson and Strowbridge 19988; Schoppa *et al,* 1998). AMPA and NMDA receptors are clustered at post-synaptic sites on granule cell dendrites in EPL (Sassoe-Pognetto and Ottersen, 2000), and AMPA, kainate and NMDA receptors are also found on granule cell bodies (Montague and Greer, 1999; Sassoe-

Pognetto *et al,* 2003; Davila *et al,* 2007). Glutamate has been reported as activating granule cell GABA release through actions at each of these receptors, but their relative contributions may depend on the subcellular location (and origin) of synaptic glutamate input to granule cells, and the frequency or duration of this input (Balu *et al,* 2007; Schoppa, 2006).

Studies employing electrical stimulation in MOB slices have concluded that it is activation of NMDA receptors that drives GABA release from granule cells (Isaacson and Strowbridge, 1998; Schoppa *et al*, 1998). Ca^{2+} entry through dendritic NMDA receptor channels may be both necessary and sufficient for synaptic GABA release from granule cells, which occurs in the absence of post-synaptic action potentials and voltage-dependent Ca^{2+} entry (Chen *et al,* 2000; Halabisky *et al,* 2000; but see Isaacson 2000). Granule cells may thus act locally, at dendrodendritic synapses, generating recurrent dendrodendritic inhibition of mitral cells from which they receive direct synaptic input. With sufficient granule cell depolarization, action potentials invading their dendrites could presumably release GABA onto surrounding mitral cells, generating more widespread lateral inhibition. Inhibition mediated by granule cells, however, is effectively disabled under physiological conditions, at least *in vitro,* by Mg^{2+} blockade of NMDA receptors (Chen *et al,* 2000; Halabisky and Strowbridge, 2003). How this voltage-dependent blockade may be released in vivo has been unclear, with separate but similar sets of experiments providing conflicting conclusions as to how granule cells may be activated by glutamate released from mitral cells during olfactory processing (Schoppa and Westbrook, 1999; Halabisky and Strowbridge, 2003; Schoppa, 2006; Arevian *et al,* 2008). This issue has recently been clarified, however, by taking into account additional sources of synaptic glutamate input to granule cell dendrites, extrinsic to the olfactory bulb (Balu *et al,* 2007, see below).

The apparent NMDA receptor-dependence of GABA release from granule cells has been explained in terms of their membrane properties. Granule cells express a prominent transient outward current, which unlike the transient outward current (I_A) described in other central neurons is not inactivated at resting potential (Schoppa and Westbrook, 1999). This current can therefore attenuate post-synaptic depolarization following synaptic input to AMPA/kainate receptors in granule cells, preventing action potential generation and voltage-dependent Ca^{2+} entry. There must be, however, sufficient depolarization to relieve the intracellular Mg^{2+} block typical of NMDA receptors, to allow GABA release to follow the longer kinetics of Ca^{2+} entry during NMDA receptor activation (Schoppa and Westbrook, 1999). Synaptic input to granule cell dendrites from mitral cell dendrites might thus elicit NMDA receptor-mediated, action potential-independent release of GABA, and local dendrodendritic recurrent inhibition of mitral cells.

NMDA receptor dependence of GABA release has been described as a general property of dendrodendritic inhibition involving mitral cells and tufted cells (Christie *et al,* 2001). The physiological significance of this inhibition, effectively disabled when the MOB is isolated from other brain regions *in vitro*, has been unclear. Indeed, a recent *in vitro* study simulating repetitive synaptic glutamate release onto granule cells such as may occur during respiration *in vivo* has suggested that it is not NMDA receptor activation, but high (gamma) frequency AMPA/kainate receptor input from mitral cells that normally drives GABA release from granule cells (Schoppa, 2006). As the transient outward current in granule cells inactivates within 50 ms, it filters AMPA/kainate iGluR-mediated inputs according to their frequency or duration. The transient outward current prevents granule cells from responding to isolated or brief ionotropic inputs from mitral cells, but if input is tetanic and outlasts transient outward

current inactivation, AMPA/kainate receptors drive granule cells to action potential threshold, providing Ca^{2+} entry for GABA release. Outward current inactivation could contribute to the frequency-dependent modulation of lateral inhibition seen following tetanic stimulation in olfactory bulb slices, mediated at least in part by granule cells, and thought to underlie activity-dependent recruitment of granule cells (Arevian *et al,* 2008). Schoppa (2006) identified two essential sources of synaptic glutamate release onto granule cells, input from mitral cell lateral dendrites to distal granule cell dendrites in EPL activating kainate receptors with slow kinetics, and input from mitral cell axon collaterals to granule cell proximal dendrites activating AMPA receptors with fast kinetics, with no apparent contribution from NMDA receptor activation. Arevian *et al* (2008), however, reconfirm the NMDA receptor-dependence of action potential-dependent Ca^{2+} entry into granule cells, and describe mitral to granule cell connections in the olfactory bulb circuit as almost 100% reciprocal, i.e., between mitral cell lateral dendrites and granule cell distal dendrites in EPL. In contrast to the conclusions of either report, another study using tetanic stimulation *in vitro* (Halabisky and Strowbridge, 2003) describes both NMDA receptor dependence and two essential sources of glutamate to granule cells. This earlier study found that synaptic activation of AMPA receptors on granule cell proximal dendrites relieves Mg^{2+} block from NMDA receptors on granule cell distal dendrites, thus enabling NMDA receptor dependent inhibition in the EPL. As in Schoppa (2006), Halabisky and Strowbridge (2003) suggest mitral cell axon collaterals as a potential source of inputs to granule cell proximal dendrites, but neither study could exclude a contribution from centrifugal axons.

Ionotropic Glutamate Synapses from Cortical Afferents to Granule Cells

The roles of AMPA/kainate receptor-mediated and NMDA receptor-mediated activation of granule cells, the origin of synaptic glutamate input to different subcellular regions of granule cells, and the requirement for tetanic synaptic activation have recently been clarified by studies using focal stimulation of specific glutamate inputs guided by two-photon imaging *in vitro* (Balu *et al,* 2007). To differentiate between mitral cell axon collaterals and centrifugal afferents as the origin of synaptic glutamate, slices including anterior piriform cortex were used to allow localized stimulation of cortical feedback projections, and the integrity of this pathway was verified by DiI tract tracing. These and other studies (Laaris *et al,* 2007) confirm that glutamate-releasing projections from piriform cortex depolarize granule cells, via AMPA and NMDA receptors on their proximal dendrites and cell bodies, relieving Mg^{2+} block from NMDA receptors on distal granule cell dendrites in EPL. Cortical feedback to deeper layers of the MOB may thus gate dendrodendritic inhibition in the EPL. In this view, the dependence of GABA release on NMDA receptors (or tetanic stimulation) simply reflects a mechanism that normally allows granule cells to act as coincidence detectors *in vivo* (Chen *et al,* 2000), responding with dendritic GABA release to appropriately timed glutamatergic inputs from two sources, mitral cell lateral dendrites, and axons from brain regions receiving mitral cell output. Both proximal and distal synapses on granule cells contain AMPA and NMDA receptors (or AMPA receptors alone), with fast overall kinetics at proximal synapses, slow kinetics at distal. Unlike the distal, dendrodendritic synapses in EPL, which showed depression with paired pulse stimulation, proximal synapses on granule cells from cortical axons showed strong facilitation, explaining the requirement for tetanic activation of this

pathway (Balu *et al,* 2007). As these authors point out, high frequency oscillations are a characteristic of populations of piriform cortical neurons in behaving animals (Freeman, 1978), and could thus represent an endogenous gating signal for dendrodendritic inhibition. Thus although lateral inhibition in the external plexiform layer can be activated under specific conditions in olfactory bulb slices *in vitro*, interactions between mitral cells and granule cells *in vivo* may not generate recurrent or lateral mitral cell inhibition (or synchronized oscillation) independently of olfactory cortex, but serve to involve cortical areas receiving mitral cell output in MOB processing.

Glutamate release from extensive mitral cell lateral dendrites may result in dynamic interactions between widely separated odorant receptor-specific neuron groups within the MOB (Margrie *et al,* 2000; Xiong and Chen, 2002; Migliore and Shepherd, 2007). As this is mediated by NMDA receptor dependent GABA release, this too may require appropriately timed synaptic glutamate release from the axons of cortical neurons. Mitral cell lateral dendrites project thousands of micrometers from the cell body, potentially releasing glutamate onto widely distributed granule cells. By controlling the ability of granule cells to respond with GABA release, glutamate released by cortical axons may control not only the strength of these interactions in the EPL, but also their distribution. Action potentials are actively propagated in mitral cell lateral dendrites, releasing glutamate in response to voltage-gated Ca^{2+} entry. In turn, their amplitude and propagation distance may be influenced by GABA released from granule cells (Margrie *et al,* 2000; Xiong and Chen, 2002; Lowe, 2002). Together with modeling studies (Migliore and Shepherd, 2007), these studies suggest that inhibitory dendritic inputs from granule cells may serve to gate action potential propagation along mitral cell dendrites, regulating the distance over which individual mitral cells can interact with granule cells, and thereby influence other mitral cells. Glutamate synapses made by cortical feedback axons are therefore intimately involved in the distributed, dynamic patterns of MOB neuronal activity that represent odors.

Metabotropic Glutamate Receptors in the MOB

While many functions of iGluRs in the MOB are now well understood, relatively little is known about the function of mGluRs. mGluRs are expressed at unusually high density in the MOB (Ennis *et al,* 2007). Recently, specific mGluR pharmacological reagents as well as mGluR-specific knockout mice have revealed a wide variety of physiological responses and ion channels that are modulated by mGluRs (Schoepp *et al*, 1999). To date, eight mGluR subtypes have been cloned and are subdivided into three groups based on second messenger linkage, sequence homology and pharmacological sensitivity of the receptors: Grp I (mGluR1 & mGluR5), Grp II (mGluR2 & mGluR3) and Grp III (mGluR4, mGluR6, mGluR7 & mGluR8). Six of these eight metabotropic glutamate receptor subtypes have been found in the MOB.

mGluRs and iGluRs are not only distinguished by their different modes of ion channel regulation but also by their distinct differences in sensitivity to glutamate (ED_{50}: mGluR1, 5: 10 μM; AMPAR: 200-500 μM; NMDAR: 2.5-3 μM (Meldrum, 2000). mGluRs can be orders of magnitude more sensitive to glutamate than iGluRs (AMPA receptors), even though the ED_{50} for glutamate at AMPA/kainate receptors is significantly influenced by subunit composition (Dingledine *et al,* 1999; Meldrum, 2000). This has important implications for

tonic mGluR activation and sensitivity to glutamate spillover under conditions where glutamate concentrations are too low to activate AMPA receptors.

Mitral and tufted cells express high levels of mGluR1 (Masu *et al*, 1991; Martin *et al*, 1992; Shigemoto *et al*, 1992; van den Pol, 1995; Sahara *et al,* 2001). mGluR1 is present on the cell body, and apical and lateral dendrites of mitral and tufted cells, and the splice variant mGluR1a has been localized postsynaptically at ON synapses (van den Pol, 1995) which suggests that mGluR1 mediates responses of mitral and tufted cells to glutamatergic inputs from olfactory nerve terminals, and/or could function as auto- or heteroreceptors for glutamate released from the apical or lateral dendrites of mitral and tufted cells. In contrast to their counterparts in the accessory olfactory bulb (where responses to pheromones are processed in many mammals), mitral and tufted cells in the MOB do not express Grp. II receptors (mGluR2/3) (Ohishi *et al*, 1993; 1998). mGluR7 and mGluR8 are present on the axon terminals of mitral and tufted cells in piriform cortex (Kinzie *et al*, 1995; Saugstad *et al*, 1997; Kinoshita *et al*, 1998; Wada *et al*, 1998).

Granule cells express high levels of mGluR5 (Grp I) (Romano *et al*, 1995), localized on their dendrites in the EPL apposed to presynaptic glutamatergic dendrites from mitral and tufted cells (van den Pol, 1995). This expression pattern suggests that mGluR5 may mediate responses of GCs to glutamatergic inputs from mitral and tufted cells. Granule cells also express low to moderate levels of mGluR2 (Grp. II, Ohishi *et al*, 1993, 1998), and low levels of mGluR4 and mGluR7 (Grp. III; Kinzie *et al*, 1995; Ohishi *et al*, 1995; Saugstad, 1997; Wada *et al*, 1998), but the subcellular localizations of these receptors are not known.

Juxtaglomerular cells express several types of mGluRs. External tufted cells appear to express the same mGluRs as mitral cells and tufted cells in the EPL, namely, high levels of mGluR1, and lower levels of mGluR7 and mGluR8 (Ohishi *et al*, 1993, 1998; Kinzie *et al*, 1995; Saugstad *et al*, 1997). Other, unidentified, juxtaglomerular cells express low to moderate levels of Grp I (mGluR5) Grp II (mGluR2) and Grp III (mGluR4/7/8) receptors (Romano *et al*, 1995; Onishi *et al*, 1993, 1998; Saugstad *et al*, 1997).

In view of their abundant expression in MOB neurons, a pressing issue in the organization and operation of the olfactory system is the analysis of mGluR actions on adult MOB neurons and upon MOB network function. The MOB is an ideal platform for investigating how mGluRs modulate neural network dynamics to enhance sensory processing. The MOB receives direct sensory input, but this input is strongly modified by intrinsic inhibitory interneurons that provide two discrete levels for lateral interactions (Aungst *et al,* 2003), discussed above. The MOB also receives extensive cortical and subcortical centrifugal inputs that modulate local circuit processing at multiple levels. This organization makes the MOB preparation significantly different from hippocampus, amygdala, neocortex, and cerebellum in addressing functional questions of mGluR modulation in the brain.

mGluR Effects on MOB Neurons

Until recently, the major contributions to our understanding of the role of mGluRs in the MOB have come from studies of dissociated frog or neonatal rat neurons. Activation of Group I mGluRs increases Ca^{2+} release from internal stores in rat and frog mitral and tufted cells and unidentified interneurons (Geiling and Schild, 1996; Carlson *et al*, 1997). An mGluR-mediated increase in Ca^{2+} in the dendrites of mitral and tufted cells can

presynaptically facilitate glutamate release (Schoppa and Westbrook, 1997). In addition, Group I mGluRs depolarize mitral and tufted cells, possibly through the closure of K^+ channels (Schoppa and Westbrook, 1997). In cultured frog olfactory bulb interneurons, activation of Group II (mGluR2/3) mGluRs inhibits high voltage-activated Ca^{2+} currents and decreases spontaneous GABAergic inhibitory postsynaptic currents (IPSCs) in mitral/tufted cells (Bischofberger and Schild, 1996). Grp. II and III mGluRs presynaptically inhibit glutamate release from putative mitral and tufted cells in disassociated cultures of rat MOB (Trombley and Westbrook, 1992; Schoppa and Westbrook, 1997). However, adult rat mitral and tufted cells do not express Grp. II mGluRs (Ohishi *et al*, 1993; 1998), and specific Group II mGluR antagonist had no effect on the inhibition of glutamate release evoked by Group II agonists (Schoppa and Westbrook, 1997).

mGluR-Mediated Modulation of Mitral Cells

Recent findings obtained in adult rodent MOB slice preparations demonstrate a distinct role of mGluR1 in modulating the excitability of mitral cells (Heinbockel *et al*, 2004). mGluR agonists potently and dose dependently depolarized and increased the firing rate of mitral cells (Figure 5). These effects on mitral cells persisted in the presence of blockers of iGluRs (APV, CNQX) and GABA receptors (gabazine) and/or tetrodotoxin (TTX). Therefore, the excitatory action of Group I mGluR agonists wais not mediated indirectly through other MOB neurons, but resultsed from a direct effect on mitral cells.

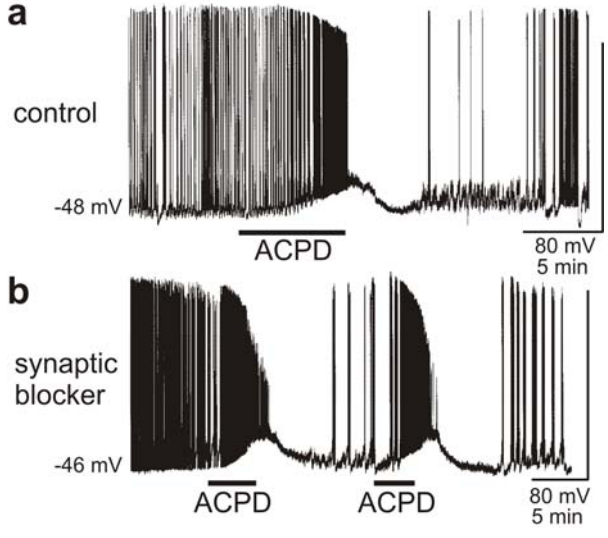

Figure 5. (a) Bath application of the Group I/II mGluR agonist ACPD [(±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid] resulted in membrane potential depolarization and increased action potential firing in this rat mitral cell. (b) A similar response to ACPD was obtained in the presence of blockers of fast synaptic transmission in another rat mitral cell; NMDA receptor blocker APV [DL-2-amino-5-phosphonopentanoic acid], AMPA receptor blocker CNQX [6-cyano-7-nitroquinoxaline-2-3-dione], $GABA_A$ receptor blocker gabazine. There was no apparent desensitization with repeated application of ACPD. Modified from Heinbockel et al., 2004 with permission of The American Physiological Society.

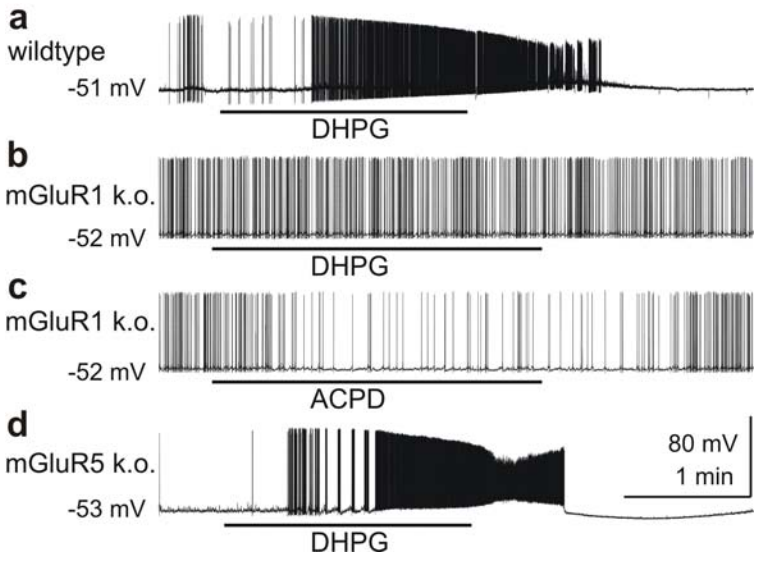

Figure 6. Excitatory actions of mGluR agonists on mitral cells are absent in mGluR1 null mutant mice. (a) Mitral cells recorded in slices harvested from wildtype mice are uniformly activated by the Group I mGluR agonist DHPG [(RS)-3,5-dihydroxyphenylglycine]. (b) DHPG was without effect in mitral cells recorded in slices from mGluR1 null mutant (k.o.) mice. (c) In contrast to its excitatory effect in wildtype mice, ACPD elicited a modest reduction in the firing rate of mitral cells recorded in slices from mGluR1 mutant (k.o.) mice. (d) The excitatory effect of DHPG on mitral cells recorded in slices from mGluR5 null mutant (k.o.) mice was similar to that observed in wildtype mice. All experiments performed in the presence ionotropic (NMDA, AMPA) and $GABA_A$ receptor blockers, CNQX, APV, and gabazine, respectively. From Heinbockel et al., 2004; with permission of The American Physiological Society.

Blockade of mGluRs by Group I or mGluR1 receptor antagonists reducesd mitral cell spontaneous and sensory-evoked excitability via modulation of membrane excitability and membrane bistability (Heinbockel *et al*, 2004) (Figure 7). Mitral cells *in vitro* exhibit intrinsic membrane potential bistability (Heyward *et al,* 2001), i.e., mitral cells spontaneously alternate between two discrete membrane potentials differing by ~10 mV: a more depolarized "upstate", perithreshold for spike generation, and a more hyperpolarized "downstate" devoid of spiking (Figure 7). Membrane bistability is an important determinant of mitral cell excitability and responses to olfactory nerve input (Heyward *et al,* 2001). In the perithreshold upstate, mitral cells generate short latency spikes in response to olfactory nerve input (Heyward *et al,* 2001; Hayar *et al,* 2001). Quite the opposite happens in the hyperpolarized downstate: olfactory nerve input often fails to elicit spikes in mitral cells or it evokes spikes at long latencies. Although bistability is generated by intrinsic membrane properties of mitral cells (Heyward *et al,* 2001) it can be modulated by extrinsic input. mGluR blockade produces a hyperpolarizing shift in membrane potential and reduces mitral cell spontaneous firing rate while it increases the time spent in the membrane downstate. The result is a reduction in sensitivity to activation of iGluRs following stimulation of olfactory receptor neurons. The functional consequence of mGluR1 blockade in mitral cells is twofold: the probability of mitral cell spiking in response to olfactory nerve stimulation is reduced, and the latency of evoked spikes is increased. Since mGluR1 blockade does not affect olfactory nerve-evoked

EPSCs in mitral cells, these effects on olfactory nerve-evoked spiking result from direct modulation of postsynaptic membrane properties, in mitral cells. Furthermore, these results indicate that endogenously released glutamate tonically modulates mitral cell excitability via activation of mGluR1 to modulate the basal and sensory-evoked discharge of mitral cells.

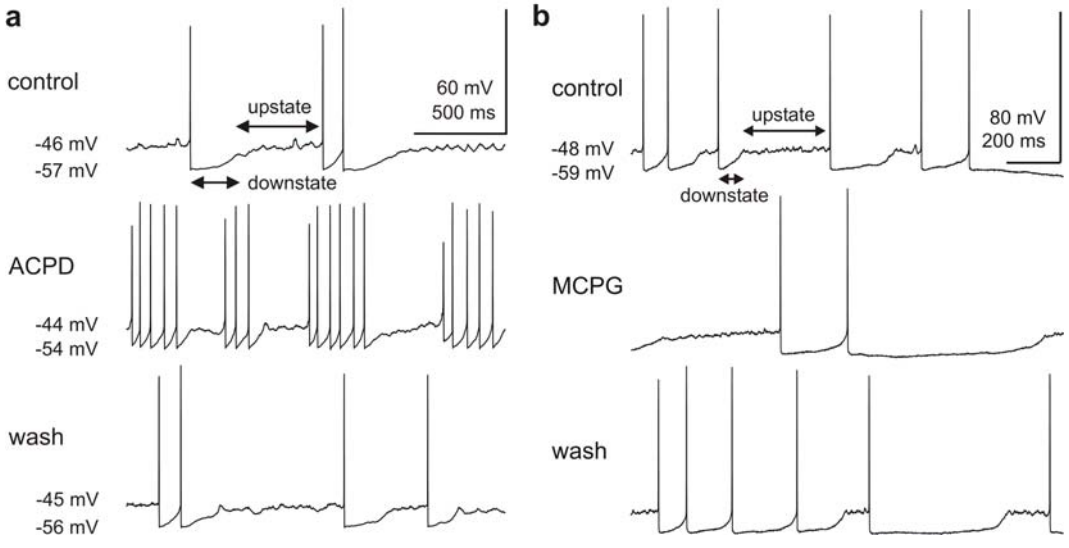

Figure 7. Activation and blockade of mGluRs modulates mitral cell spontaneous activity and membrane bistability. (a) The response of a rat mitral cell is shown at an expanded time scale to illustrate the effects of ACPD on mitral cell bistability. Note that downstate potential stability is absent in the presence of ACPD. (b) Upper trace: current clamp recording showing membrane bistability in another rat mitral cell. Middle trace: bath application of the mGluR antagonist MCPG [(RS)-α-methyl-4-carboxyphenylglycine] reduced the firing frequency of the mitral cell and prolonged the up- and downstates. Lower trace: The effects of MCPG were reversible upon washout. Modified from Heinbockel et al., 2004 with permission of The American Physiological Society.

mGluR-Mediated Modulation of Granule Cells

Recent studies of MOB slices demonstrate that excitability of granule cells in the MOB is increased by mGluR5 activation, and decreased by mGluR5 blockade (Heinbockel *et al*, 2007a, b). Group I mGluR agonists robustly depolarize (~20 mV) and increase the firing rate of granule cells. In contrast to findings in mitral cells, the depolarizing response to mGluR agonists persists in granule cells in the presence of synaptic blockers only with reduced amplitude (6-8 mV). Group I mGluR agonists thus directly depolarize granule cells through activation of mGluR5 receptors, and indirectly depolarize them through increased glutamate release following activation of mGluR1 on mitral cells (Heinbockel *et al*, 2007a). The direct actions of Group I mGluR agonist on granule cells are mediated by the Group I receptor subtype mGluR5 rather than mGluR1, the second Group I receptor subtype. A selective mGluR1 antagonist does not block the effect of a Group I agonist on granule cells, whereas it completely eliminates mGluR1-mediated excitation of mitral cells at identical concentrations (Heinbockel *et al*, 2004). In mGluR5 knockout mice, in synaptic blockers Group I mGluR

agonists have no effect on membrane potential or firing rate of granule cells whereas the same agonists activate granule cells in mGluR1 knockout mice (Heinbockel *et al*, 2007a, b) (Figure 8). Group II and Group III mGluR agonists do not elicit currents in granule cells at holding potentials of -70 mV.

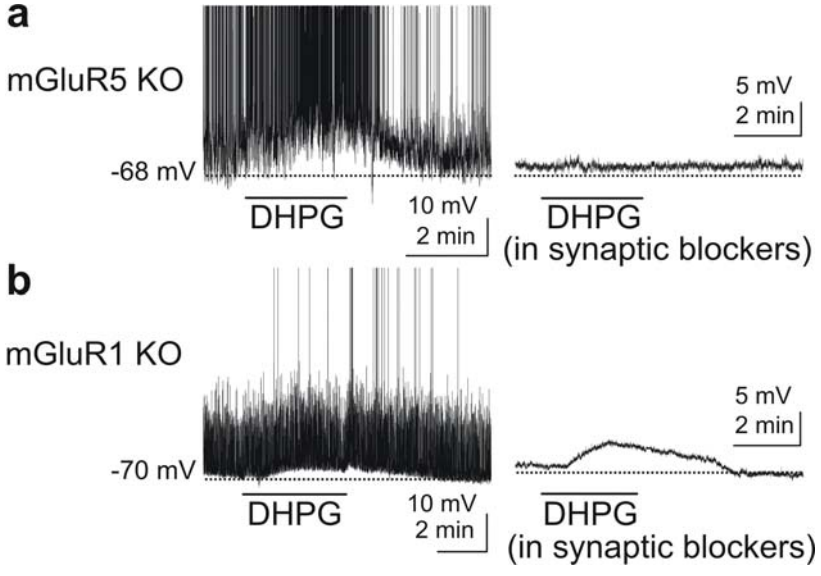

Figure 8. DHPG-evoked excitation of granule cells is absent in mGluR5 null mutant mice. A: Left trace shows that DHPG excites a granule cell recorded in a slice harvested from an mGluR5$^{-/-}$ mouse. The right trace shows that in the presence of synaptic blockers (CNQX, APV, and gabazine), DHPG had no effect on the same granule cell. B: In normal media (left trace), DHPG depolarizes and moderately increases the firing rate of a granule cell from an mGluR1$^{-/-}$ mouse. Subsequent application of DHPG to the same cell in the presence of synaptic blockers (right trace) elicited a comparable depolarization although evoked spikes are absent. Note that application of synaptic blockers (right traces) in (A) and (B) decreases baseline noise compared to left control traces, which include spontaneous EPSPs. Modified from Heinbockel et al., 2007a with permission of The American Physiological Society.

In voltage clamp recordings, activation of mGluR5 induces an inward current in granule cells. The I-V relationship determined as the steady-state current amplitude at the end of a 500-ms voltage step indicates that the mGluR5-mediated current varies linearly with voltages negative to -70 mV, reverses in polarity at about –100 mV, and decreases at potentials positive to -60 mV. Like the mGluR1-mediated current in mitral cells, the mGluR5 current in granule cells probably involves multiple components (Anwyl, 1999). Closure of K^+ channels is likely to contribute to the inward current as the current is abolished by K^+ channel blockers. This is further supported by an increase of granule cell membrane input resistance in response to Group I mGluR agonists, while no resistance change is observed in the presence of K^+ channel blockers. One possible mechanism for the mGluR-evoked current is a decrease of a leak K^+ current which has also been observed in response to Group I mGluR activation in other systems (Glaum and Miller, 1992; McCormick and von Krosigk, 1992; Guerineau *et al*, 1994; Takeshita *et al*, 1996). The prominent A-like K^+ current in granule cells, I_A (Schoppa and Westbrook, 1999), cannot explain the mGluR inward current, as I_A is strongly voltage dependent, activates at potentials positive to -45 mV and inactivates rapidly (Schoppa and

Westbrook, 1999). At more positive potentials, the mGluR5-mediated current may involve voltage-gated Ca^{2+} channels, Ca^{2+}-dependent nonselective cation conductances (Guerineau *et al,* 1995, Congar *et al,* 1997, Crepel *et al,* 1994; Raggenbass *et al,* 1997) or electrogenic Na^{+}/Ca^{2+} exchangers (Keele *et al,* 1997; Lee and Boden 1997; Staub *et al,* 1992).

mGluR5 may modulate the strength of granule cell responses to glutamatergic inputs from mitral/tufted cells and granule cell-mediated dendrodendritic feedback inhibition. Activation of mGluR5 on granule cells increases GABA release from these cells (Heinbockel *et al,* 2007a). In wildtype mice and mGluR1 knockout mice, in the presence of iGluR antagonists, a Group I mGluR agonist increases the frequency of GABAergic IPSCs in mitral cells, i.e., GABAergic inhibition of mitral cells (Heinbockel *et al,* 2007a). This suggests that direct activation of mGluR5 is sufficient to drive GABA release from granule cells. Experiments using field potential and current source density analysis as well as voltage-sensitive dye imaging show that mitral-tufted cell activation results in depolarization within the EPL (Aroniadou-Anderjaska *et al,* 1999a, b; Laaris *et al,* 2007). This depolarization primarily reflects glutamatergic excitation of granule cell dendrites. Inactivation of mGluRs reduces the magnitude of this depolarization (Heinbockel *et al*, 2007a), suggesting that activation of mGluRs by endogenously released glutamate tonically modulates the excitability of granule cells. This is also confirmed by the following experiment: application of an mGluR antagonist reducesd the feedback IPSP evoked by intracellular depolarization of individual mitral cells. These effects doid not appear to be mediated by presynaptic suppression of glutamate release as the mGluR antagonist doides not reduce the mitral/tufted cell-evoked EPSP or EPSC in granule cells. The mGluR antagonist also reducesd synaptic responsiveness in granule cells, i.e., it reducesd spiking responses elicited by stimulation of mitral/tufted cells (Figure 9). The reduction in mitral/tufted cell-evoked spiking and the feedback IPSP may to be due to a small tonic hyperpolarization in some GCs elicited by mGluR antagonists, which would bias granule cells away from spike threshold and reduce evoked spiking (Heinbockel *et al,* 2007a).

mGluR-Mediated Synaptic Inhibition of Mitral Cells by Periglomerular Cells

GABAergic periglomerular cells in the GL also express high levels of mGluRs. mGluR-mediated activation of periglomerular cells could, therefore, also contribute to mGluR-driven GABAergic input to mitral cells. Experiments in rat MOB slices indicate that this is indeed the case (Dong *et al*, 2007). Measurements of spontaneous IPSCs and TTX-insensitive miniature IPSCs in mitral and external tufted cells show that a Group I mGluR agonist evokes GABA release from periglomerular cells via both spike-dependent and spike-independent presynaptic mechanisms. In contrast, mGluR-mediated GABA release from granule cells occurs in a spike-dependent manner (Dong *et al*, 2007) suggesting different functional roles for mGluRs in these two cell types and levels of processing. mGluRs on periglomerular cells may play a role in facilitating intraglomerular inhibition after subthreshold input, whereas mGluRs on granule cells may amplify spike-driven lateral inhibition more globally through the mitral-to-granule cell circuit (Dong *et al*, 2007).

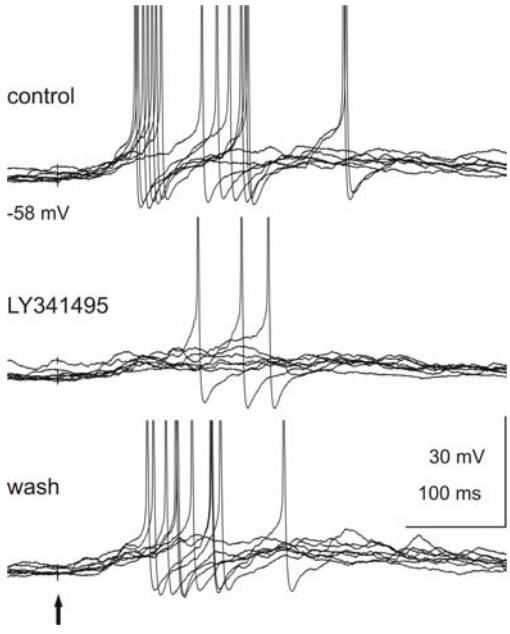

Figure 9. Blockade of mGluRs attenuates mitral/tufted cell-evoked excitation of granule cells. Upper trace - single shocks (marked by arrow in lower trace) applied to the glomerular layer to directly activate mitral/tufted cells evoked EPSPs and spikes in a mouse granule cell. Middle trace: Application of the mGluR antagonist LY341495 markedly reduced evoked excitation of the same granule cell in a reversible manner (lower trace). Twelve stimulus trials are shown in each trace. Modified from Heinbockel et al., 2007a with permission of The American Physiological Society.

Functional Considerations of mGluR Activation in the MOB

The density of mGluR1 in mitral cells and tufted cells and mGluR5 in granule cells is higher than in most other regions of the brain. This suggests that mGluRs play particularly important roles in the operations of the MOB network. Furthermore, the localization of mGluR1 to mitral cell apical dendrites suggests a role for mGluR1 at glutamatergic synapses of olfactory nerve terminals in the glomeruli (van den Pol 1995) and/or glutamate-mediated recurrent excitation (i.e., spillover) among mitral cell apical dendrites (Aroniadou-Anderjaska *et al,* 1999b; Isaacson 1999; Carlson *et al,* 2000; Friedman and Strowbridge 2000; Schoppa and Westbrook 2001; Salin *et al,* 2001, De Saint Jan and Westbrook, 2007). mGluR1 is also found on mitral cell lateral dendrites, presynaptic to granule cell dendrites (van den Pol 1995). Here, mGluR1 may function to facilitate and/or prolong depolarization of the lateral mitral cell dendrite following glutamate release from neighboring mitral and tufted cells. mGluR5 is found on granule cell dendrites in the external plexiform layer, where they receive glutamatergic synapses from mitral/tufted cell lateral dendrites (van den Pol, 1995). In agreement with their high level of expression and pattern of localization, new findings discussed above demonstrate that the excitability of mitral and granule cells is directly and potently modulated via activation of mGluRs by endogenously released glutamate in MOB

slices (Heinbockel *et al*, 2004; 2007a, b). Ambient glutamate acting at mGluRs tonically modulates neurons in the MOB and hence the operation of the MOB neural network. The results show that mGluR activation in the main olfactory bulb has potent effects on key features of mitral cells and granule cells, including intrinsic membrane properties, excitatory drive, action potential discharge, and release of neurotransmitter.

Olfactory processing in mitral cells involves oscillatory and synchronous temporal firing activity (Kauer 1998; Kay and Laurent 1999; Friedrich and Laurent 2001; Luo and Katz 2001; Spors and Grinvald 2002). mGluR1 on mitral cell apical dendrites plays a role in olfactory nerve-evoked excitation of mitral cells (Heinbockel *et al*, 2004; De Saint Jan and Westbrook 2005, 2007; Ennis *et al*, 2006; Yuan and Knopfel, 2006). mGluR1 mediates mitral cell responses to ON input such that mGluR1 agonists depolarize mitral cells while mGluR antagonists attenuate ON-evoked spiking and slow oscillations in mitral cells (Schoppa and Westbrook 2001; Heinbockel *et al,* 2004). ON-evoked excitatory postsynaptic potentials in a subset of mitral cells exhibit a small mGluR1-mediated component in normal physiological conditions (De Saint Jan and Westbrook, 2005, 2007). mGluR1-mediated EPSPs elicited by single ON pulses are limited by glutamate uptake mechanisms (De Saint Jan and Westbrook, 2005). To determine the specific contribution of mGluR1 in mitral cell responses, Ennis *et al* (2006) stimulated the olfactory nerve over a range of frequencies comparable to odor-evoked firing frequencies of olfactory receptor neurons *in vivo* (Duchamp-Viret *et al,* 1999). Their strategy demonstrated that glutamate transporters tightly regulate access of synaptically-evoked glutamate from ON terminals to postsynaptic mGluR1s on mitral cell apical dendrites (Ennis *et al*, 2006). mGluR1 may not play a major role in phasic responses to ON input (Ennis *et al,* 2006), but instead may play an important role in shaping slow oscillatory activity in mitral cells and/or activity-dependent regulation of plasticity at ON-mitral cell synapses (Schoppa and Westbrook 2001; Yuan and Knopfel, 2006). This idea is supported by the finding that a single stimulation of the ONL can evoke an EPSP in mitral cells lasting several seconds, which results from dendritic glutamate release from the intraglomerular network activating mGluR1 receptors along with NMDA receptors (De Saint Jan and Westbrook, 2007). Glutamate-spillover mediates recurrent activation of mGluR1: the long-lasting responses in mitral cells to which the spillover contributes would amplify incoming olfactory information and facilitate odor-evoked slow temporal activity in the MOB. Hence, mitral cells that are strongly activated following odorant stimulation of the nasal epithelium release glutamate that can lead to spillover-mediated activation of mGluRs among mitral and tufted cells. Furthermore, such spillover-mediated excitation may, in turn, presynaptically enhance glutamate release onto granule cells and increase lateral inhibition and contrast between strongly and weakly activated mitral cells (van den Pol 1995; Kinzie *et al,* 1998).

As has been shown at several CNS synapses, mGluR-mediated excitation typically has a slow time-course and is maximally activated by high frequency activity (Batchelor and Garthwaite, 1977; Shen and Johnson, 1997; Tempia *et al,* 1998, 2001; Anwyl, 1999; Reichelt and Knopfel, 2002; Huang *et al,* 2004; Karakossian and Otis, 2004). This is important both for sensory responses of mitral cells to odorant stimulation as well as for dendrodendritic GABA release from granule cells onto mitral/tufted cells. mGluR1 responses in mitral cells might be preferentially activated over successive sniff cycles during odor inhalation. Continuous activation of mGluR1 by glutamate could thus serve to facilitate or amplify sensory responses of mitral cells. Consistent with this, a mGluR antagonist has been found to

reduce olfactory nerve-evoked rhythmic oscillations in mitral cells (Schoppa and Westbrook 2001).

Long-term synaptic plasticity has been described for glutamate synapses between olfactory afferents and mitral cells. Ennis *et al* (1998) reported long-term potentiation (LTP) at the level of the olfactory nerve to mitral cell synapses. A short high frequency tetanus applied to the olfactory nerve resulted in LTP of NMDA receptor-mediated excitation of mitral cells. Furthermore, experience-dependent modification through olfactory deprivation can alter the strength of primary olfactory synapses with juxtaglomerular neurons (Tyler *et al,* 2007). Odor deprivation triggered compensatory modifications through both pre- and postsynaptic mechanisms by increasing (a) the glutamate release probability from olfactory afferents in MOB glomeruli, (b) the amplitude of quantal synaptic currents mediated by AMPA- and NMDA-type glutamate receptors, and (c) the abundance of ionotropic glutamate receptors in glomerular circuits. Recently, Mutoh *et al* (2005) using a low frequency stimulation paradigm at olfactory afferent to mitral cell synapses observed mGluR-mediated long-term depression (LTD) that is expressed presynaptically and does not require fast synaptic transmission. LTP at this synapse could be involved in rapid olfactory memory formation whereas LTD at this synapse could be relevant for the establishment, maintenance, and experience-dependent refinement of odor maps in the MOB (Mutoh *et al*, 2005).

Conclusion

Glutamate receptors thus have multiple roles at all levels of processing in the MOB. Primary sensory input is brought to the brain through synaptic glutamate release from terminals of olfactory receptor neurons, and olfactory information is processed through glutamatergic activation of local interneurons by MOB output neurons. While communication at these synapses is primarily mediated by iGluRs, specific mGluR subtypes increase the excitability of mitral cells and interneurons via modulation of distinct ionic conductances in these cells. Importantly, the net effect of these mGluR actions is to increase excitatory drive from mitral and tufted cells, and in turn, to facilitate inhibitory feedback from granule cells and periglomerular cells (Heinbockel *et al*, 2007a; Dong *et al*, 2007). Patch clamp recording and voltage-sensitive dye imaging experiments (Heinbockel *et al*, 2007a) suggest that a major functional consequence of mGluR activation is to increase both the spatial spread and temporal duration of lateral inhibition in the MOB. Emerging evidence suggests that the dynamics of iGluR activation of inhibitory interneurons, from multiple sources, influences their inhibitory regulation of MOB output (Balu *et al*, 2007; Schoppa, 2006). Activation of mGluRs on mitral cells, periglomerular cells and granule cells may serve to prolong the temporal window of lateral inhibition beyond the activation of iGluRs and amplify lateral inhibition during repeated sniff cycles. iGluRs and mGluRs thus act at the MOB network level to dynamically regulate output to higher olfactory centers, and integrate feedback from these centers into olfactory processing within the MOB.

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