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Molecular diversity of glutamate receptors and their physiological functions

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Summary

Glutamate receptors play an important role in many integrative brain functions and in neuronal development. We report the molecular diversity of NMDA receptors and metabotropic glutamate receptors on the basis of our studies of molecular cloning and characterization of the diverse members of these receptors. The NMDA receptors consist of two distinct types of subunits. NMDAR1 possesses all properties characteristic of the NMDA receptor-channel complex, whereas the four NMDAR2 subunits, termed NMDAR2A-2D, show no channel activity but potentiate the NMDAR1 activity and confer functional variability by different heteromeric formations. The NMDA receptor subunits are considerably divergent from the other ligand-gated ion channels, and the structural architecture of these subunits remains elusive. The mGluRs form a family of at least seven different subtypes termed mGluR1-mGluR7. These receptor subtypes have seven transmembrane segments and possess a large extracellular domain at their N-terminal regions. The seven mGluR subtypes are classified into three subgroups according to their sequence similarities, signal transduction mechanisms and agonist selectivities: mGluR1/mGluR5, mGluR2/mGluR3 and mGluR4/mGluR6/mGluR7.

On the basis of our knowledge of the molecular diversity of the NMDA receptors and mGluRs, we have studied the physiological roles of individual receptor subunits or subtypes. We have shown that K^+-induced depolarization or NMDA treatment in primary cultures of neonatal cerebellar granule cells induces the functional NMDA receptor and specifically up-regulates NMDAR2A mRNA among the multiple NMDA receptor subunits through the increase in resting intracellular Ca^{2+} concentrations. Our study demonstrates that the regulation of the specific NMDA receptor subunit mRNA governs the NMDA receptor induction that is thought to play an important role in granule cell survival and death. Analysis of an agonist selectivity and an expression pattern of mGluR6 has indicated that mGluR6 is responsible for synaptic neurotransmission from photoreceptor cells to ON-bipolar cells in the visual system. We have also investigated the function of mGluR2 in granule cells of the accessory olfactory bulb by combining immunoelectron-microscopic analysis with slice-patch recordings on the basis of the identification of a new agonist selective for this receptor subtype. Our results demonstrate that mGluR2 is present at the presynaptic site of granule cells and modulates inhibitory GABA transmission from granule cells to mitral cells. This finding indicates that the mGluR2 activation relieves excited mitral cells from GABA inhibition but maintains the lateral inhibition of unexcited mitral cells, thus resulting in enhancement of the signal-to-noise ratio between the excited mitral cells and their neighboring unexcited mitral cells.

Introduction

Glutamate receptors mediate excitatory neurotransmission and play an important role in neuronal plasticity and neurotoxicity in the central nervous system (Nakanishi, 1992; Nakanishi and Masu, 1994). These receptors are essential for inducing long-lasting changes in neuronal responsiveness which are thought to underlie learning, memory, and neuronal development. They also play a

critical role in pathophysiological processes such as epilepsy and ischemic neuronal cell death. The diverse functions of glutamate neurotransmission are mediated by a variety of glutamate receptors that are classified into two major groups termed ionotropic and metabotropic receptors (Nakanishi, 1992; Nakanishi and Masu, 1994). The former receptors can be subdivided into N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors, both of which contain glutamate-gated, cation-specific ion channels. The latter receptors (mGluRs) are coupled to intracellular signal transduction through G proteins. For the past few years, we have studied the properties, functions and regulation of the NMDA receptors and mGluRs, which were molecularly isolated by using our novel receptor cloning strategy that combined electrophysiology and a *Xenopus* oocyte expression system (Masu *et al.*, 1987; Masu *et al.*, 1991; Moriyoshi *et al.*, 1991). This article deals with the molecular nature and functions of the diverse members of the NMDA receptors and mGluRs and discusses some of the physiological roles of different glutamate receptors.

Results and Discussion

Molecular diversity of NMDA receptors and metabotropic glutamate receptors

Our molecular cloning studies demonstrated that the NMDA receptors consist of two distinct types of subunits, one termed NMDAR1 and the other four termed NMDAR2A-2D (Moriyoshi *et al.*, 1991; Sugihara *et al.*, 1992; Ishii *et al.*, 1993). NMDAR1 possesses all properties characteristic of the NMDA receptor-channel complex, including agonist and antagonist selectivity, glycine modulation, voltage-dependent Mg^{2+} blockade, Ca^{2+} permeability and Zn^{2+} inhibition (Moriyoshi *et al.*, 1991; Sugihara *et al.*, 1992; Karp *et al.*, 1993). NMDAR2A-2D show no channel activity in their homomeric structures but potentiate the NMDA receptor activity in combined expression with NMDAR1 (Ishii *et al.*, 1993). These subunits also confer functional variability by different heteromeric subunit configurations (Ishii *et al.*, 1993). The NMDAR1 mRNA is ubiquitously expressed throughout the brain regions, whereas individual NMDAR2 mRNAs are distinctly distributed in different brain regions (Moriyoshi *et al.*, 1991; Ishii *et al.*, 1993). The functional heterogeneity of the NMDA receptors in different neuronal cells is thus produced by the functional and anatomical differences of the NMDAR2 subunits. All of the NMDA receptor subunits, like the other ligand-gated ion channels, were initially thought to comprise four membrane-spanning domains preceded and followed by extracellular domains at both the N-terminal and C-terminal sides. However, the NMDA receptors are by

far divergent from the other ligand-gated ion channels in their amino acid sequences, and several lines of recent evidence suggested that the C-terminal tail is located on the intracellular side rather than on the extracellular side. A transmembrane model of the NMDA receptors is thus illustrated in Fig. 1 under the assumption that the N-terminal and C-terminal portions are located

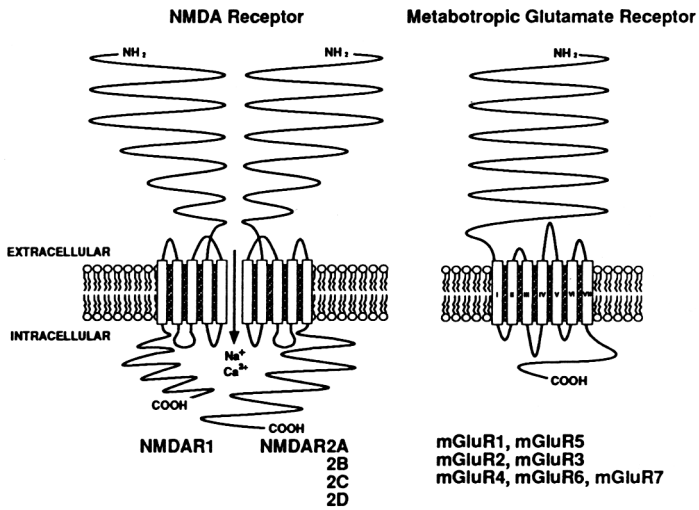

Fig. 1 Transmembrane models of the NMDA receptors and metabotropic receptors. It has generally been accepted that the mGluR subtypes comprise seven membrane-spanning domains with a large extracellular N-terminus, whereas the number of transmembrane segments of the NMDA receptors and their topology relative to the membrane remain unsettled.

on the extracellular and intracellular sides, respectively. However, the number of transmembrane segments and the transmembrane topology of the NMDA receptors still remain elusive. The mutational analysis of NMDAR1 indicated that asparagine at the second transmembrane segment is within a channel pore and is responsible for governing a high Ca^{2+} permeability and the blockades of Mg^{2+} and other channel blockers (Sakurada *et al.*, 1993).

The mGluRs form a family of at least seven different subtypes termed mGluR1-mGluR7 (Masu *et al.*, 1991; Tanabe *et al.*, 1992, 1993; Abe *et al.*, 1992; Nakajima *et al.*, 1993; Okamoto *et al.*, 1994). These receptor subtypes have seven transmembrane segments common to the other members of G protein-coupled receptors but possess a large extracellular domain at their N-terminal regions and thus represent a novel family of G protein-coupled receptors (Fig. 1). The seven mGluR subtypes differ in their agonist selectivities and signal transduction mechanisms as analyzed in CHO cells by DNA transfection (Masu *et al.*, 1991; Tanabe *et al.*,

1992, 1993; Aramori *et al.*, 1992; Abe *et al.*, 1992; Nakajima *et al.*, 1993; Okamoto *et al.*, 1994). mGluR1 and mGluR5 are coupled to the IP_3/Ca^{2+} signal transduction and efficiently respond to quisqualate. The other five are all linked to the inhibitory cyclic AMP cascade, but their agonist selectivities are totally different between the mGluR2/mGluR3 subgroup and the mGluR4/mGluR6/mGluR7 subgroup. The former effectively interacts with trans-1-aminocyclopentane-1, 3-dicarboxylate (trans-ACPD), whereas the latter potently reacts with L-2-amino-4-phosphonobutyrate (L-AP4). The seven subtypes of the mGluR family are thus classified into three subgroups according to their sequence similarities, signal transduction mechanisms and agonist selectivities: mGluR1/mGluR5, mGluR2/mGluR3 and mGluR4/mGluR6/mGluR7 (Nakanishi, 1992; Nakanishi and Masu, 1994). All but mGluR6 mRNA are widely but distinctly distributed in various brain regions (Tanabe *et al.*, 1992, 1993; Abe *et al.*, 1992; Shigemoto *et al.*, 1992; Ohishi *et al.*, 1993a, 1993b; Okamoto *et al.*, 1994). These findings strongly indicate that the individual mGluR subtypes have their own functions by specializing the signal transduction and expression patterns in different nerve cells. The chimeric experiments between mGluR1 and mGluR2 demonstrated that the amino-terminal extracellular domain serves as a glutamate binding site of mGluR (Takahashi *et al.*, 1993). Thus, the mode of agonist binding of mGluR is different from that of the other G protein-coupled receptors for small molecule transmitters.

Physiological functions of different glutamate receptors

In order to understand the physiological role of glutamate transmission in integrative brain functions and development, it is essential to characterize the functions and regulation of individual glutamate receptors on the basis of our knowledge of the diverse members of these receptors. The NMDA receptors are known to cause neuronal degeneration and neuronal cell death but are also required for survival of certain neuronal cells. The involvement of the NMDA receptors in neuronal cell death and survival has been well characterized in primary cultures of neonatal cerebellar granule cells (Balázs *et al.*, 1992). K^+ depolarization or NMDA treatment promotes the survival of cultured granule cells, and these cells become sensitive to NMDA toxicity after prolonged K^+ depolarization (Cox *et al.*, 1990; Balázs *et al.*, 1992). The above treatments are thought to produce an influence that mimics the physiological stimulation of immature granule cells during cerebellar development. Primary cultures of cerebellar granule cells thus provide a useful system to study the role of the glutamate receptors in neuronal

functions. We investigated the function and regulation of the glutamate receptors in cultured cerebellar granule cells (Bessho *et al.*, 1993; Bessho *et al.*, 1994).

Primary cultures of cerebellar granule cells were prepared from 6-day-old rats, and these cells were treated with high KCl or NMDA. Both treatments induced functional NMDA receptors as assessed by fura-2 fluorescence analysis of NMDA receptor-mediated intracellular Ca^{2+} increase. When the effects of K^+ depolarization and NMDA treatment on mRNA levels of individual NMDA receptor subunits were examined by Northern blot analysis, both treatments were found to specifically up-regulate the NMDAR2A mRNA among the multiple NMDA receptor subunits. Furthermore, antisense oligonucleotides specific for NMDAR2A mRNA prevented the induction of functional NMDA receptors, thus confirming the contribution of the specific up-regulation of NMDAR2A mRNA in the induction of the NMDA receptor. When Ca^{2+} influx was blocked during K^+ depolarization by the Ca^{2+} channel blocker nifedipine or during NMDA treatment by the NMDA receptor antagonist D-2-amino-5-phosphonovalerate, this block abolished both the NMDA receptor induction and the up-regulation of NMDAR2A mRNA. Thus, our investigation demonstrates that the increase in intracellular Ca^{2+} governs the up-regulation of the specific NMDA receptor subunit mRNA that is responsible for the NMDA receptor induction. It has been supposed that modestly elevated intracellular Ca^{2+} promotes neuronal cell survival, whereas substantially elevated intracellular Ca^{2+} leads to neuronal cell death (Franklin and Johnson, 1992). Thus, it is very likely that the induction of the NMDA receptor is involved in the survival of granule cells by maintaining moderate levels of intracellular Ca^{2+}, but excess stimulation of the elevated NMDA receptor results in substantial increase in intracellular Ca^{2+} and causes granule cell death. However, we also found that NMDA is very toxic after prolonged (5 days) K^+ depolarization, but not so after short-term (1 day) depolarization, although the NMDA receptor markedly increases under both conditions. It is thus plausible that the NMDAR2A mRNA up-regulation is necessary, but may not be sufficient, for the NMDA toxicity in granule cells after K^+ depolarization.

Because studies of mGluRs have been initiated only recently, the physiological roles of this receptor family largely remain unknown. One interesting system of mGluRs is glutamate-mediated synaptic transmission within the visual system. Recent electrophysiological studies indicated that the L-AP4-sensitive mGluR is responsible for synaptic transmission between photoreceptor cells and ON-bipolar cells through the coupling to the cyclic GMP cascade (Nawy and Jahr, 1990; Shiells and Falk, 1990). To investigate the mGluR in the visual system, we

screened a retinal cDNA library. We identified mGluR6 from the retinal library and characterized its properties and expression in detail (Nakajima *et al.*, 1993). When the agonist selectivity of mGluR6 was examined in CHO cells after DNA transfection, the selective and potent response to L-AP4 and L-serine-O-phosphate was found, thus consistent with the property of the mGluR reported in ON-bipolar cells. The expression of mGluR6 mRNA is restricted in the retina. Furthermore, *in situ* hybridization signals of mGluR6 mRNA were exclusively observed in the inner nuclear layer where ON-bipolar cells are known to be distributed in the retina. When the cellular localization of mGluR6 was examined by immunocytochemical analysis using a polyclonal antibody raised against the mGluR6 protein, the mGluR6 immunoreactivity was confined to the boundary between the photoreceptor cell layer and the bipolar cell layer, indicating that mGluR6 is located at the postsynaptic site. It is, however, to be noted that mGluR6 expressed in CHO cells is coupled to the inhibitory cyclic AMP cascade and is thus different in signal transduction from the cyclic GMP-coupled mGluR in ON-bipolar cells. However, it is also known that when a preferred G protein or subsequent effectors are absent in transfected cells, the receptor expressed in a heterologous system is capable of modulating different second messengers. It is thus plausible that mGluR6 corresponds to the L-AP4 recepor in ON-bipolar cells.

On the basis of the above findings of mGluR6, together with the electrophysiological characterization of mGluR-mediated signal transduction in ON-bipolar cells (Nawy and Jahr, 1990; Shiells and Falk, 1990), the following model can be discussed for synaptic transmission between photoreceptor cells and ON-bipolar cells. When light activates the photoreceptor, intracellular concentrations of cyclic GMP decrease as a result of the stimulation of phosphodiesterase through the activation of transducin. The decrease in cyclic GMP concentrations leads to the closure of the cyclic GMP-gated ion channel and hyperpolarizes the photoreceptor cells. This hyperpolarization reduces glutamate release. Under this situation, the mGluR6-G protein-phosphodiesterase system in ON-bipolar cells becomes inactive, and high concentrations of cyclic GMP are maintained in ON-bipolar cells and stimulate the cyclic GMP-gated ion channel, thus resulting in depolarization of ON-bipolar cells. Glutamate release from ON-bipolar cells is augmented and in turn excites ganglion cells, and this excitation is transmitted to the brain. Our investigation thus raises an interesting possibility that a specific mGluR subtype plays a critical role in synaptic transmission in the visual system.

We also defined the physiological role of mGluR2 in sensory transmission of the accessory

olfactory bulb (AOB) by combining immunoelectron microscopy and slice-patch recording on the basis of the identification of a new selective agonist for this receptor subtype (Hayashi *et al.*, 1993). Our previous study indicated that two extended forms of 2-(carboxycyclopropyl)glycines (CCGs) are potent and selective agonists for the mGluR family (Hayashi *et al.*, 1992). Recently, Ohfune and co-workers developed a new CCG derivative, (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine, abbreviated as DCG-IV (Ishida *et al.*, 1993). We tested the agonist potency and selectivity of this compound for different mGluR subtypes expressed in CHO cells. DCG-IV was found to be a very potent and specific agonist for mGluR2/mGluR3 among the mGluR family members. DCG-IV is also capable of binding to the NMDA receptor, but this potency is much less than that for the activation of mGluR2/mGluR3. DCG-IV has no effects on the AMPA/kinate receptors. DCG-IV is thus a very useful agonist for mGluR2.

Because the prominent expression of mGluR2 mRNA in granule cells of the AOB was observed by *in situ* hybridization analysis, the subcellular localization of mGluR2 was examined immunoelectron-microscopically with the aid of a polyclonal antibody raised against the mGluR2 protein expressed in *E. coli*. This analysis indicated that an immunoreactivity of mGluR2 is confined to dendrites of granule cells of the AOB. In the AOB, mitral cells receive afferent inputs from the vomeronasal nerve and transmit excitatory outputs to various brain regions. Granule cells are inhibitory interneurons that form typical dendrodendritic synapses with mitral

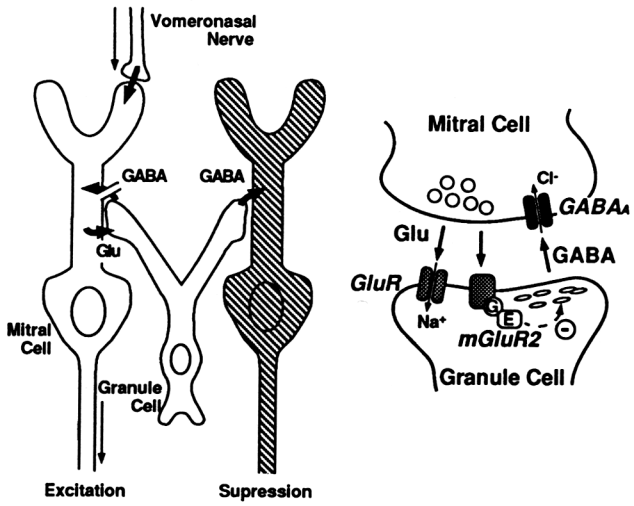

Fig. 2. A model of a regulatory role of mGluR2 in the olfactory transmission of the accessory olfactory bulb. $GABA_A$, $GABA_A$ receptor; GluR, ionotropic glutamate receptor. For detailed explanation, see the text.

cells. These synapses undergo reciprocal regulation, in which the granule cell is excited by glutamate from the mitral cell and exerts an inhibition onto the mitral cell by GABA (Fig. 2). We investigated the role of mGluR2 in synaptic transmission between the mitral cell and granule cell by examining the effect of a newly identified agonist DCG-IV on the GABA transmission from the granule cell to the mitral cell (Hayashi *et al.*, 1993). Whole cell recording of a mitral cell was performed by the slice-patch method, and extracellular stimuli were applied to a granule cell. As expected, when a granule cell was electrically stimulated, GABA-mediated inhibitory postsynaptic currents (IPSCs) were evoked in a mitral cell. When mGluR2 was activated by the addition of DCG-IV in this system, IPSCs were markedly reduced in a reversible manner.

These observations as well as others lead to the conclusion that glutamate released from the mitral cell activates mGluR2 at the presynaptic site of the granule cell and relieves a GABA-mediated inhibition onto the mitral cell (Fig. 2). The granule cell lacks an axon and forms divergent synaptic contacts with not only the original mitral cell but also a large number of neighboring mitral cells. The excitation of mitral cells thus evokes lateral inhibition in the neighboring mitral cells through the divergent synaptic formations with trans-synaptically excited granule cells. Under the mechanism revealed in our study, however, it can be postulated that the GABA inhibition is relieved in excited mitral cells by the activation of mGluR2. Furthermore, this activation is thought to be confined to the synapses of the excited mitral cells and would thus maintain the lateral inhibition of unexcited neighboring mitral cells. This mechanism would evidently enhance the signal-to-noise ratio between the excited mitral cells and their neighboring mitral cells (Fig. 2). It is thus tempting to speculate that the mGluR2-mediated modulation in the microcircuitry between the mitral cell and granule cell plays an important role in discrimination and resolution of the olfactory sensory transmission in the AOB.

It has now been revealed that both NMDA receptors and mGluRs are more diversified than previously envisioned. Our investigations discussed here demonstrate that different glutamate receptors have their own functions and play important roles in the regulation of glutamate or other transmissions involved in integrative brain functions and neuronal development. Further study of not only the physiological roles of multiple glutamate receptors but also the cooperative functions of different glutamate receptors will undoubtedly be interesting and important for understanding glutamate-mediated brain functions.

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