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Synaptic transmission and plasticity in the spinal cord substantia gelatinosa: the role of GluR2, GluR5 and GluR6 glutamate receptor subunits

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Synaptic transmission and plasticity in the spinal cord substantia gelatinosa: The role of GluR2, GluR5 and GluR6 glutamate receptor subunits

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ATPA	(RS)-2-Amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid
CGP55845	(2S)-3[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phosphinic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
ConA	concanavalin A
DA	domoic acd; (2S,3S,4R,5'R)-2-carboxy-4-(5'-carboxy-1'-methyl-1Z,3E-hexadienyl)-3-pyrrolidineacetic acid
DAP5	D-(-)-2-amino-5-phosphonopentanoic acid
DH	dorsal horn
DNQX	6,7-dinitroquinoxaline-2,3-dione
DR	dorsal root
DRG	dorsal root ganglia
EPSC	Excitatory postsynaptic current
EPSP	fast excitatory postsynaptic potentials
ES	embryonic stem
GluR	Glutamate receptors
GYKI52466	(1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine)
GYKI53655	(1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine).
HFS	high-frequency stimulation
HVAC	high voltage activated Ca^{2+} (HVAC) channels
iGluR	Ionotropic GluRs
I-V	current-voltage
JSTX	Joro Spider toxin
KA	kainic acid; (2S,3S,4R)-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid
LTD	long-term depression
LTP	long-term potentiation
LY382884	(3S,4aR,6S,8aR)-6-(4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid

MCPG	(S)-α-methyl-4-carboxyphenyl glycine
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotropic GluRs
MK801	(5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5, 10-imine maleate
NBQX	2,3-dioxy-6-nitro-1,2,3,4 tetrahydrobenzero-7-sulphanoylbenzo[f]quinoxaline-2,3-dione
NK1	neurokinin 1
NMDA	N-methyl-D-aspartate
RI	rectification index
RS-PCR	reverse transcription polymerase chain reaction
SDH	superficial spinal dorsal horn
SG	substantia gelatinosa
SYM 2081	((2*S*-4*R*) diastereomer of 4-methylglutamate)
SYM 2206	((±)-4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine)
TTX	tetrodotoxin

ABSTRACT

To understand the physiological role of the AMPA-type or kainate-type ionotropic glutamate receptors and their participation in sensory information processing, including pain, it will be necessary to develop a comprehensive description of their actions in the adult mouse spinal cord substantia gelatinosa (SG) region. Without selective antagonists of the AMPA and kainate receptors, however, pharmacology has provided little assistance in this endeavor. In this study, gene-targeted mice lacking GluR2 AMPA subunit and GluR5 or GluR6 kainate receptor subunits were used to identify the receptor subunits that comprise the AMPA and KA receptors responsible for modulation of primary afferent neurotransmission.

AMPA receptors are not thought to be involved in the induction of LTP of excitatory synaptic transmission in the SG region, but they may be involved in the expression via several messenger pathways. However, one subunit of the AMPA receptors, GluR2, is known to control Ca^{2+} influx. To test whether GluR2 plays any role in the induction of LTP, the mice lacking the subunit were used in the present work. In GluR2 mutants, LTP in the SG region of spinal slices was markedly enhanced. These results suggest an important role for GluR2 subunit of AMPA receptors in regulating synaptic plasticity and pain behavior.

In this study, gene-targeted mice lacking GluR5 or GluR6 kainate receptor subunits have also been used to identify the receptor subunits that comprise the kainate receptors responsible for presynaptic modulation of primary afferent neurotransmission. In the presence of synaptic inhibition, both GluR5 and GluR6 subunits contribute to the depressant action of kainate at the C-fiber and Aδ-fiber-activated polysynaptic pathways. In the absence of synaptic inhibition, the GluR6 subunit is critically involved in inhibiting transmission at both Aδ- and C-fiber monosynaptic pathways, whereas GluR5 plays a lesser role in inhibiting the C-fiber-activated pathway. Both GluR5 and GluR6 KA receptor subunits contribute to the KA receptor-mediated facilitation of excitatory synaptic transmission at synapses on the SG neurons. These results indicate that AMPA and kainate receptors play multiple and complex roles in regulation of excitatory synaptic transmission in the spinal cord SG region with potentially significant implications for pain control.

CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

This dissertation consists of five chapters (general introduction, materials and methods, results (AMPA receptor and kainate receptor), and general conclusion) and a list of references cited. The chapters, AMPA receptor and kainate receptor, contain research results which were formulated as introduction, results and discussion for future publications.

The dissertation contains a large part of the experimental results obtained by the author during the course of his graduate study under the supervision of Dr. Mirjana Randić.

Research Objective

The dorsal horn (DH), especially substantia gelatinosa (SG) of the spinal cord, is known to play an important role in the modulation of nociceptive transmission through fine myelinated and unmyelinated primary afferent fibers arising from the periphery. Glutamate, or a related amino acid, is the major excitatory neurotransmitter at primary afferent fiber-spinal DH neuron synapses (so called glutamatergic synapses). Elucidating the function of glutamate and their receptors has traditionally relied on pharmacological approaches using compounds that act as agonists or antagonists at specific receptors on the pre- and postsynaptic membranes. Recently, a new tool has been added to the armamentarium for studying the synaptic function, namely genetic manipulations (esp. null mutations or 'knockouts') that delete specific subunits comprising glutamate receptors.

The objective of this research was to investigate the role(s) of glutamate receptor subunits (GluR2, GluR5, or GluR6) in synaptic transmission and plasticity in the spinal DH region by using the genetic pharmacological tools. The specific purpose of the conducted experiments was to study physiological consequences resulting from the genetic deletion of specific glutamate receptor subunits at primary afferent fiber-spinal DH neurons synapses by recording electrical properties of passive (e.g. resting membrane potential, input resistance of SG neuronal membranes) and active (e.g. spontaneously or electrical stimulation-evoked excitatory postsynaptic potentials/currents) membranes.

The experiments used current-clamp intracellular recordings with sharp microelectrode, and also whole-cell voltage-clamp recordings from *in vitro* adult mouse spinal cord slices with/without attached dorsal roots. Mice with targeted mutations deleting the GluR2, GluR5, or Glur6 have kindly provided by professor S. F. Heinemann (The Salk Institute, San Diego).

Literature Review

This section reviews the anatomical and functional organization of spinal dorsal horn, and also glutamate receptors-mediated synaptic transmission and plasticity, in order to provide a background information for the study of the role(s) of glutamate receptor subunits in the modulation of excitatory synaptic transmission.

1. Anatomical and functional organization of the spinal cord dorsal horn

Information from sensory receptors in skin, muscle, joints and viscera is transmitted to central nervous system (CNS) via primary sensory neurons (Campbell et al., 1989; Gardner and Martin, 2000; Gardner et al., 2000). The cell bodies of the primary sensory neurons are located in dorsal root ganglia (DRG). Each ganglion cell gives off an axon that branches into a peripheral process and a central process (Ramón y Cajal, 1909). The peripheral process contributes to a peripheral nerve and terminates peripherally as a sensory receptor. The central process gives rise to a numerous collateral branches (Langford and Coggeshall, 1979, 1981) and enters into spinal cord through a dorsal root. Together these two processes form primary afferent fibers that transmit the encoded stimulus information to the spinal cord or brain stem.

A) Primary sensory neurons in dorsal root ganglia

Primary sensory neurons have been classified into three groups on the basis of the perikaryal size, distribution of cellular organelles, neurochemistry and chemosensitivity (Harper and Lawson, 1985a, b; Sugiura et al., 1986). Large DRG cells (Type A, 30-70 μm in diameter) have short-duration action potentials (0.49 -1.35 ms at the base) that are tetrodotoxin-sensitive, and these cells give off large diameter myelinated axons (Aα and Aβ). Small DRG cells (Type B, 25 -30 μm in diameter) produce action potentials of long duration (0.5 - 8.0 ms at the base), which in some cases are tetrodotoxin-insensitive. They usually give off fine myelinated (Aδ) and unmyelinated (C) fibers. In addition to these two cell groups, a number of studies describe the existence of intermediate-size cells, which are associated with Aδ-fibers (Mastuura, 1967; Honda et al., 1983; Harper and Lawson, 1985a). The conduction velocity and the threshold for exciting fibers are inversely related to axonal diameter (Table 1). The larger the fiber, the faster the conduction velocity and the lower the threshold.

Sensory neurons with A- or C-fibers may have differences in function. It is well known that stimulation, from the external environment, of mechanoceptive neurons with A-fibers gives rise to a

well-localized sharp pricking sensation, while stimulation of nociceptive neurons with C-fibers is said to give rise to poorly localized unpleasant sometimes burning-type or 'unbearable' sensation (Törebjork and Ochoa, 1990).

B) Sensory receptors at peripheral terminals of primary sensory neurons

The peripheral processes of the DRG cells have different morphological and functional properties. The encapsulated processes of them have mechanoreceptors and proprioceptors, which are responsible for mediating the somatic modalities of touch and proprioception, respectively (Gardner et al., 2000). These receptors are innervated by DRG with large-diameter, myelinated axons that conduct action potentials rapidly. On the contrary, the processes with bare nerve endings contain nociceptors and thermal receptors which mediate painful or thermal sensations, respectively (Gardner et al., 2000). These receptors are innervated by DRG neurons with either unmyelinated or thinly myelinated axons that conduct impulses more slowly. Table 2 demonstrates relationships among sensory receptor types, afferent fiber types, and sensory modalities. This knowledge states that individual DRG neurons respond selectively to specific types of stimuli because of morphological and molecular specialization of their peripheral terminals, further supporting the notion of "laws of specific nerve energies", proposed by Johannes Müller in 1826, that morphologically distinct receptors transduce particular forms of energy and transmit information to the brain through distinct pathways dedicated to that modality (Gardner and Martin, 2000).

C) Central terminals of primary sensory neurons

Generally, the central processes of primary sensory neurons enter the spinal cord through the dorsal roots (However, see also Coggeshall et al., 1980 for exception; i.e. there are 30% of the total unmyelinated fibers entering through the ventral roots in L6-S1 segments). Primary afferent fibers, once entering dorsal root entry zone, give off most of their collaterals in their spinal cord segment of entry, but rostrocaudal spread is also significant. The spread is larger for the fibers in the medial (Aδ) than those in the lateral part (C) of Lissauer's tract (Chung et al., 1979); the tract that is located between the dorso-lateral edge of the dorsal horn (DH) and the surface of the spinal cord.

The distribution of primary afferent fibers in the spinal DH is in an orderly way based on fiber size and sensory modality. Most small afferents with either fine myelinated (Aδ) or unmyelinated (C) end predominantly in laminae I and II, although a few reach lamina III-VI laminae (Light and Perl, 1979a and b). In detail, high threshold Aδ mechanoreceptors terminate in laminae I and V, while low threshold Aδ mechanoreceptors only terminate in lamina III (Light and Perl, 1979b). Most large (Aβ)

cutaneous afferents, which function as low threshold mechanoreceptors, have a characteristic pattern of arborization in the deeper laminae (III-VI) of the DH (LaMotte, 1977; Brown, 1981). Cutaneous C fibers, the majority of which are polymodal high-threshold nociceptors in the rat (Lynn and Carpenter, 1982), terminate in lamina II (Ralston and Ralston, 1979; Beal and Bichnell, 1981; Nagy and Hunt, 1983), although there is also a contribution to lamina I (Gobel et al., 1981)

In terms of 'neurochemical markers' of primary afferent fibers, many of calcitonin gene-related peptide-immunoreactive peptidergic afferents contain substance P and are nociceptors (Lawson et al., 1997). Substance P-containing afferents (which include both A and C fibers) end mainly in lamina I and the outer layer of lamina II ('IIo'; *see below*), although some penetrate deeper into the DH. Some C fiber-containing somatostatin terminate in lamina IIo (Sakamoto et al., 1999). Approximately, half of the C fibers in a somatic nerve do not contain peptides, but most of these can be revealed by their ability to bind the lectin Bandeiraea simplicifolia isolectin B4 (Silverman and Kruger, 1990). Functions of non-peptidergic C fibers are poorly understood; however, it is believed that this population also includes many nociceptors (Guo et al., 1999; Gerke and Plenderleith, 2001). Although there are no intrinsic neurochemical markers that will specifically label myelinated low-threshold mechanoreceptors, these can be identified by transganglionic transport of cholera toxin B subunit (Robertson and Grant, 1985). If CTb is injected into a peripheral somatic nerve, it is normally transported only by afferents with myelinated axons and this results in labeling of terminals in lamina I (Aδ nociceptors) and in laminae III-VI (Aδ down-hair afferents and Aβ afferents).

D) Cytoarchitecture of the spinal dorsal horn (Laminae I-VI)

The spinal DH essentially consists of the central terminals of primary sensory neurons, projection neurons, intrinsic DH neurons, and descending nerve fibers from the brainstem and other cerebral structures. Anatomically the DH of the spinal cord can be subdivided into six distinct layers (laminae) in the dorsal-ventral direction of the gray matter, which was proposed in cat (Rexed, 1952), as well as in rat (Molander et al., 1984). Rexed's Lamina I and II comprise superficial spinal dorsal horn (SDH) (Laired and Cervero, 1989).

Lamina I, the marginal zone at the edge of the spinal DH, was first described as "thin veil of gray substance, forming the dorsal most part of the spinal gray matter" by Rexed (1952). This area contains from small (5 – 10 μm) to medium (10 - 15 μm) and large (30 - 50 μm) size of neurons in diameter, receiving direct small-diameter Aδ and C primary afferent input with different stimulus modalities from most tissues of the body (Christensen and Perl, 1970) and providing a major output pathway from the spinal cord to higher structures (brainstem and thalamus) (Craig, 1996).

Morphologically, neurons in lamina I can be grouped under basic categories of fusiform, pyramidal and multipolar (cf. there is also 'flattened' which is not always distinguished from multipolar) based on somatic shape and dendritic arborization (Lima and Coimbra, 1986; Zhang et al., 1996). These three morphological types are relatively evenly distributed in the cervical and lumber enlargements (Lima and Coimbra, 1986; Zhang et al., 1996; Zhang and Craig, 1997). Recent intracellular labeling evidence in cats has revealed a direct correspondence between the morphological characteristics of lamina I neurons and their functional responses to natural cutaneous stimuli (Han et al., 1998). Fusiform cells appear to be nociceptive-specific (NS) neurons, responsive only to noxious heat and pinch, whereas multipolar cells are polymodal nociceptive (HPC) neurons responsive to noxious heat, pinch and cold, and pyramidal cells are innocuous thermoreceptive (COLD) neurons, responsive only to cooling (Craig and Kniffki, 1985; Craig and Bushnell, 1994; Dostrovsky and Craig, 1996; Han et al., 1998). The NS cells are dominated by Aδ fiber input, and they can respond tonicallly to maintained noxious mechanical stimuli, so they may be important for 'first pain' (Andrew and Greenspan, 1999). The HPC cells are dominated by C-fiber input and can have slow ongoing discharge; they evince responses that match the psychophysical responses evoked by 'repeated brief contact heat' stimuli (Craig and Andrew, 1999), which are characterized as 'second pain' (Vierck et al., 1997). Golgi studies showed that fusiform cells have unmyelinated axons, but pyramidal and multipolar cells have myelinated axons (Gobel, 1978a; Lima and Coimbra, 1986). Therefore, nociceptive-specific cells may have slow conduction velocities whereas polymodal nociceptive and innocuous thermoreceptive cells faster conduction velocities (Craig and Kniffki, 1985; Craig and Serrano, 1994). On the other hand, by functional features, lamina I neurons can be divided into two groups, ventrolateral tract (VL)-projection and non-projection neurons (Grudt and Perl, 2002). VL-projection neurons are characterized by a relatively thick axon that runs ventrally and medially toward the contralateral spinal cord. Non-projection neurons have axons that are directed ipsilaterally and that frequently exhibit extensive branching within the SDH.

Lamina II is parallel to lamina I, and covered by that layer dorsally and laterally, but not medially. Due to the concentration of small neurons and their processes plus a relative paucity of myelinated axons (McClung and Castro, 1978; Molander et al., 1984), lamina II is observed as a translucent band under the naked eye, or light microscope and is called 'substantia gelatinosa (SG)'. This area is of particular interest since the sensory inputs to this area are entirely undertaken by Aδ and C fibers in nature (Light and Perl, 1979a and b). As in the cat, lamina II can be subdivided into an intensely-stained outer zone with densely packed cells and a less compact inner zone (Molander et al., 1984). These zones are now commonly referred to as the lamina IIo and IIi. The outer zone contains

more small myelinated fibers than the inner zone. In the neuropil of lamina II, the most prominent structures are glomeruli (~5% of total synapses in lamina II) (Coggeshell and Willis, 1991). A glomerulus consists of a central terminal, which is a primary afferent ending, in synaptic contact with several surrounding dendrites and axonal terminals. In the middle of lamina II, type I glomeruli are predominant. The core of type I glomerulus consists of a small, electron-dense axonal terminal that has a corrugated contour and is filled with densely packed spherical vesicles. In the ventral portion of lamina II, type II glomeruli are predominant. The core of this glomerulus has an electron-lucent terminal with a regular contour, and it contains fewer synaptic vesicles. The central terminals in type I glomeruli are thought to be derived from unmyelinated primary afferent fibers, whereas those in type II glomeruli from myelinated fiber (Ribeiro-da-Silva and Coimbra, 1982). The glomeruli are regarded as key structures of the DH because they offer a morphologic basis for a more complex modulation of information transfer than do the more common axodendritic synapses. In lamina II, almost all neurons are intrinsic interneurons (both excitatory and inhibitory). The two predominant cell types in lamina II are 'stalked' and 'islet' cells (Gobel 1975, 1978a, 1978b) (there are also some other types of interneurons, e.g. arboreal cells, II-III border cells and spiny cells). Stalked cells, similar to the 'limitroph' neurons described by Ramón y Cajal (1909), have cell bodies that are generally located in the lamina IIo, dendrites which fan out ventrally and are often covered with spines and stalk-like branches and axons that arborize in lamina I (Todd and Spike, 1993). This type of cells have been thought of excitatory interneurons (Gobel, 1978b) that receive input from at least some Aδ primary afferent fibers (Grudt and Perl, 2002). Islet cells are present throughout lamina II and have dendrites that extend along the rostrocaudal axis of the spinal cord, usually remain within lamina II and often possess characteristic recurrent branches. The axons of islet cells arborize close to the cell body and dendritic tree. An interesting feature of this type of cells is that their dendrites form synapses on nearby dendrites and axonal terminals, i.e. "dendrodendric" and "dendroaxonic" synapses, respectively. It has earlier been suggested that islet cells are inhibitory interneurons (Gobel, 1978b). This proposal was supported by recent findings that neurons with features of islet cell morphology contain GABA and glycine (Todd and Mckenzie, 1989; Todd and Sullivan, 1990). In addition, a recent morphological and electrophysiological study shows that input to the islet cells stemmed from C-afferent fibers (Grudt and Perl, 2002).

Laminae III, IV, V and VI, i.e. deep to substantia gelatinosa, comprise the nucleus proprius. Neurons of the nucleus proprius are either interneurons or projection neurons, receiving from inputs from primary afferent fibers, substantia gelatinosa interneurons, and descending spinal tracts.

Therefore, the main role of the nucleus is to integrate sensory input with information that descends from the brain.

Lamina III forms a broad band across the DH, parallel to both lamina I and II. In cytoarchitectonic studies, lamina III has slightly larger and more widely spaced cells than lamina II (Rexed, 1952). Lamina III is distinguished from lamina II by several features. First, lamina III is characterized by a dense meshwork of fine as well as coarse myelinated axons that are absent or rare in lamina II. This suggests that lamina III is the target of large caliber fibers that reach the DH by way of the dorsal funiculus. Second, whereas synapses with round vesicles (presumably with excitatory action) predominate in lamina II, synapses with flat vesicles (presumably with inhibitory action) are more numerous in lamina III (Ralston, 1979). Third, in contrast to lamina II, where type I glomeruli are most common, type II glomuruli are present exclusively in lamina III (Bennett et al., 1980). Although a large population of lamina III cells are not identified as yet in terms of cell types, two particular cell types, spinocervical tract cells and postsynaptic dorsal column cells which project to the spinocervical nucleus and into the dorsal columns, respectively, have been demonstrated (Brown et al, 1977; Brown and Fyffe, 1981; Brown, 1981). The spinocervical tract cells have their dendrites that are oriented more in the longitudinal than in the transverse plane, that do not travel into lamina I or IIo (Brown, 1981) and that would not get direct input from many fine afferent fibers (Brown, 1981). By contrast, dendrites of the postsynaptic dorsal column cells do travel dorsally into laminae II and I, their dendritic trees are not restricted mediolaterally, and they could have extensive monosynaptic contacts from fine afferent fibers. Although axons from some of the lamina III cells are restricted in this lamina, many other cells have axonal ramifications at least partially outside lamina III, for example, laminae IV-VI, the ipsilateral dorsolateral funiculus and the contralateral ventral funiculus (Light and Kavookjian, 1988). Primary afferent input into lamina III comes from the flame-shaped arbors, which have recently been shown to carry information from hair follicles (Coggeshall and Willis, 1991). Other types of coarse primary afferents that enter lamina III arise from Pacinian corpuscles and rapidly and slowly adapting fibers. On the other hand, although there is fine primary afferent input into lamina III, e.g. unmyelinated axons-forming synapses in lamina III (Sugiura et al., 1986, 1989), it seems to be much less important than the coarse fiber input. Therefore, any C-fiber input in lamina III may be relayed by neurons in the superficial laminae with axons that pass ventrally ('polysynaptic') (Light and Kavookjian, 1988) or by some neurons in lamina III with dendrites that pass dorsally ('monosynaptic') (Szentagothai, 1964; Surmeier et al., 1988; Todd, 1989; De Koninck et al., 1992; Ma et al., 1996).

Lamina IV is a relatively thick layer that extends across the DH. It forms a band beneath laminae II and III but does not possess their characteristic lateral curvature. Lamina IV is different from the substantia gelatinosa by several features: (1) the presence of a larger, fair concentration of medium-sized neurons and of a larger, pyramidal type neuron (Ramón y Cajal, 1909; Scheibel and Scheibel, 1968; Rethelyi, 1984); (2) the absence of glomeruli (Ralston, 1968); and, finally, (3) the fact that the dendrites of typical lamina IV neurons radiate in all planes rather that sagittally (Scheibel and Scheibel, 1968), with a preferential spread in the lateral direction (Proshansky and Egger, 1977). Lamina IV can also be distinguished from lamina III by the heterogeneity of neuronal sizes and the presence of some very large cells compared with more homogeneous, smaller cells that characterize lamina III. The neurons in this layer are of various sizes, ranging from small (approximately 8 by 11 μm) to large (35 by 45 μm) (Coggeshall and Willis, 1991). The largest cells are relatively infrequent, but they are so prominent that there is a general impression that this is a layer with large cells. The dendrites of lamina IV neurons penetrate the overlying laminae II and III, and their axons have two components, a rich local plexus in lamina V (Rastad et al., 1977; Brown et al., 1977) and a main branch that proceeds towards the lateral funiculus. The main branch, which gives off numerous collaterals along its course (Maxwell and Koerber, 1986), has been traced to the spinocervical tract (Brodal and Rexed, 1953; Craig, 1978; Rastad et al., 1977), the dorsal funiculus (Brown, 1981), the spinothalamic tract (Willis et al., 1979) and the spinohypothalamic tract (Burstein et al., 1990). This indicates that the larger lamina IV neurons are long-distance projection neurons. The major input into lamina IV neurons is thought to be terminal ramifications of large myelinated primary afferent fibers. Experimental studies failed to show a fine primary afferent input into lamina IV (LaMotte, 1977; Light and Perl, 1977). Supportively, at the electron-microscopic level, the degeneration of primary afferent fibers and terminals in lamina IV is characterized by neurofilamentous and electron-lucent degeneration, which corresponds to large and fine myelinated afferents, respectively, in contrast to the electron-dense (unmyelinated afferent fibers) degeneration that characterizes laminae I and II (Ralston and Ralston, 1982).

Lamina V forms the neck of the DH, medially limited by white matter and laterally transformed gradually through a mesh of myelinated fibers into white matter. The neurons show even more variability than in the lamina IV (8 by 10 μm to 30 by 40 μm). The dendrites of lamina V neurons, in contrast to those of lamina IV, are planar, extending mediolaterally and dorsoventrally but not sagittally (Scheibel and Scheibel, 1968). Their dendritic arbor forms stacked discs lined up transversely along the length of the cord. The inputs to this lamina are predominantly C fibers from viscera, Aδ fibers from skin and group IV fibers from muscle. In addition, there are also descending

inputs from corticospinal and rubrospinal tract fibers. The projections of lamina V neurons are similar to the ones of lamina IV.

Lamina VI exists only in the cervical and lumbosacral enlargements (Rexed, 1952), where it appears as a distinct band, darker than laminae V and VII, in Nissl stained sections (Coggeshell and Willis, 1991). The cells in this layer are smaller (8 by 8 μm) and more regular in their arrangement than those in lamina V. The dendrites of lamina VI neurons are less regular (Scheibel and Scheibel, 1968). Neurons in this area have complex input from many collaterals of primary afferent axons, which destine to reach ventral horn cells in this area (Raymón y Cajal 1909; Scheibel and Scheibel, 1968)

2. Synaptic transmission in the spinal cord DH

A number of studies indicate that glutamate and aspartate are the major candidates for the fast excitatory neurotransmitters (Mayer and Westbrook, 1987; Kangrga et al., 1988; Yoshimura and Nishi, 1993), whereas tachykinins appear to be involved in the slow excitatory synaptic transmission (Urban and Randić, 1984).

It is presently well known that glutamate, or a related amino acid, is the major excitatory neurotransmitter mediating the fast excitatory transmission in mammalian CNS (Mayer and Westbrook, 1987; Wroblewski and Danysz, 1989) including the spinal DH (Jahr and Jessell, 1985; Kangrga et al., 1988; Gerber and Randić, 1989a; Yoshimura and Jessell, 1990). Glutamate has been found in the DRG neurons (De Biasi and Rustioni, 1988), from which it is released upon activation of the primary afferents (Kangrga and Randić, 1990, 1991). Glutamate produces fast excitatory postsynaptic potentials (EPSPs) in the spinal DH cells via activation of postsynaptic glutamate receptors (Gerber and Randić, 1989a; Yoshimura and Jessell, 1990).

Glutamate receptors (GluRs) fall into two distinct classes: ionotropic and metabotropic receptors. Ionotropic GluRs (iGluRs) are glutamate-gated ion channels, which could be further subdivided into the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate (KA) subtypes according to their agonist profiles (Watkins et al., 1990). Metabotropic GluRs (mGluRs) are coupled to GTP-binding proteins. They appear to modulate the excitatory neurotransmission by the synthesis of second-messenger molecules and play a role in the regulation of the opening of ion channels (Pin and Duvoisin, 1995). At most central synapses, both AMPA and NMDA receptors are activated during synaptic transmission. AMPA receptors mediate fast neurotransmission, whereas NMDA receptors contribute to the late components of fast EPSPs.

A) Ionotropic GluRs

NMDA receptors, having subunits of NR1, NR2A-D subunits and NR3A-B, mediate excitatory neurotransmission in the CNS in different ways from AMPA receptors. In the spinal synaptic transmission, NMDA receptors appear to contribute to the generation of slow EPSPs (Gerber and Randić, 1989b; Gerber et al., 1991), and underlie the wind-up phenomenon (Dickenson, 1990) that can be important in pain. Functional NMDA receptors are heteromeric assemblies composed of multiple NR1 subunits in combination with at least one type of NR2. The NR3 subunits can also co-assemble with NR1 (Das et al., 1998; Perez-Otano et al., 2001; Chatterton et al., 2002) to form unique excitatory glycine receptors (Chatterton et al., 2002). At present, NMDA receptors have notoriously been characterized by a high permeability to Ca^{2+} ion (MacDermott et al., 1986), voltage-dependent block by Mg^{2+} ion (Mayer et al., 1984) and slow 'activation/deactivation' kinetics (Lester et al., 1990). Further, different combination of subunits and splice variants by alternative splicing confer the delicate functional properties to NMDA receptors (Cull-Candy et al., 2001). For example, diheteromeric NMDA receptors containing NR2A or NR2B subunits are featured by high-conductance channel openings with a high sensitivity to block by Mg^{2+} ion, whereas receptors composed of NR2C or NR2D subunits give rise to low-conductance openings with a lower sensitivity to extracellular Mg^{2+} ion. Moreover, once glutamate was applied briefly, NR1/NR2A-containing NMDA receptors generate a macroscopic current with a deactivation time constant of tens of milliseconds. However, the current generated by NR1/NR2D-containing NMDA receptors has a deactivation time constant of several seconds due to their high affinity to glutamate (Monyer et al., 1994; Wyllie et al., 1998; Vicini et al., 1998). These unique properties provide the NMDA receptors with critical roles in excitatory synaptic transmission/plasticity and pathology.

In the spinal cord, *in situ* hybridization studies show high expression of NR1 and NR2D mRNA throughout all laminae (Tölle et al., 1993; Luque et al., 1994). In addition, electrophysiological studies, recording of single-channel (outside-out) and synaptic currents, address the precise distribution of each NMDA receptor subunit, i.e., both NR1/NR2B (high conductance; 57 pS) and NR1/NR2D (low conductance; 44 pS and 19 pS) receptors are present extrasynaptically, whereas NR2A receptors predominate at primary afferent fiber-spinal DH synapses, which was determined by the kinetic and pharmacological properties of the NMDA receptor-mediated EPSCs (Momiyama, 2000).

Most of selective NMDA receptor agonists available are based on NMDA, the diagnostic ligand for these receptors. NMDA itself is an analogue of aspartate. Although this compound acts selectively at NMDA receptors, it cannot discriminate between receptor subtypes. Recently, a conformationally

constrained analogue of glutamate, homoquinolinic acid, has been shown to have higher affinity for NMDA receptors that contain NR2B subunit (Prado de Carvalho et al, 1996). As the most commonly used antagonist, D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5) acts at the glutamate binding site, and shows tendency to selectivity for NR2A and NR2B- containing NMDA receptor. On the other hand, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5, 10-imine maleate (MK801) did not bind to the glutamate recognition site (Wong et al., 1986) and appeared to block current flow by binding to a site within the activated NMDA receptors (Huettner and Bean, 1988). This accounts for the use-dependent, or uncompetitive, antagonism by MK801. Ifenprodil, which has been known as NR2B subunit selective ligand acting at NMDA receptor polyamine site, may prove beneficial in the treatment of chronic pain (Boyce et al., 1999; Chizh et al., 2001; Wei et al., 2001).

AMPA receptors are composed of heteromeric assemblies of the GluR1, 2, 3 and/or 4 subunits; different assemblies of subunits confer specific functional properties on the channel (Dingledine et al., 1999). GluR2 subunit has important role in determining the ion selectivity of the AMPA receptor-channel because its presence in an edited form renders the channel impermeable to Ca^{2+} ions. GluR2 transcripts undergo RNA editing, producing a one-amino acid change (e.g. positively charged arginine (R) at position 586 of the transmembrane segment 2, instead of the neutral glutamine (Q) in the other subunits; the so called 'Q/R site') in the channel pore that decreases Ca^{2+} permeability. AMPA receptors lacking GluR2 have high Ca^{2+} permeability ratio (P_{Ca}: $P_{Na} \cong 3$) and display a strong inward rectification in a current-voltage relationship (Hollmann and Heinemann, 1994; Dingledine et al., 1999).

The study of agonists on AMPA receptors has been examined in detail for both recombinant and native AMPA receptors. In both experimental systems, agonist-activated currents desensitize rapidly for AMPA, quisqualate and glutamate, and to a limited degree for KA as an agonist. Rank orders of potency for steady-state currents follow quisqualate>AMPA>glutamate> KA for recombinant AMPA receptor subunits (flip). A series of quinoxalinedione compounds are known as competitive antagonists for AMPA receptors. Although these compounds, e.g. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 2,3-dioxy-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), have significant inhibitory activity at KA receptors, they have become standard tools for the study of AMPA receptors. In a study with heteromeric complexes of recombinant GluR1/2 and GluR2/4 AMPA receptors, NBQX was more potent than CNQX (IC_{50} values 60 nM and 400 nM, respectively (Stein et al., 1992). NBQX, but not CNQX, has been shown to be dependent upon the agonist used, with activity greater for KA > glutamate > AMPA (Lambolez et al., 1992; Stein et al., 1992). Another class of compounds, the 2,3-

benzodiazepine, that have enabled pharmacological separation of AMPA and KA receptor-mediated events is known. The most widely studied of these are GYKI52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine) and GYKI53655 (1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine). The IC_{50} of GYKI53655 for AMPA receptors is less than 1 μM (c.f. 9.8 μM for GYKI52466), and it has no effect on KA receptors at concentration as high as 100 μM (Paternain et al., 1995; Wilding and Huettner, 1995). A diastereomer of 4-methylglutamate, SYM 2206 ((±)-4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7- methylenedioxyph thalazine), has been reported to be a potent non-competitive AMPA receptor antagonist (IC_{50} = 1.6 μM), working at the same site as GYKI52466 and GYKI53655 (Pelletier et al., 1996). In addition, Joro Spider toxin (JSTX) and philanthotoxin have been shown to selectively attenuate AMPA receptor-mediated responses of spinal neurons in vivo (Jones and Lodge, 1991) and appear to do so by acting as open channel blockers (Priestley et al., 1989). Studies using recombinant AMPA receptor subunits show that JSTX has high affinity for GluR1, 3 and 4 (IC_{50} of 30 nM) but has no effect on GluR2(R) (Blaschke et al., 1993; Herlitze et al., 1993)

AMPA receptors mediate fast excitatory synaptic transmission in most of the synapses in the CNS. Their rapid openings at resting membrane potential are suitable for this purpose. The functional significance of Ca^{2+}-permeable AMPA receptors has been studied in a population of hippocampal, neocortical non-pyramidal and spinal DH neurons (Gu et al., 1996; Isa et al., 1996). Particularly, in the spinal DH neurons, Ca^{2+}-permeable non-NMDA receptors have been demonstrated *in vitro* by Ca^{2+} detection using indicator dyes (Reichling and MacDermott, 1993), ion permeability studies (Goldstein et al., 1995), pharmacology (Gu et al., 1996) and agonist-induced cobalt loading immunocytochemistry (Engelmann et al., 1999). These receptors participate in synaptic transmission *in vitro* (Gu et al., 1996) and may also be present at synaptic sites *in vivo*. A higher percentage of postsynaptic AMPA receptor clusters, in DH C1 (also called 'type I') glomeruli (C1 terminals are mainly endings of unmyelinated afferent fibers), are immunopositive for GluR1 than for GluR2. However, more of GluR2 are in DH C2 (also, called 'type II') terminals (considered endings of small myelinated afferent fibers) (Popratiloff et al., 1996), suggesting that AMPA receptors lacking GluR2 may participate in synaptic transmission between DRG and DH neurons. Moreover, immunocytochemical evidence indicates that nearly all (97%) of the GluR2/3-immunoreactive neurons are not GABA- or glycine-immunoreactive, the finding indicating the prominent expression of Ca^{2+}-permeable AMPA receptors on the inhibitory interneurons (Spike et al., 1998). These results raise the possibility that Ca^{2+}-permeable AMPA channels may play a special role in the mediation of sensory input by unmyelinated fibers (Popratiloff et al., 1996) and especially transmission of

nociceptive information (Engelmann et al., 1999). Work by the MacDermott's group (Gu et al., 1996), and others (Jia et al., 1996; Mahanty and Sah, 1998; Youn et al., 2000; Heinemann et al., 2001; Randić and Youn, 2001), suggests that activation of Ca^{2+}-permeable AMPA receptors, may lead to synaptic strengthening. A recent finding indicates that Ca^{2+}-permeable AMPA receptors may relate to mechanical allodynia after a mild thermal injury (Sorkin et al., 1999).

KA receptors have five subunits, termed GluR5 - 7, KA 1 and 2 (For reviews see: Hollmann and Heinemann, 1994; Dingledine et al., 1999; Lerma et al., 2001). They are believed to share the same transmembrane topology and stoichiometry as AMPA and NMDA receptors. Thus they are thought to be a tetramer in which each monomer carries its own ligand-binding site and contributes with a specific amino acid stretch to form the channel lumen. Radioligand binding assays have identified two subclasses of KA receptors with different affinity. GluR5, 6 and 7 may represent the low affinity KA-binding site with a dissociation constant (K_D) of 50 - 100 nM (Bettler et al., 1992), whereas KA 1 and 2 correspond to the high affinity KA-binding site (K_D of ~4 - 15 nM) in neuronal membranes (Werner et al., 1991; Herb et al., 1992).

There are two important sources to confer structural variability to KA receptors: alternative splicing and RNA editing (Lerma et al., 2001). The former has been reported exclusively in GluR5 (GluR5-1, 5-2a, 5-2b and 5-2c; Sommer et al., 1992) and GluR7 (GluR7a and 7b; Schiffer et al., 1997) in rat. However, the mouse GluR6 has been found to exist as two splice variants that differ in their COOH-terminal domains (Gregor et al., 1993). The role of the different KA receptor splice variants is unknown. The latter is a post-transcriptional modification occurring at a Q/R site, like GluR2 subunit, of M2 segment only in GluR5 and GluR6 subunit (but not in GluR7, KA1, and KA2). As is the case for the GluR2 AMPA receptor, it has been shown that the Q-to-R substitution in GluR6 homomeric KA receptors decreases the permeability to Ca^{2+} (Burnashev et al., 1995; Egebjerg et al., 1993) whilst increasing their chloride permeability (Burnashev et al., 1996). At the same time, the presence of a R at this site transforms the rectification properties of these receptors from inwardly rectifying to linear or slightly outwardly rectifying and reduces the unitary conductance of the channels. This RNA editing process is developmentally regulated. The majority of GluR6 editing occurs earlier than for GluR5, and it is more thoroughly completed in the mature nervous system: up to 95% of GluR6 transcripts are edited, compared with 50-60% observed for the GluR5 subunit (Bernard et al., 1994; Paschen et al., 1994, 1995, 1997; Schmitt et al., 1996). In addition, it may be important to note that the RNA editing of KA receptors seems to be site- and cell-specifically regulated. The functional implications of this process are not well understood as yet. For instance, mice with mutations at the Q/R editing site in GluR5, so that all GluR5 was edited, have been found

to exhibit a reduction of KA receptor-mediated currents in their sensory neurons (Sailer et al., 1999), but the responses of these animals to painful stimuli are not altered. Besides of Q/R editing site, two additional positions prone to RNA editing have been identified in the M1 segment of this subunit: the I/V site, where a valine can substitute for an isoleucine, and the Y/C site, where a tyrosine can be replaced by a cysteine (Kohler et al., 1993). Editing at these positions modulates the effect of the Q/R site in Ca^{2+} flow, such that the fully edited subunit exhibits null passage of this cation. The mechanism of interaction among these three sites remains to be elucidated.

A major hindrance to understanding of KA receptors has been the lack of specific agonists and antagonists. Although a clear pharmacological boundary has been traced between NMDA and the other ionotropic glutamate receptor classes, the separation between AMPA and KA receptors has only been vaguely sketched and, for the longest time, the two types have been pooled together into what has been called the non-NMDA receptor subtype. KA, albeit showing a clear preference for KA receptors, has a very significant effect on AMPA receptor channels at relatively low doses. The difference in EC_{50} between the two receptors lies around a mere 5- to 30-fold higher affinity for KA receptors (Clarke et al., 1997; Huettner, 1990; Lerma et al., 1993; Wilding and Huettner, 1996). It has been suggested that only a concentration range (300 nM –3 μM) of KA selectively activates KA receptors in CA1 hippocampal neurons (Müller et al., 2000). Domoate, one of the first AMPA/KA receptor agonist to be identified, is 20- to 25-fold more effective than KA on DRG cells and on recombinant GluR5 subunits (Huettner, 1990; Sommer et al., 1992). SYM 2081 ((2*S*-4*R*) diastereomer of 4-methylglutamate) is one of the latest additions to the list of specific KA receptor agonists. It displays a selectivity three orders of magnitude larger for KA than for AMPA receptors both in binding and in functional assays, but the selectivity of this molecule for KA over NMDA receptors is significantly lower, only 200-fold (Gu et al., 1995). Although its pharmacological profile is incomplete, SYM2081 does not seem to show the subunit specificity, as it elicits rapidly desensitizing currents on GluR5 and GluR6 homomeric channels. SYM 2081 has also been used as a functional antagonist of the KA receptors (Li et al., 1999) because its presence at low concentrations drives the receptor to an inactive state, preventing its subsequent opening by other agonists. (RS)-2-Amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid (ATPA), which was originally developed as AMPA analogue, is highly potent (K_i= 4.3 nM in radio-labeled competitive inhibition; Hoo et al., 1999) and exclusively selective for GluR5 receptors over other KA receptor subunits, or AMPA receptor subunits (EC_{50} = 2.1 μM for recombinant GluR5 subunits; Clarke et al., 1997). However, it has been recently shown (Paternain et al., 2000) that ATPA can also act on GluR5/KA2 (EC_{50} = 6.3 μM), GluR6/KA2 (EC_{50} = 84 μM), and GluR5/GluR6 heteromers (EC_{50} = 12 μM). The

quest for specific KA receptor antagonists has not been as successful as the search for agonists. Initially, NS-102 was proposed as a selective antagonist, but it was later found that its IC_{50} for KA receptors is similar as that for AMPA receptors, which limited its usage. One of recent achievement for selective antagonists is LY382884. It has been shown to have an even higher selectivity for GluR5 over the other KA receptor subunits and, more importantly, over AMPA receptors (Bortolotto et al., 1999; Simmons et al., 1998).

The differential expression of the subunits of KA receptors in the DRG and the spinal DH has been reported. The expression of GluR5 was detected strongly in DRG neurons, particularly small neurons (Huettner, 1990; Partin, 1993), and weakly in the spinal DH (Tölle et al., 1993; Hwang et al., 2000). The expression of other subunits has also been detected within DRG, including GluR6, GluR7, KA1 and KA2 (Partin et al., 1993; Petralia et al., 1994; Hwang et al., 2000), and in the spinal DH with moderate signal of GluR7 and KA1, strong signal of KA2, and no signal of GluR6 (Tölle et al., 1993). However, a recent finding that the expression of GluR6 was up-regulated by inflammatory condition is of interest (Zou et al., 2000). In contrast to AMPA receptors, the mediation of fast excitatory synaptic transmission by KA receptors at synapses between primary afferents and the spinal DH neurons has only recently been demonstrated (Li et al., 1999). In the spinal DH (Li et al., 1999), like in hippocampus (Castillo et al., 1997; Vignes and Collingridge, 1997), the KA receptor-mediated excitatory postsynaptic currents (EPSC) have much smaller peak amplitude and slower decay kinetics than AMPA receptor-mediated EPSCs. As KA receptors containing GluR5 subunit are present at high level on C primary afferents (Huettner, 1990; Partin et al, 1993), they have been suggested to contribute to synaptic transmission of nociceptive signals. Recent findings that LY 382884, a selective GluR5 subunit antagonist, has analgesic action in formalin-injected rats (Simmons et al., 1998) and that a desensitizing KA receptor selective agonist SYM 2081 has antinociceptive effect on behavioral responses to nociceptive heat stimuli (Li et al., 1999) suggested that nociceptive transmission can be regulated by KA receptors.

B) Metabotropic GluRs

The eight presently described mGluRs are grouped into three classes based on structural homology, pharmacology, and signal transduction mechanisms: Group I (mGluR 1 and 5) are coupled to phospholipase C (PLC) and stimulate phosphoinositide hydrolysis and intracellular Ca^{2+} signal transduction, whereas Group II (mGluR 2 and 3) and Group III (mGluRs 4 and 6–8) are negatively coupled to adenylate cyclase (Nakanishi, 1994; Conn and Pin, 1997). Group II and III mGluRs are also known to inhibit the function of voltage-dependent Ca^{2+} channels and activate or potentiate

potassium channels, both of which could lead to modulation of synaptic transmission. Out of the eight mGlu receptors cloned, high levels of staining for mGlu1 (mainly mGlu1a), mGlu5 (mGlu 5a and b), and mGlu7 receptors are found in laminae I and II of the DH in the rats (Shigemoto et al., 1992; Vidnyánszky et al., 1994; Ohishi et al., 1995; Boxall et al., 1998; Berthele et al., 1999), with the mGlu5 receptor being predominantly located on the soma and dendrites of DH neurons (Vidnyánszky et al., 1994; Jia et al., 1999). Levels of mRNA coding for mGlu2 and mGlu4 receptors are very low in the spinal cord with a moderate signal for mGluR3 receptors in the DH, much of this being in glia (Ohishi et al., 1993, 1995; Boxall et al., 1998). No mRNA labeling for mGluR6 and mGluR8 has been detected in the rat spinal cord (Valerio et al., 1997). Evidence supports the possibility of the presence of autoreceptors belonging to all three groups of mGluRs on central endings of primary afferents in the spinal cord, though their presence in primary afferent fibers appeared sporadic (Vidnyánszky et al., 1994; Li et al., 1997; Valerio et al., 1997; Jia et al., 1999). Although the physiological role of mGluRs is not clear, it has recently been shown that the activation of mGluRs can modulate (both depress and potentiate) glutamatergic transmission (Chen and Sandkühler, 2000; Zhong et al., 2000; Gerber et al., 2000a,b). Studies of the actions of mGluR agonists and antagonists on responses of spinal cord DH neurons (Neugebauer et al., 1994, 1998; Young et al., 1994, 1995, 1997) to noxious and non-noxious stimuli indicate that mGluRs are primarily involved in mediating nociceptive inputs.

3. Synaptic plasticity in the spinal cord

The efficiency of synaptic transmission in the CNS is not constant and can be modulated by the rate of activity in synaptic pathways. The leading experimental model for such a change has been long-term potentiation (LTP), an increase in synaptic strength that lasts for hours to days. More recently, a long-term depression (LTD), a decrease in synaptic efficacy, has also been described (Bliss and Collingridge, 1993; Malenka, 1995; Linden and Connor, 1995; Malenka and Nicoll, 1997; for a recent review, Malenka and Nicoll, 1999). Although the biochemical mechanisms involved in the induction and expression of LTP/LTD have not been definitely identified, leading candidates include subunit-specific AMPA receptor cycling at synapses (Shi, et al., 1999; Zhu et al., 2000; Liu and Cull-Candy, 2000), as well as diffusible intercellular messengers and phosphorylation/dephosphorylation processes involving specific protein kinases and protein phosphatases (Lisman, 1997; Barria et al., 1997; Lisman et al., 1997; Lee et al., 2000). These

dynamic changes in synaptic strength are thought to provide a cellular basis for information storage in the CNS.

The existence of long-term modifications of primary afferent neurotransmission following repetitive stimulation of a dorsal root (DR), or peripheral nerve, has been reported both *in vitro* and *in vivo* in the rat spinal cord, and there are some indications of having essentially the same mechanisms as it does in the brain (Randić et al., 1993; Pockett, 1995; Randić, 1996; Liu and Sandkühler, 1995, 1997, 1998; Sandkühler et al., 1997; Svendssen et al., 1997, 1998, 1999; Sandkühler and Liu, 1998; Chen and Sandkühler, 1999; Gerber et al., 2000a). Both the AMPA and the NMDA receptor-mediated components of afferent neurotransmission can exhibit LTP and LTD (Randić et al., 1993; Svendsen et al., 1998). The cellular mechanisms underlying LTP and LTD in the DH are still not well understood. There is evidence for involvement of NMDA receptor and postsynaptic Ca^{2+} since NMDA receptor antagonists or loading of DH cells with a Ca^{2+} chelator BAPTA block the induction of LTP and LTD (Randić et al., 1993; Liu and Sandkühler, 1998). In addition, recent studies suggest the potential role of neurokinin 1 (NK1), Group I and II metabotropic glutamate and opioid receptors in the generation and maintenance of LTP and LTD (Randić, 1996; Liu and Sandkühler, 1997, 1998; Zhong et al., 1998, 2000; Chen and Sandkühler, 1999; Gerber et al., 1999, 2000a; Randić et al., 1999). Evidence supports a role for several Ca^{2+}-sensitive protein kinases in induction of LTP, and protein phosphatases in induction of high-frequency stimulation (HFS)-induced LTD. Although at present it is not certain whether pre- or postsynaptic factors, or both, are responsible for expression of LTP of the primary afferent neurotransmission, a finding that a brief HFS of primary afferent fibers at C-fiber strength produced a sustained enhanced release of endogenous glutamate and aspartate in the slice superfusate, suggests the involvement of presynaptic factors (Randić, 1996). Although transduction mechanisms involved in the generation of LTD in the spinal DH are not well known, it has been suggested that the synaptic activation of protein phosphatases plays a role in the generation of HFS-induced LTD (Randić, 1996). In contrast, the induction of low-frequency stimulation-induced LTD in the DH appears not to require synaptic activation of protein phosphatases (Sandkühler et al., 1997). However, there is evidence for the involvement of metabotropic glutamate receptors (Zhong et al., 1998, 2000; Chen and Sandkühler, 1999; Gerber et al., 2000a,b) and opioid receptors (Randić, 1996). Whereas, in the brain, LTP and LTD are associated with the processes of learning and memory, their principal roles in the superficial spinal DH may be related to plasticity of spinal nociception.

Table 1. Afferent Fiber Groups (Gardner et al., 2000)

	Cutaneous nerve	*Fiber diameter (μm)*	*Conduction velocity (m/sec)*
Myelinated			
Large	Aα	13-20	80-120
Small	Aβ	6-12	35-75
Smallest	Aδ	1-5	5-30
Unmyelinated	C	0.2-1.5	0.5-2

Table 2. Receptor types active in somatic sensation (Gardner et al., 2000)

Receptor type	*Fiber group*	*Modality*
Cutaneous and subcutaneous mechanoreceptors		Touch
Meissner's corpuscle	Aα, β	Stroking, fluttering
Merkel disk receptor	Aα, β	Pressure, texture
Pacinian corpuscle	Aα, β	Vibration
Ruffini ending	Aα, β	Skin stretch
Hair-tylotrich, hair-guard	Aα, β	Stroking, fluttering
Hair-down	Aδ	Light stroking
Field	Aα, β	Skin stretch
Thermal receptors		Temperature
Cool receptors	Aδ	Skin cooling (25°C)
Warm receptors	C	Skin warming (41°C)
Heat nociceptors	Aδ	Hot temperature (>45°C)
Cold nociceptors	C	Cold temperature (<5°C)
Nociceptors		Pain
Mechanical	Aδ	Sharp, pricking pain
Thermal-mechanical	Aδ	Burning pain
Thermal-mechanical	C	Freezing pain
Polymodal	C	Slow, burning pain
Muscle and skeletal mechanoreceptors		Limb proprioception
Muscle spindle primary	Aα	Muscle length and speed
Muscle spindle secondary	Aβ	Muscle stretch
Golgi tendon organ	Aα	Muscle contraction
Joint capsule mechanoreceptors	Aβ	Joint angle
Stretch-sensitive free endings	Aδ	Excess stretch or force

CHAPTER 2. MATERIALS AND METHODS

1. Genetic background of mutant mice

All mutant mice used for experiments were provided by Professor S.F. Heinemann, Molecular Neurobiolog Lab, The Salk Institute, San Diego. Some of mice used for control (129SvEv or B6129F1 strain) were purchased from Taconic Inc, ME. Mouse strains originating from embryonic stem (ES) cell lines and blastocysts, used to generate chimeras, are listed in the table 1.

All experiments were approved by the University Animal Care and Use Committee and were consistent with the ethical guidelines of the National Institutes of Health and of the International Association for the Study of Pain. Moreover, all efforts were made to minimize the number of animals used, and their suffering. Developmental compensation could be a concern in knockout experiments; however, we feel this is unlikely because levels of mRNA expression of other kainate receptor subunits were unchanged in GluR6 mutant (Mulle et al., 1998) or GluR5 mutant mice (A. Sailer, unpublished observations). Furthermore, functional replacement of whole-cell kainate receptor currents does not occur in CA3 pyramidal neurons (Mulle et al., 1998), cerebellar Purkinje neurons (Brickley et al., 1999), or dorsal root ganglion neurons (G. T. Swanson, unpublished observations). Each knockout mouse used in this study was genotyped by Southern blot analysis of tail DNA.

2. Spinal cord slice preparation

Under deep isoflurane anesthesia, segments of the lumbosacral (L4-L6) spinal cord were removed with long (8-15 mm) dorsal roots. Several transverse slices (400-450 μm thick) were cut with attached dorsal roots in an oxygenated (95% O_2, 5% CO_2) Krebs-bicarbonate solution (4°C) on a vibratome and placed in a holding chamber (33 ± 1°C) to recover for at least 1 hr. Slices were cut and mounted in a medium comprising (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 10; pH 7.4, 310 to 320 mOsm. A single slice was then transferred into a recording chamber where it was submerged beneath an oxygenated superfusing medium (flow rate of about 3 ml/min, 33-34°C) containing (in mM): NaCl, 128; KCl, 1.9; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 10; pH 7.4, 310 to 320 mOsM, and was equilibrated with 95% O_2, 5% CO_2. This solution was usually used for conventional intracellular recordings and some whole-cell patch-clamp recordings, if not specified.

3. Electrophysiology

Dorsal root stimulation and conventional intracellular recording. Intracellular recordings with sharp microelectrodes were made from substantia gelatinosa (SG, lamina II) neurons. When viewed under a dissecting microscope at a magnification of ×10-40 with transmitted illumination, the SG was distinguishable as a translucent bend in the spinal SG, although it was difficult to discern with certainty the border between laminae I and II. Under visual control, a single fiberglass (#6010; o.d. and i.d., 1.0 and 0.58 mm, respectively; AM Systems, Carlsborg, WA, USA) microelectrode filled with 4 M potassium acetate (pH 7.2) (DC resistance: 140-220 MΩ) was placed in the SG, and neurons were impaled by oscillating the capacity compensation circuit of a high-input impedance bridge amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA, USA). A DC pen-recorder was used to record membrane potentials continuously, and a Digidata 1200 system with pCLAMP (version 5.5 or 6) software (Axon Instruments) was used for data acquisition and analysis. Most recordings were obtained from cells with a stable resting membrane potential (more negative than -55 mV) and with overshooting action potentials. The protocol for assessing the effects of KA receptor agonists and antagonist on synaptic responses was as follows. Monosynaptic and polysynaptic excitatory postsynaptic potentials (EPSPs) in SG neurons were evoked by orthodromic electrical stimulation of primary afferent fibers in the lumbar dorsal root (L4 and/or L5) using a bipolar platinum wire electrode or glass suction electrode (with the cathode internal). Single shocks at a fixed suprathreshold strength (0.01-0.5 ms pulses, 3-35 V), repeated at 2-min intervals, were given for at least 10 min before, during (2 min), and for a 20-30-min period after bath administrations of chemicals. This frequency of stimulation was chosen for sampling data because it did not result in response facilitation or depression. A stimulus intensity that yielded a 5-15 mV EPSP was chosen to standardize the baseline synaptic strength across slices, and it was below threshold for eliciting an action potential in most of the slices chosen for study. The stimulus intensity necessary to activate Aδ and C fibers and the afferent fiber conduction velocity, were determined by extracellular recording of compound action potentials from longitudinal spinal slice-dorsal root-dorsal root ganglia preparations in the previous experiments (Kangrga and Randić, 1991). The classification of EPSPs in relation to the primary afferents activated, was done solely on the basis of conduction velocity, which was calculated either by measuring the distance between the stimulating electrode and the recording site on the dorsal root and dividing by the conduction latencies of action potentials recorded, or from the latency of evoked EPSPs and the distance from the stimulating electrode to the recording site. Primary afferents conducting at velocity above 15 m/s were classified as Aβ (Park et al., 1999),

whereas those conducting between 1.5 and 15 m/s were classified as Aδ, and those conducting below 1.5 m/s as C fibers. The minimum stimulus intensities and durations used to activate Aδ and C fibers were 3 V/0.1 ms and 5 V/0.5 ms, respectively. Stimulation of dorsal roots led to generation of an EPSP. With small stimulus strength this EPSP was graded in amplitude, had a fixed latency and monophasic decay. As the stimulus strength was increased, however, a later slow polysynaptic component(s) was apparent. In order to discriminate between monosynaptic and polysynaptic EPSPs, two experiments were carried out. (1) Identification of the Aδ- or C-fiber-evoked EPSPs as monosynaptic was based on their constant latencies and absence of failures with a repetitive stimulation at frequency of 10-20 Hz (Randić et al., 1993) (2) The latency of these EPSPs remained constant in the presence of a high concentration of divalent cations (4 mM Ca^{2+}, 8 mM Mg^{2+}), the procedure that has been shown to suppress polysynaptic EPSPs by decreasing neuronal excitability. These findings contrast with the properties of dorsal root-evoked polysynaptic EPSPs. The presumably polysynaptic EPSPs had variable latencies and showed failures with high-frequency stimulation and with an external solution containing high divalent cation concentrations. Moreover, the shapes and amplitudes of polysynaptic EPSPs were variable in different trials when dorsal roots were stimulated at a constant intensity. Input resistance was measured at 2-min intervals by passing a hyperpolarizing current pulse of 0.05 nA across the cell membrane and measuring the voltage deflection produced. The current values were of sufficient duration (200-300 ms) to fully charge the membrane capacitance. Bridge balance was monitored throughout experiments and corrected when necessary. To reduce the increased spontaneous synaptic activity and subsequent action potential firing due to the removal of synaptic inhibition, the Mg^{2+} concentration in the superfusing solution was increased to 3 mM in some of the experiments where bicuculline and strychnine were applied to block the $GABA_A$ and glycine receptors.

In the synaptic plasticity study, evoked-EPSPs, test pulses were repeated at 2 min intervals during control period (10-30 min) giving half maximal amplitude of EPSPs, and the 30 - 100 min period after conditioning high-frequency stimulation (HFS; 3 tetani of 1 s duration each at 100Hz and 10s intervals; at 30 V and 0.5 ms duration). Neurons showing long-term potentiatin (LTP) or long-term depression (LTD), respectively, were grouped on the basis of at least 20% increase or decrease, in the amplitude of the synaptic response, following high-frequency stimulation (Randić et al., 1993). Summary graphs were obtained by normalizing each experiment according to the average value of all points on the 10–30 min baseline prior to HFS.

Whole-cell voltage-clamp recording. For recording synaptic currents from substantia gelatinosa (SG,

recognized as a translucent band in lamina II of spinal DH) neurons, whole-cell voltage-clamp recordings were made with an Axopatch 200B (Axon Instruments) or EPC-7 (HEKA) amplifier under blind condition in external solution (in mM: 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, pH 7.4; perfusion rate of 3-4 ml/min) at 33-34°C. Bicuculline methoiodide (BMI, 10-20 μM), strychnine (2 μM) and D-(-)-2-amino-5-phosphonopentanoic acid (DAP5, 50-100 μM) are included to the external solution in experiments indicated. Borosilicate glass patch pipettes (6-12 MΩ) were filled with internal solution (mM: 140 $CsMeSO_4$, 10 Na-HEPES, 10 EGTA, 2 NaCl, 1 $CaCl_2$, 2 Tris-ATP, 0.3 Tris-GTP, 5 QX314, pH 7.2, 295-300 mOsm). For voltage-clamp recordings, the capacitative current was electronically canceled, and the series resistance was measured (about 8-20 MΩ) directly from amplifier and compensated by 70%. The recording was terminated if the series resistance changed by more than 20%. Excitatory postsynaptic currents (EPSCs), evoked by electrical stimulation of dorsal roots (0.1-0.5 ms pulses of 3-30 V at 0.033 Hz) using bipolar platinum wire electrode, were filtered at 2 kHz and sampled at 10 kHz (Digidata 1200A) and stored on a computer programmed with pCLAMP (version 8, Axon Instruments) software. For I-V relationships of synaptic responses, EPSCs were evoked at each potential ranging from –60 mV to +40 mV (20-mV increments), and the peak amplitude of EPSCs was measured to plot against each potential. If EPSC amplitude at positive potentials fell below the extrapolated line, it was considered an inwardly rectifying I-V relationship. The rectification index (RI) of the current-voltage (I-V) relationship was defined as the ratio of the EPSC peak amplitude at + 40 mV to the predicted linear value at +40 mV (extrapolated from linear fitting of the current at the negative potentials; Liu and Cull-Candy, 2000). Junction potentials were not corrected.

For miniature EPSC (mEPSC) recordings, 0.5 μM tetrodotoxin (TTX), 50-100 μM D-AP5 (in some experiments), 5 μM bicuculline methoiodide, and 2 μM strychnine were included in the perfusing solution. Borosilicate glass patch pipettes (6-12 M MΩ) were filled with internal solution (mM: 125 K^+-gluconate, 5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, and 5 HEPES; pH 7.2, 295-300 mOsm). Whole-cell patch-clamp recordings from SG cells were performed using the 'blind method' (Blanton et al., 1990). Neurons were voltage clamped at -70 mV using the Axopatch 200B amplifier (Axon Instruments), and series resistance was compensated by 60-70%. Access resistance was monitored continually and neurons discarded if this parameter changed by more than 20%. mEPSCs were filtered at 2 kHz, sampled at 10 kHz (Digidata 1200) and stored onto a Gateway EV700 computer programmed with pCLAMP (version 8, Axon Instruments) software. Analysis of mEPSCs was performed by the use of the Mini Analysis Program (Synaptosoft, Leonia, NJ). All records were fitted manually by screening through and picking events from the digitized data. The whole data file

was fitted to check for stability of the recordings. Two- to 3-min stretches of data were used for mEPSC frequency analysis. Data are presented as the mean ± SEM. Parameters were compared by the use of the Student's unpaired *t* test or the Wilcoxon signed rank test.

Chemicals. Chemicals used and their sources were as follows: [(RS)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)] propionic acid (ATPA) or (RS)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl) propionic acid (Vignes et al. 1998), (-)-bicuculline methoiodide, and strychnine hydrochloride from Sigma (St. Louis, MO, USA); (2S)-3[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phosphinic acid (CGP 55845A), a gift from Novartis Pharma AG Research, Basel, Switzerland; 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 53655), a gift from Dr. Antal Simay (IVAX Drug Research Institute, Budapest, Hungary); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5), (2S,3S,4R)-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid (kainic acid), (S)-α-methyl-4-carboxyphenyl glycine (MCPG), and 2,3-dioxy-6-nitro-1,2,3,4 tetrahydrobenzero-7-sulphanoylbenzo[f]quinoxaline-2,3-dione (NBQX), all obtained from Tocris Cookson (Bristol, UK) (3S,4aR,6S,8aR)-6-(4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid (LY 382884) was a gift of Lilly Research Laboratories, Eli Lilly and Co. (Indianapolis, IN, USA). All solutions were freshly prepared every day from stock solutions that were stored at -20°C. Drugs were dissolved in oxygenated recording solution immediately prior to use and applied to the slices in known concentrations by addition to the superfusing medium. All compounds were applied by addition in the perfusing medium, and each neuron served as its own control. Drug-containing solution entered the recording chamber within 30 s of changing solutions, with complete exchange occurring within 3 min.

Data analysis. mEPSC and events in the baseline noise were detected and measured using Mini Analysis Software (Synaptosoft). mEPSCs were detected automatically using a threshold-crossing algorithm, and their frequency, amplitude, and kinetic parameters analyzed. At least 250 events were analyzed for each cell under each condition. Kinetic analyses of mEPSC properties included: peak amplitude, rise time from 10 to 90% peak amplitude, decay time constant (decay τ), and duration. mEPSC duration was measured as the time from 10% of peak current to 90% return to baseline. A noise histogram was generated from the baseline current during periods containing no synaptic events, and a Gaussian distribution was fitted to the histogram to determine the SD of baseline noise. Synaptic events were selected automatically by a threshold-crossing algorithm, with detection level set at, or greater than, two times the SD of baseline noise (Datapac III, Run Technologies, Irvine,

CA). To test whether kainate modifies the amplitude or frequency of miniature synaptic currents, cumulative probability histograms for amplitude and interevent intervals were generated. A paired Student's *t*-test was used for comparison of EPSC amplitudes and statistical significance of the results. The nonparametric Kolmogorov-Smirnoff test (SYSTAT 7.0, SPSS, Chicago) was used to assess the significance of shifts in cumulative probability distribution of interevent interval. For comparison of amplitude distributions, histograms were constructed with events categorized into 2 pA bins. Data are presented as mean ± standard error of meant (s.e.m.), and statistical significance assessed using Student's *t* test ($p<0.05$ and $p<0.01$ were considered significant and indicated in the figures by * and **, respectively). χ^2 tests were used to evaluate significance in differences between means or distributions, respectively. $P<0.05$ was considered as significant.

Table 1. Some information on mutant mice used.

Mutant Mice	*ES cell line*	*Blastocysts*	*Providers*	*References*
GluR2	129 SvEv	C57BL6J	Salk Institute	Personal communication with Dr. B. Vissel
GluR5	129 SvEv	129 SvEv	Salk Institute	Mulle et al., 2000
GluR6	129 SvEv	C57Bl6J	Salk Institute	Mulle et al., 1998
GluR5/6/KA2	129 SvEv	C57Bl6J	Salk Institute	Personal communication with Dr. G. T. Swanson

CHAPTER 3. AMPA RECEPTOR

Enhanced LTP of primary afferent neurotransmission in mice deficient in the AMPA receptor GluR2

1. Introduction

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-type glutamate receptors (AMPARs) mediate the majority of fast excitatory neurotransmission in the mammalian central nervous system, including the spinal cord (Gerber and Randić, 1989; Yoshimura and Jessell, 1990). AMPARs are assembled from the four subunits, GluR1 through GluR4, either alone or in various combinations (Hollmann and Heinemann, 1994). Different assemblies of subunits confer specific functional properties on the channel: for example, AMPARs assembled without GluR2 subunit or with its unedited form exhibit inwardly rectifying current-voltage (I-V) relation and high Ca^{2+} permeability (Hollmann et al., 1991; Burnashev et al., 1992).

Ca^{2+}-permeable AMPARs have been demonstrated in a subpopulation of the cultured embryonic spinal dorsal horn (DH) neurons (Gu et al., 1996) or of young rat spinal DH neurons using agonist-induced cobalt loading immunocytochemistry (Engelmann et al., 1999). In addition, a higher percentage of postsynaptic AMPARs were immunopositive for GluR1 than for GluR2 in DH C1 glomeruli (main endings of unmyelinated afferent fibers; Popratiloff et al., 1996), suggesting that AMPARs lacking GluR2 may participate in synaptic transmission between primary afferent fibers and DH neurons. However, an *in situ* hybridization study showing high expression of GluR2 subunit mRNA in the spinal DH indicated a prominent existence of Ca^{2+}-impermeable AMPARs in the spinal SG neurons (Tölle et al., 1993).

Therefore, to study the role of Ca^{2+}-permeable AMPARs in the neurotransmission and the plasticity of sensory excitatory synaptic transmission in the spinal DH, we used mutant mice lacking the GluR2 subunit.

2. Results

Rectifying Properties of AMPA Receptors in the Spinal Cord Neurons

Whole-cell patch-clamp recordings from SG neurons in transverse spinal slices revealed no significant difference between wild-type and GluR2 mutant mice in resting membrane potential (-61.8

± 2.7 mV versus 59.9 ± 1.7 mV; P = 0.89) and input resistance (204.2 ± 29.3 versus 230.9 ± 49.3; P = 0.62), respectively (Table 1).

To examine the effect of genetic deletion of GluR2 subunit on synaptic membrane properties, we established I-V relationships of AMPAR-mediated EPSCs evoked by electrical stimulation of dorsal roots in wild-type or GluR2 mutant slices. Also, to quantify the degree of rectification, we calculated the RI of EPSCs, defined as the ratio of EPSC amplitude at +40 mV divided by the predicted (linear) value at +40 mV extrapolated from linear fitting of EPSCs (Liu and Cull-Candy, 2000). In a summary graph (Fig. 1A) from six wild-type SG neurons (five mice) recorded in control bath solution, I-V relation of EPSCs was almost linear (mean RI = 0.85 ± 0.08). However, a graph summarized from eight GluR2-/- SG neurons (four mice; Fig. 1B) recorded in the same control solution exhibited significantly stronger inward rectification (mean RI = 0.37 ± 0.06, P < 0.01). To further eliminate the contribution of $GABA_A$, glycine and N-methyl-D-aspartate receptors (NMDARs), we established I-V relationships of EPSCs in the presence of 10-20 μM BMI, 2 μM strychnine and 50-100 μM DAP5. Under this condition, six wild-type SG neurons (five mice) showed from slight (RI = 0.6) to strong (RI = 0.05) inward rectification, resulting in inwardly rectifying I-V relationship in the summary graph (mean RI = 0.41 ± 0.08, Fig. 1C). However, all six GluR2-/- SG neurons (four mice) produced strong inward rectifications in I-V relation (Fig. 1D). The mean RI from them (0.07 ± 0.03) was significantly decreased when compared with that of wild-type group in the presence of BMI, strychnine and DAP5 (P < 0.01), or that of GluR2-/- group in the control bath solution (P < 0.01) (Fig. 1E). Because the I-V relationship and the RI are closely correlated with the Ca^{2+}-permeability (Isa et al., 1996) and the subunit composition of AMPAR channels (Seeburg et al., 1998), these results indicate that the Ca^{2+}-permeability through AMPARs expressed on the postsynaptic membrane of SG neurons may efficiently be increased due to the genetic deletion of GluR2 subunit.

Enhanced LTP of primary afferent neurotransmission in mice deficient in the AMPA receptor GluR2 subunit

It has been shown that AMPARs can mediate a transient change in synaptic strength via postsynaptic Ca^{2+} influx through the receptor channels in the cultured spinal cord DH neurons, and LTP in hippocampal and amygdala slices (Gu et al., 1996; Jia et al., 1996; Mahanty and Sah, 1998; Randić et al., 1993). Since mRNA transcripts of GluR2 subunit are highly expressed in the spinal SG area (Tölle, 1993), we decided to study the role of Ca^{2+}-permeable AMPARs in synaptic plasticity at primary afferent fiber-SG neuron synapses by using mice deficient in the AMPAR GluR2 subunit. On the basis of previous indication, that LTP at the primary afferent fiber synapses onto SG cells is

mediated postsynaptically by Ca^{2+} influx through NMDARs (Randić et al., 1993), we hypothesized that Ca^{2+} influx through Ca^{2+}-permeable AMPARs may be sufficient for the induction of NMDAR-independent LTP. In order to test this idea, we studied LTP induction at the primary afferent fiber-SG synapses in spinal slices of wild-type or GluR2 mutant mice either in the absence or in the presence of the NMDAR antagonist DAP5.

To investigate the synaptic plasticity in the spinal cord, we recorded EPSPs evoked by electrical stimulation of dorsal roots. Tetanic stimulation of primary afferents (100Hz for 1s, delivered 3 times at 0.1 Hz, HFS) was applied to induce long-term potentiation (LTP) or long-term depression (LTD) in the spinal cord slices obtained from adult (2-4 months old) wild-type and GluR2 mutant mice. As shown in Fig. 2A-B, tetanic stimulation induced a long-lasting increase in synaptic strength in the wild-type slices. The normalized EPSP peak amplitude for wild-type mice at 20 min after tetanus was 126 ± 9 % of the averaged baseline amplitude before stimulation (ten slices from ten mice). In slices obtained from GluR2 mutants, the degree of long-lasting increase in the synaptic strength was enhanced (The normalized EPSP amplitude for GluR2 mutant mice at 20 min after tetanus was 177 ± 22 % of averaged baseline amplitude, twelve slices from twelve mice, $P<0.01$). In addition to LTP, subpopulation of neurons showed LTD of EPSPs peak amplitude following HFS in both wild-type (Fig. 2C-D; +/+, nine slices from nine mice; at 20 min after HFS, 57 ± 11 % of the averaged baseline amplitude; $P<0.05$) and GluR2 mutant (-/-, seven slices from seven mice; at 20 min after HFS, 62 ± 9 % of the averaged baseline amplitude; $P<0.05$). However, the magnitude of LTD was not significantly different between two groups ($P>0.05$).

As the induction of LTP at primary afferent synapses critically depended on the activation of NMDARs (Randić et al., 1993), we investigated whether the Ca^{2+} permeable AMPARs can substitute for NMDARs during LTP induction. As shown in Fig. 3A, in the presence of 50–100 μM DAP5, a competitive NMDAR antagonist, HFS failed to induce LTP in wild-type slices, rather producing significant LTD (+/+, eight out nine slices from nine mice; at 20 min after HFS, 68 ± 15 % of the averaged baseline amplitude; $P<0.05$). However, most of GluR2 mutant slices (-/-, seven out of eight slices from eight mice) exhibited significant LTP (at 20 min after HFS, 123 ± 15 % of the averaged baseline amplitude; $P<0.05$), indicating NMDAR-independent LTP

Although NMDARs are the primary source of Ca^{2+} entry into dendritic spines, activation of dendritic voltage-dependent Ca^{2+} channels also substantially raises Ca^{2+} levels and can generate LTP, short-term potentiation, or LTD. Hence, we investigated whether high voltage activated Ca^{2+} (HVAC) channels are involved in the NMDAR-independent LTP in GluR2 mutant mice. Fig. 3B summarizes LTP induced in the presence of 50 μM DAP5 and 10-20 μM nifedipine, a HVAC channel antagonist,

or nimodipine in five out of six slices obtained from six GluR2 mutant mice (-/-: at 20 min after HFS, 125 ± 7 % of the average baseline amplitude; $P<0.05$), and LTD in seven out of nine slices from nine wild-type mice (+/+: at 20 min after HFS, 81 ± 9 % of the averaged baseline amplitude; $P<0.05$), indicating that HVAC channels do not play a role in the NMDAR-independent LTP. On the other hand, further addition of 10 μM NBQX or 10-20 μM CNQX to perfusing solution abolished all synaptic responses, implying that the residual LTP in GluR2 mutant mice is likely mediated by the Ca^{2+} influx through the synaptic activation of AMPARs. Figure 3C is a histogram showing the distribution of number of SG neurons responding to tetanic stimulation in the presence of DAP5 or DAP5 + HVAC antagonists (nifedipine/nimodipine) with LTP and LTD, obtained from eighteen wild-type (+/+) neurons and fourteen mutant neurons. The probability of the distribution between wild-type and GluR2 mutant mice was significantly different in both DAP5 alone and DAP5 + HVAC antagonists (χ^2 test, $P<0.01$).

3. Discussion

Our data obtained from adult GluR2 mutant mouse slices are compatible with the suggestion that Ca^{2+}-permeable AMPARs can induce LTP of excitatory synaptic transmission in the spinal cord DH on the basis of the finding that Ca^{2+}-permeable AMPARs are expressed at synapses on cultured embryonic DH neurons and can trigger synaptic potentiation there (Gu et al., 1996). Hence, in the absence of GluR2, LTP and Ca^{2+} permeability are increased. The data also show that Ca^{2+} influx via L-type Ca^{2+} channels is not involved in LTP induction in GluR2 mutant mice. Therefore, the GluR2 subunit may play a crucial role in regulating both Ca^{2+} influx and LTP. The fact that GluR2 mutant mice exhibit widespread impairment in behavior suggests that GluR2 is also critical for normal brain function. A previous finding demonstrated that isolated hippocampal CA1 neurons increased Ca^{2+} permeation and that LTP was markedly enhanced in the CA1 region of hippocampal slices (Jia et al., 1996). These data raised the possibility that Ca^{2+} influx via AMPARs alone, might be able to induce long-lasting increases in synaptic efficacy at primary afferent-SG neuron synapses, suggesting an important and unexpected role in synaptic plasticity (Jia et al., 1996; Vissel, 2001).

Relative Ca^{2+} Permeability of AMPA Receptors

It has been shown *in vitro* that AMPARs containing the Q/R edited GluR2 subunit exhibit lower Ca^{2+} permeability and distinct gating properties, compared with receptor channels assembled without this subunit (Burnashev et al., 1992; Hollmann and Heinemann, 1994). Although we have not

performed ion permeability studies, a previous report (Jia et al., 1996) showed that the loss of the GluR2 subunit in individual CA1 pyramidal neurons from GluR2 mutant mice resulted in a nine-fold increase in a relative Ca^{2+} permeability following kainate application when compared with that of control mice. Indeed, this shift was similar in magnitude to that observed for Ca^{2+}-permeable NMDARs (Koh et al., 1995). These results, together with observations in hippocampal slices from GluR2 editing-deficient mice (Brusa et al., 95), support a crucial role for the GluR2 subunit in inhibiting Ca^{2+} influx via AMPARs *in vivo*.

Naturally, the degree of the expression of Ca^{2+}-permeable AMPARs in the spinal DH is variable, depending on DH sub-regions, types of terminating fibers, or neuronal properties (Gu et al., 1996; Popratiloff et al., 1996; Spike et al., 1998; Engelmann et al., 1999). Therefore, spinal slices prepared from mice genetically engineered to delete functional GluR2 subunit may have even constant or maximized condition for the expression of Ca^{2+}-permeable AMPARs: i.e., virtually, all AMPARs are Ca^{2+}-permeable. This expectation was likely confirmed by our result that all SG neurons obtained from GluR2 mutant mice showed strong inward rectification in I-V relationship of AMPAR-mediated synaptic currents.

GluR2 Subunit and Synaptic Plasticity

The magnitude of the rise in postsynaptic Ca^{2+} during LTP induction, and the level of NMDAR function, have been shown to have significant effect in the generation of LTP (Malenka and Nicoll, 1999). Previous reports also showed that trans-ACPD, a metabotropic glutamate receptor agonist, enhanced NMDA currents in SG cells, and when applied with a weak tetanus, incapable of inducing LTP by itself, generated LTP (Aniksztejn et al., 1992; Bashir et al., 1993; O'Connor et al., 1994, 1995). Since NMDAR function is strongly modulated by kinase activity (Gerber et al., 1989; MacDonald et al., 1989; Chen and Huang, 1991, 1992; Kelso et al., 1992), it is conceivable that in GluR2 mutants the Ca^{2+} influx from AMPARs could modify the NMDAR function through protein phosphorylation or direct binding to calmodulin (Ehlers et al., 1996). However, this is unlikely, since in GluR2 mutant mice, our data did not reveal any change in the NMDAR function (Jia et al., 1996). The enhanced LTP in GluR2 mutants was likely reconstituted by the addition of NMDA-independent LTP in GluR2 mutants to NMDA-dependent LTP in wild-type mice. In addition, LTD, another NMDA-dependent phenomenon, was not altered. These findings strongly support the hypothesis that Ca^{2+} influx, via AMPARs lacking the GluR2 subunit, is not modifying the NMDA component, and is sufficient for producing LTP.

Several studies have suggested the presence of NMDAR-independent LTP in the CA1-Schaeffer collateral synapses. Perfusion of a high extracellular Ca^{2+} solution induces LTP (Turner et al., 1982), which is not blocked by DAP5. NMDAR-independent LTP was also induced in CA1 by using a 200 Hz, rather than the usual 100 HZ, tetanic stimulation (Grover and Taylor, 1990). Postsynaptic injection of BAPTA, a Ca^{2+} chelator, or nifedipine, the L-type Ca^{2+} channel antagonist, blocked this form of LTP, suggesting that in the presence of DAP5, HVAC channels provided the necessary Ca^{2+} influx required for LTP at the Schaffer collateral synapses. Such a mechanism could underlie the NMDAR-independent enhancement in GluR2 mutants. However, when we perfused slices with nifedipine, in the presence of DAP5, the residual LTP was not affected, thereby indicating the lack of L-type HVAC channel involvement in NMDA-independent LTP in GluR2 mutant mice. In addition, the prevention of membrane depolarization during HFS, by keeping cell at holding potential of –60 mV in the whole-cell patch clamp recordings was not able to block the residual NMDAR-independent LTP of EPSCs recorded from GluR2 mutant neurons, in the presence of DAP5 (121.1 ± 5.4 % of averaged baseline at 16-20 min, n=4; data not shown), also indicating no involvement of any other membrane depolarization-activated channels in the NMDAR-independent LTP. Together, our data support the possibility that Ca^{2+} influx via AMPARs devoid of GluR2 subunit, is sufficient to induce LTP in a NMDAR-independent manner.

On the other hand, the locus of the action of Ca^{2+}-permeable AMPARs in the induction and expression of LTP in GluR2 mutant mice, i.e. 'presynaptic' or 'postsynaptic' is questionable. Although the presynaptic mechanism can not be completely excluded, the finding that there is no difference between wild-type and GluR2 mutant mice in a paired-pulse depression (the ratios of 2^{nd} EPSP to 1^{st} EPSP spaced 100 ms were 0.62 ± 0.10 (n=6) and 0.62 ± 0.06 (n=8) for wild-type and GluR2 mutants, respectively), which is one of parameters frequently examined in testing for presynaptic locus of an action (Gerber et al., 2000), indicates that the presynaptic locus of Ca^{2+} influx through the Ca^{2+}-permeable AMPARs is not a liable mechanism.

Furthermore, it is interesting to note that LTP in GluR2 mutant slices was not saturable, thus indicating that the mechanisms underlying LTP induction are altered. One reason for normal saturable LTP could be that Ca^{2+} influx through the NMDAR channel activates calmodulin, which is known to bind and inactivate the NMDAR channel. Therefore normal LTP may be self-limiting. However, in GluR2 mutant mice, the additional Ca^{2+} influx will not inactivate the AMPAR channel, thereby leading to an ever-increasing LTP at higher stimulation intensities. Alternatively, downstream signaling components may limit LTP in normal animals. In GluR2 mutant mice, the additional Ca^{2+} could activate kinases that are normally rate-limiting to generate non-saturable LTP.

Finally, it is noteworthy in relation to the functional relevance of Ca^{2+}-permeable AMPARs in nociceptive transmission, that the recent findings from behavioral tests indicated that tactile allodynia after thermal injury, and hyperalgesia after formalin injection or carrageenan injection, are blocked or decreased by Ca^{2+}-permeable AMPAR antagonists, Joro spinder toxin or philanthotoxin, without any side effects (Sorkin et al., 1999 and 2001). In addition, a decrease in the GluR2 expression in the spinal DH lamina I-II following dorsal rhizotomy, which can cause radicular pain, was reported (Carlton et al., 1998). Consistent with these behavioral data, our result of the presence of the enhanced LTP and the NMDAR-independent LTP, may be able to provide the underlying cellular mechanism for the certain types of pain behavior, and further, a useful clue for a molecular target to treat chronic pain.

Table. 1. Properties of membrane or synaptic responses recorded by intracellular or whole-cell recordings from the spinal dorsal horn neurons

Intracellular recordings	+/+		-/-	
	Mean ± s. e. m.	n	Mean ± s. e. m.	n
Resting membrane potential (mV)	-72.2 ± 1.3	39	-73.4 ± 1.4	31
Input resistance (MΩ)	212.9 ± 25.1	23	215.6 ± 31.2	27
$EPSP_{threshold}$ (V)	4.7 ± 0.3	30	5.4 ± 0.5	20
Test Stimulus for EPSP (V)	6.2 ± 0.4	34	7.0 ± 0.5	30
monoEPSP (mV)	9.8 ± 1.1	17	8.2 ± 0.9	16
Conduction velocity of A-EPSPs (m/s)	4.4 ± 0.6	16	3.3 ± 0.4	16
Conduction velocity of C-EPSP (m/s)	0.5 ± 0.05	6	0.3 ± 0.02*	3
Whole-cell recordings				
Resting membrane potential (mV)	-61.8 ± 2.7	8	59.9 ± 1.7	7
Input resistance (MΩ)	204.2 ± 29.3	10	230.9 ± 49.3	11

Statistical significance, which compared with the value in +/+, is indicated by an asterisk: *P<0.05. Voltage (V) to determine the threshold of EPSPs and for test stimulus was given at the duration of 0.1 msec.

Fig. 1. Rectification properties of synaptic currents in substantia gelatinosa (SG) neurons.

(A and C) Examples of primary afferents-evoked EPSCs from two wild type (+/+) SG neurons at +40 and -60 mV, showing reduced currents at positive potentials (*left panels*). Electrical stimulation of primary afferents-evoked EPSCs that showed a continuum of I-V relationships (*right panels*), from essentially linear (A, rectification index (RI) = 0.85 ± 0.08, n = 6 slices from 5 mice) in a control solution to inwardly rectifying I-V plots (C, RI = 0.41 ± 0.08, n = 6 slices from 4 mice) in the presence of 50 μM DAP5, 10 μM BMI and 2 μM strychnine. EPSCs were evoked at each potential (20 mV increments). Solid line follows the data points; dashed line at positive potentials represents extrapolated fit for EPSCs behaving ohmically.

(B and D) Synaptic currents (*left panels*) recorded from GluR2 mutant (-/-) SG neurons in control solution (B) and in the presence of 50-100 μM DAP5, 10 μM BMI and 2 μM strychnine (D). By contrast with wild-type neurons (A and C), the I-V relation for the peak of evoked EPSCs in mutant SG neurons (B and D) is strongly inwardly rectifying. In mutant mice showing predominantly inwardly rectifying I-V relationship, RIs were significantly reduced in both control bath solution (B, RI = 0.37 ± 0.06, n = 8 slices from 4 mice, $P < 0.01$) and DAP5 + BMI + strychnine solution (D, 0.07 ± 0.03, n = 6 slices from 4 mice, $P < 0.01$), when compared with wild-type mice (A and C).

(E) A scatter diagram representing RIs of twelve wild-type and fourteen GluR2 mutant (-/-) neurons. *Open circles* indicate neurons examined in a control solution, whereas *triangles* show neurons in the presence of DAP5, BMI and strychnine. The *closed circles* and *triangles* show the mean RI ± SEM for each group.

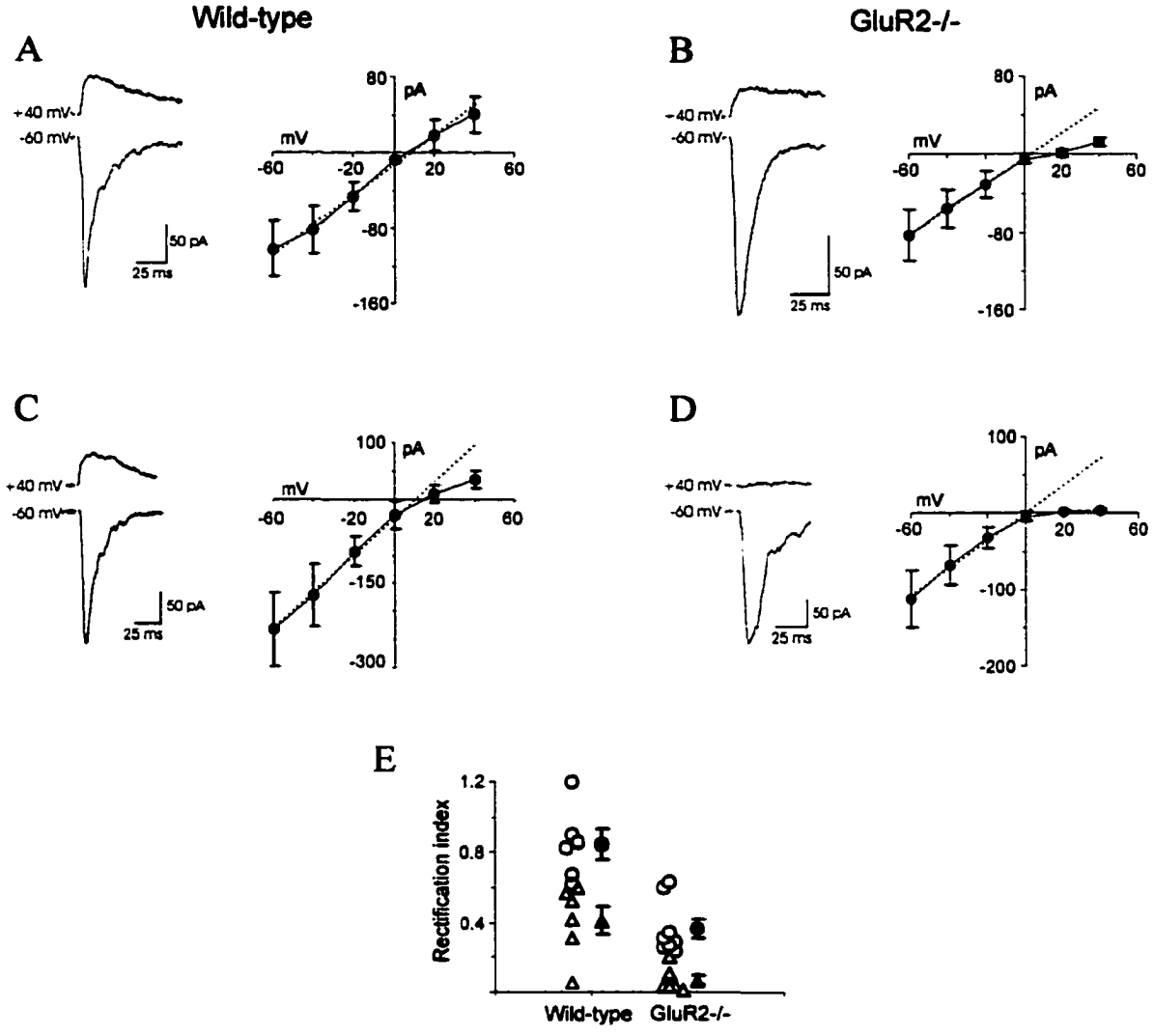

Wild-type
GluR2-/-
A
B
C
D
E
+40 mV
-60 mV
50 pA
25 ms
pA
mV
80
-80
-160
100
-150
-300
-100
-200
-60
-20
20
60
Rectification index
1.2
0.8
0.4
Wild-type
GluR2-/-

Fig. 2. LTP and LTD of excitatory synaptic transmission at primary afferent synapses with neurons in the substantia gelatinosa (SG).

EPSPs recorded intracellularly (sharp microelectrodes) in the SG of spinal cord slices obtained from 2-4 month-old wild type (+/+) and mutant (-/-) GluR2 littermates. *Inset* shows the approximate location of tested SG cells.

(A) The superimposed traces displayed are individual synaptic responses in wild-type and GluR2 mutants taken before (*trace 1*) and during the long-term potentiation (*trace 2*). Sampled traces are the averages of four to six consecutive EPSPs around the corresponding time point indicated by the arabic numeral on the graph.

(B) Summary graphs (mean ± SEM) showing the magnitude and the time course of LTP induced by stimulation consisting of a burst of 100 pulses at 100 Hz, repeated three times at 10 s intervals. The result was averaged from twelve slices from twelve GluR2 mutant (*closed circles*) and ten slices from ten wild type mice (*open circles*). In the absence of GluR2, LTP was increased.

(C) The superimposed traces are individual synaptic responses in wild-type and GluR2 mutants taken before (*trace 1*) and during the long-term depression (*trace 2*).

(D) The graphs show summarized results (mean ± SEM) averaged from seven slices from seven GluR2 mutant (*closed circles*) and nine slices from nine wild-type mice (*open circles*) that upon tetanic stimulation of high intensity developed LTD. However, the magnitude and time course of LTD was not different between mutant mice and wild-type mice.

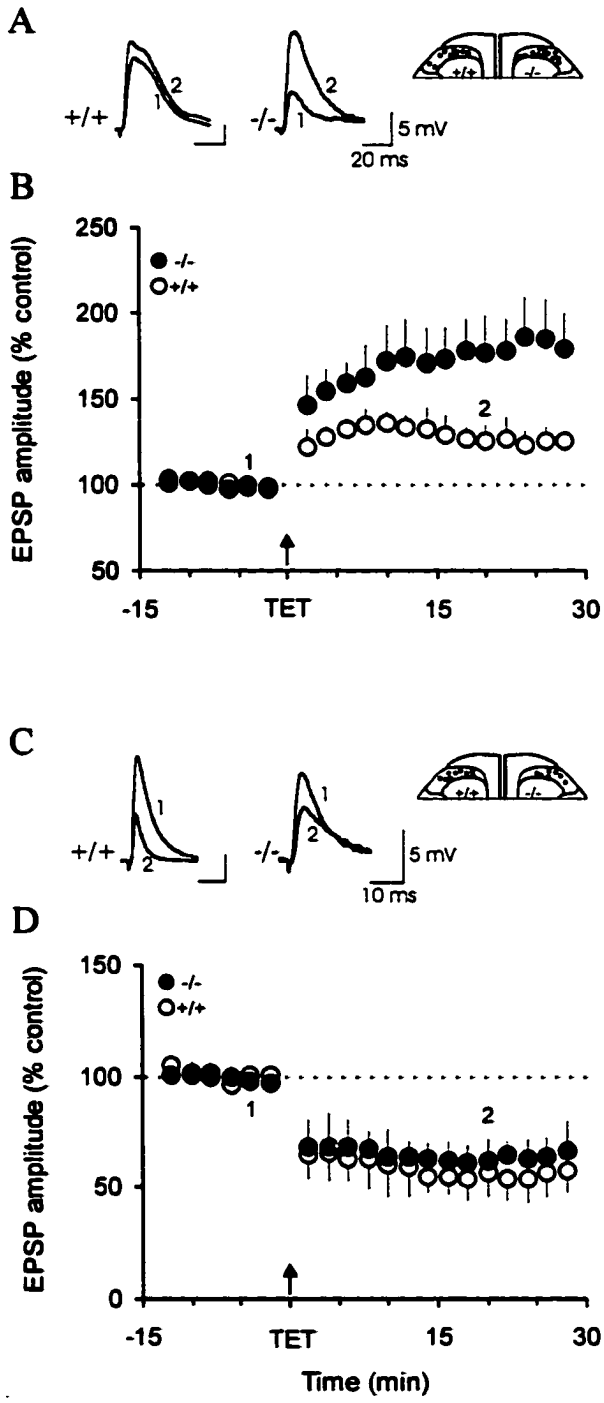
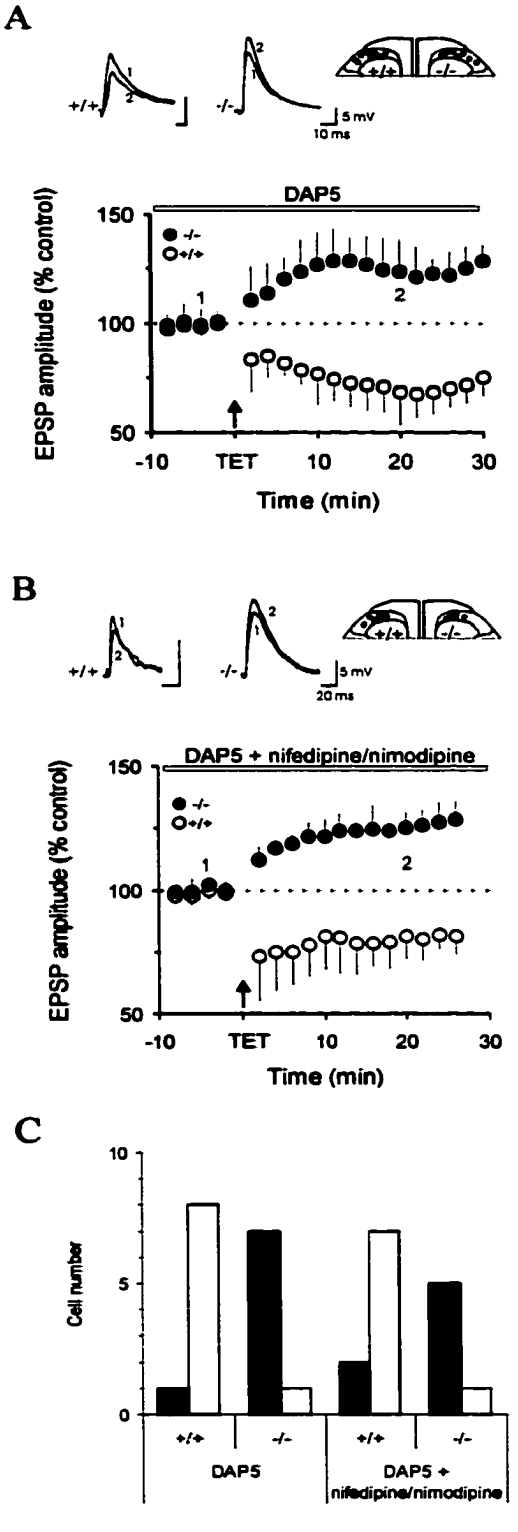

A
+/+
1
2
-/-
1
2
5 mV
20 ms
B
EPSP amplitude (% control)
250
200
150
100
50
-/-
+/+
1
2
-15
TET
15
30
C
+/+
1
2
-/-
1
2
5 mV
10 ms
D
EPSP amplitude (% control)
150
100
50
0
-/-
+/+
1
2
-15
TET
15
30
Time (min)

Fig. 3. NMDA-independent LTP in GluR2 mutant mice.

(A) GluR2 mutant (-/-) mice exhibit NMDA receptor-independent LTP, whereas wild-type (+/+) mice display LTD. The plot summarizes LTP (*closed circles*) induced at time zero (*arrow*) in the presence of 50-100 μM DAP5 from seven slices (seven -/- mice) and LTD (*open circles*) (recorded in the same solution) from eight slices (eight +/+ mice). The superimposed traces displayed above the graph are individual synaptic responses in wild-type and GluR2 mutants taken at the times indicated by arabic numerals. *Inset* shows the approximate location of tested SG cells.

(B) The plot summarizes LTP induced in the presence of 50 μM DAP5 and 10-20 μM nifedipine or nimodipine in five slices obtained from five GluR2 mutant mice, and LTD recorded in seven slices from seven wild-type mice. Data are expressed as mean ± SEM. Sampled traces (*insets*) are averages of four to six consecutive records of EPSPs obtained at the times indicated in the graph by the corresponding numbers. Bars indicate periods of application of each drug.

(C) Summary histogram showing number of SG neurons responding to tetanic stimulation in the presence of DAP5 or DAP5 + HVAC antagonists (nifedipine/nimodipine) with LTP (*closed bars*) and LTD (*open bars*), obtained from eighteen wild-type (+/+) neurons and fourteen mutant (-/-) neurons.


A
+/+
-/-
5 mV
10 ms
DAP5
EPSP amplitude (% control)
150
100
50
-/-
+/+
1
2
-10
TET
10
20
30
Time (min)
B
+/+
-/-
5 mV
20 ms
DAP5 + nifedipine/nimodipine
EPSP amplitude (% control)
150
100
50
-/-
+/+
1
2
-10
TET
10
20
30
Time (min)
C
Cell number
10
5
0
+/+
-/-
+/+
-/-
DAP5
DAP5 + nifedipine/nimodipine

CHAPTER 4. KAINATE RECEPTOR

Modulation of excitatory synaptic transmission in the spinal cord substantia gelatinosa region in mice deficient in the kainate receptor GluR5 or GluR6 subunit

(A part of this chapter was submitted to The Journal of Neuroscience, as a paper by Dong-ho Youn, Stephen F. Heinemann and Mirjana Randić, 2002)

1. Introduction

The substantia gelatinosa (SG), lamina II of the gray matter of the dorsal horn (DH) is the preferential site of termination of small-diameter primary afferent fibers that respond to noxious stimuli (Kumazawa and Perl, 1978; Light and Perl, 1979a, b; Sugiura et al, 1986, 1989; Yoshimura and Jessell, 1989, 1990; Randić et al, 1996; Gerber et al., 1989a,b, 2000a; Moore et al., 2000). The most superficial laminae of the DH are of fundamental importance for nociceptive transmission by virtue of the fact that it is here that most small-caliber myelinated (Aδ) and unmyelinated (C) fibers terminate. Lamina II is of particular interest as the sensory input to this area is almost entirely C-fiber in nature. The primary function of neurons in the SG is to integrate noxious afferent information arriving to this region via the high-threshold Aδ and C fibers. The SG cells function as excitatory and inhibitory interneurons and regulate the output of projection neurons in other laminae of the DH (Willis and Coggeshall, 1991).

Kainate receptors are composed of homomeric and heteromeric configurations of five cloned subunits: GluR5-7 and KA1 and KA2 (Herb et al., 1992; Chittajallu et al., 1999). Kainate receptors are localized at both presynaptic and postsynaptic sites in the superficial laminae of the spinal cord DH (Tölle et al., 1993; Petralia et al., 1994; Hwang et al., 2001). Kainate receptors in the postsynaptic membrane were shown to contribute to excitatory postsynaptic currents (Li et al., 1999), whereas kainate receptors on primary afferent neurons have been proposed to regulate glutamate release and excitatory synaptic transmission (Gerber et al., 1999; Kerchner et al., 2001b).

It is assumed that the relative abundances of the mRNAs for the kainate receptor subtypes will reflect likely composition of the receptor complexes already expressed in the membrane. All five kainate receptor subunits are expressed to some extent in spinal cord neurons. While the vast majority of the high-affinity kainate receptors was accounted for by KA2 (79% for spinal cord neurons), these do not form functional receptor complexes on their own (Herb et al., 1992; Hollmann and Heinemann, 1994; Swanson et al., 1996). However, they can assemble with low-affinity

subunits, such as GluR5 and GluR6, to form functional heteromeric channels with unique properties (Herb et al., 1992; Howe, 1996; Swanson et al., 1996).

In a recent report (Dai et al., 2002), responses of cultured rat spinal neurons to kainic acid (KA) have been related to the expression of kainate receptor subunits revealed by single-cell reverse transcription polymerase chain reaction (RT-PCR). Single-cell RT-PCR showed relative abundances of mRNAs for the kainate receptors GluR5, 6, and 7 of 38, 10, and 54% for spinal cord neurons, respectively. The relative abundance of KA1 and KA2 was 19 and 79% for spinal cord neurons, respectively. The most striking feature of results (Dai et al., 2002) is the relative abundance of GluR6 and paucity of GluR5 in spinal cord neurons. Interestingly, it has recently been shown that, while PCR could detect the mRNAs for all kainate receptor subunits in cultured cortical neurons, only GluR6 and KA2 could be detected by Western blot (Janssens and Lesage, 2001).

In spinal cord neurons, GluR5 and GluR7 were expressed to comparable extents (38 and 54%, respectively), though 4-5 times greater than GluR6 (10%). The likely combination is, therefore, GluR5 and/or GluR7 together with KA2. Although homomerically expressed GluR7 receptors are thought to be either not functional (Lomeli et al., 1992), or KA is of very low potency (Schiffer et al., 1997), Cui and Mayer (1999) have recently demonstrated that heteromeric combinations of GluR5, 6, and 7 can form channels with distinct pharmacological and functional properties. The expression of GluR5 together with KA2 is, therefore, likely to dominate the functional receptors in spinal cord neurons, while the presence of GluR7 is likely to exert a modulatory influence.

Developmental regulation of kainate-type glutamate receptor expression in the rat spinal cord using *in situ* hybridization (Stegenga and Kalb, 2001) showed that the expression of many (GluR5-7, KA1) KA-type glutamate receptor subunits was higher during development and was either not detected or at low abundance levels by adulthood (Stegenga and Kalb, 2001). Low to moderate levels of GluR5 mRNA were detected throughout the P2 spinal cord with slightly more robust expression in the SG. By P10, the signal was selectively present in the SG. GluR5 mRNA levels were not detected in spinal cord tissue at age P22, or adult. Moderate levels of GluR6 signal were present throughout the P2 spinal cord, again with a slightly higher signal in the SG. At P10, 22 and adult ages, however, a definitive signal was not discernible for the GluR6 transcript. The mRNA signal for GluR7 was detectable at low levels throughout the spinal grey mater with a moderate to low signal around the central canal at the P2 spinal cord. By P10 the GluR7 signal was hardly detectable in the SG with all other regions being negative for GluR7 mRNA at P22. Moderate levels of the KA1 transcript were found at P2 spinal cord. The dorsal half of the spinal cord and the region surrounding the central canal display significant levels of KA1 mRNA. No mRNA from the KA1 transcript was detected in

the P22 spinal cord. The KA2 transcript was expressed widely within the developing spinal cord with moderate to high level expression detected within the SG. By P10 and 22, SG retained a moderate level of KA2 mRNA expression and no detectable signal throughout the remaining spinal grey matter. KA2 mRNA was not detected in the adult spinal cord. The precise spatio-temporal of expression of individual kainate receptor subunits implies an important role for specific combinations of subunits in developing neuron function. The ways in which such diversity is employed by neurons is not clear.

In the spinal cord DH, kainate receptors play an important role in sensory transmission. At primary afferent synapses in the rat spinal cord DH, in addition to the modest number of postsynaptic kainate receptors that contribute to EPSPs evoked by high-intensity primary afferent fiber stimulation (Li et al., 1999), there are kainate (GluR5/6/7) receptors expressed presynaptically by dorsal root ganglion (DRG) neurons (Sato et al., 1993a; Petralia et al., 1994; Hwang et al., 2001) that have been proposed to suppress AMPA- and NMDA-mediated excitatory synaptic transmission through a presynaptic action that reduces glutamate release (Gerber et al., 1999; Kerchner et al., 2001b). Small-diameter DRG neurons (Huettner, 1990), which include cells that carry nociceptive information to the spinal cord, are known to express functional kainate receptors (Davies et al., 1979; Agrawal and Evans, 1996; Lee et al., 2001). In addition, it was recently shown that DH inhibitory neurons express presynaptic kainate receptors that stimulate GABA and glycine release that may lead to suppression of inhibitory transmission in the DH (Kerchner et al., 2001a). The first of these functions in the brain has been well established, but the second remains controversial (Frerking and Nicoll, 2000; Lerma et al., 2001).

In the present study, we used gene-targeted mice lacking GluR5, GluR6, or GluR5/6/KA2 kainate receptor subunits to determine the identity of the subunits comprising kainate receptors in the adult mouse spinal cord SG region. We have investigated the regulation by kainate receptors of the spinal excitatory synaptic transmission at Aδ- and/or C-primary afferent fiber-SG synapses, where we found that kainate receptors depress or potentiate the excitatory transmission. These studies lead us to conclude that the GluR5, but more importantly, GluR6 receptor subunit comprises kainate receptors that depress or potentiate primary afferent synaptic transmission to the spinal SG region.

2. Results

Conventional intracellular recordings of up to 7h were obtained from 179 substantia gelanitnosa (SG) neurons in transverse spinal cord slices of wild-type and mutant adult mice. In most of the experiments, each of the neurons recorded from was in a different spinal cord slice preparation. No

significant differences were revealed between wild-type and mutant mice in passive (resting membrane potential, input resistance) or active (stimulus threshold to evoke synaptic response, amplitude of evoked synaptic response, and conduction velocity) membrane properties (but see also Table 1). Single shock electrical stimulation of the primary afferent Aδ and/or C fibers in a L4 or L5 dorsal root elicited monosynaptic and/or polysynaptic excitatory postsynaptic potentials (EPSPs) in SG cells that were suppressed by 50-100 μM GYKI 53655, GYKI 52466 or LY 300164, the AMPA receptor-selective antagonists, and 50 μM DAP5, an NMDA receptor antagonist, in a reversible manner, suggesting that they were primarily mediated by the AMPA/NMDA subtypes of glutamate receptor (Gerber et al., 1989a; Randić et al., 1993; Yoshimura and Jessell, 1990) (Fig. 1).

Activation of kainate receptors inhibits Aδ- and C-primary afferent fiber-evoked excitatory postsynaptic potentials

In agreement with the previous studies (Gerber et al., 1999; Kerchner et al., 2001b), we found that superfusion of slices with KA reduced the amplitude of primary afferent fiber-evoked EPSPs in a dose-dependent manner (0.1–10 μM; Fig. 2A, B). The depression of EPSPs in individual cells was consistently produced at the concentration of 3 μM KA (Fig. 2A, n = 19 slices from 18 wild-type mice). The peak depression had a latency of 3-5 min and the effect persisted for more than 20-30 min after the application of KA was terminated (Fig. 2A). When recording at resting membrane potential, application of 3 μM KA (2 min) caused a slow, dose-dependent and reversible membrane depolarization (5.6 ± 0.7mV) in 14 out of 19 neurons (Table 3). KA-induced depolarization recovered to control levels within 3-10 min after washout of KA. The depolarization also occurred in the presence of 1 μM tetrodotoxin (TTX) (6.5 mV, n = 2), a voltage-dependent Na^+ channel blocker, indicating a direct postsynaptic action of kainate receptors on SG neurons.

Besides KA, we also examined the effects of a low (0.3 μM) and moderate dose (1 μM) of domoic acd (DA), another more potent AMPA/kainate receptor agonist on Aδ- or C-primary afferent stimulation-evoked monosynaptic and polysynaptic EPSPs. Previous studies in the CA1 area of the hippocampus have reported that DA, when applied at a low dose of 0.2 μM, specifically activates kainate receptors (Bureau et al., 1999; Huettner, 1990). We, therefore, tested the effect of DA (0.3-1 μM, 2 min) on the evoked AMPA receptor-mediated EPSPs recorded in SG cells. The decrease in the peak amplitude of EPSPs in the SG cells, receiving input from A and/or C-primary afferents, produced by DA was a dose-dependent in the concentration range tested (Fig. 2C). The DA-induced depression of EPSPs was associated with a dose-dependent and reversible membrane depolarization (3.26 ± 0.8mV for 0.3 μM, n = 3; 7.0 ± 2.3mV for 1 μM, n = 3).

In this set of experiments, we have also used a newly developed putative GluR5 subunit-specific antagonist, LY382884, to test its ability to reverse the depressant action of KA (3 μM, 2 min) on A- and/or C-primary afferent fiber-evoked EPSPs. It has been shown that LY382884 antagonizes responses mediated by kainate receptors at concentrations below those that affect synaptic processes mediated by AMPA or NMDA receptors in the hippocampus (Bortolotto et al., 1999). In contrast to the result from the hippocampus, the inclusion of LY382884 (10 μM) to the bath solution depressed A- and/or C-primary afferent fiber-evoked EPSPs by 23% (n = 7 slices; Fig. 2D). Moreover, the evoked EPSPs, recorded in the solution containing LY382884, were depressed by KA (3 μM, 2 min) to a similar degree (18.1 ± 5.0 % inhibition in LY382884, n = 6 slices) when compared to the depression of the baseline EPSPs by this agent alone, but to a lesser degree when compared to the KA-induced depression in normal Krebs solution ($P < 0.01$; Fig. 2D). In the presence of LY382884, the KA depression of EPSPs was accompanied by a similar degree of membrane depolarization (4.2 ± 1.5mV, n = 4) to that seen in the Krebs solution.

Although we have shown that KA depresses excitatory glutamatergic transmission in the SG, it is not known which type of primary afferents and interneurons are the target of the KA action, because the SG neurons receive glutamatergic inputs from both of these sources (Yoshimura and Jessell, 1989). To determine which synaptic inputs may be regulated by kainate receptors, we next examined the effects of KA (3 μM, 2 min) on Aδ-fiber-evoked monosynaptic and polysynaptic EPSPs and EPSPs evoked in SG neurons by stimulating C-afferent fibers. All three types of EPSPs examined, were depressed in peak amplitude by KA, in all cells examined (Fig. 3A-C). However, the Aδ-fiber polysynaptic and C-fiber-evoked EPSPs were depressed in peak amplitude by KA (3 μM) to a greater extent than the Aδ-fiber monosynaptic EPSP (Aδ-fiber monosynapic EPSP: to 69.3 ± 5.7% of control, n = 8 slices from 8 mice; Aδ-fiber polysynatpic EPSP: to 45.8 ± 5.1%, n = 5 slices from 5 mice, $P<0.01$; C-fiber-evoked EPSP: to 55.8 ± 5.0%, n = 7 slices from 7 mice, $P<0.05$; Fig. 3D, Table 2). Moreover, we observed that activation of kainate receptors, besides depressing the amplitude of Aδ-fiber polysynaptic and/or C-fiber-evoked EPSPs, caused a marked depression of the area of Aδ-fiber polysynaptic EPSP (Fig. 3E) and C-fiber-evoked EPSP (Fig. 3F). In addition, activation of kainate receptors enhanced the likelihood of synaptic failure in C-fiber EPSPs, which we defined as a stimulation event in which an EPSP was not detected above the baseline noise. An example of the synaptic failures caused by 3 μM KA on C-fiber-evoked presumed monosynaptic EPSP is illustrated in Fig. 4. In this cell, the effect of KA involved a reduction of the peak amplitude of the EPSP and also complete failures of transmission (Fig. 4, trace 2).

Effects of bicuculline and strychnine on modulation of synaptic responses by KA

Given the importance of inhibitory processes in the temporal and spatial control of sensory responses in the DH (Malcangio and Bowery, 1996; Coggeshall and Carlton, 1997; Hwang et al., 2001; Ribeiro de Silva and Coimbra, 1982; Todd, 1996), and a recent evidence of the presence of presynaptic kainate receptors in GABAergic and glycinergic interneurons in the superficial laminae of the DH (Kerchner et al., 2001; Lee et al., 2002), in the present study we have investigated possible interaction of KA with inhibitory processes, mediated by $GABA_A$ and glycine receptors. Glycine and GABA are probably co-packaged in and co-released from spinal interneurons (Burger et al., 1991; Christensen and Fonnum, 1991; Jonas et al., 1998). We tested the possibility that KA-induced depression of Aδ-fiber-evoked monosynaptic, polysynaptic, and C-fiber EPSPs is caused by a long-term change in the strength of synaptic inhibition by performing experiments in the presence of bicuculline (5 μM), CGP 55845 (10 μM), and strychnine (2 μM) to eliminate the $GABA_{A,B}$ and glycine receptor-mediated synaptic inhibition, respectively (Figs. 5-7). Moreover, by adding the group I and II metabotropic glutamate receptor antagonist (S)-α-methyl-4-carboxyphenyl glycine (S-MCPG; 500 μM) to the perfusing medium, we also tried to exclude the possibility that KA-induced interneuronal activity in the SG region could cause the release of a neuromodulator that acts heterosynaptically to depress excitatory synaptic transmission.

First, we established dose-response curve for the effects of KA on primary afferent-evoked EPSPs, in the presence of 5 μM bicuculline and 2 μM strychnine ('cocktail'; Fig. 5A). As seen in Fig. 5A, KA modulated primary afferent–evoked excitatory transmission in a dose-dependent biphasic manner. Biphasic effect of KA was revealed with facilitation apparent at a low concentration (30 nM for 2 min; Fig. 19) and depression at a higher concentration (3 μM for 2 min; Fig. 5B, Table 2). The degree of KA (3 μM)-induced depression of EPSPs was significantly reduced when compared with that in a normal Krebs solution (Krebs solution: to 58.0 ± 3.9% of control; bic + strych cocktail: to 74.7 ± 4.3% of control, n = 16 slices from 9 mice; $P<0.01$; Fig. 5C). Following washout of KA from the bath solution, thirteen out of sixteen slices showed almost full recovery in EPSPs, whereas in three slices no recovery was seen. In a representative neuron receiving polysynaptic input from Aδ-primary afferent fibers, the second application of 3 μM KA, in the presence of 5 μM bicuculline and 2 μM strychnine, showed a reduced depressant effect when compared to the effect produced by the first application of KA in a normal Krebs solution (Fig. 6A). Similar as in the case of the KA effect on EPSPs in the Krebs solution, the KA-induced depression was accompanied by the membrane depolarization (4.6 ± 1.1 mV, n = 11 slices; Table 3) when slices were perfused with bicuculline and strychnine. The ability of KA to suppress primary afferent-evoked EPSPs in the absence of synaptic

inhibition-mediated by $GABA_A$ and glycine receptors was not affected by successive addition of CGP 55845 (10 μM), the $GABA_B$ receptor antagonist, and S-MCPG (500 μM), the group II an III metabotropic glutamate receptor antagonist (Fig. 5C), suggesting that in the presence of bicuculline and strychnine these receptors did not contribute to the KA-induced depression of excitatory transmission.

We next examined the possibility that the blockade of synaptic inhibition by bicuculline and strychnine may differentially affect the KA-induced depression of Aδ-fiber-evoked monosynaptic, polysynaptic and C-fiber-evoked EPSPs. Although, in each of 18 slices bathed in this solution, the KA (3 uM) decreased the peak amplitude of Aδ-fiber-evoked monosynaptic (by 24.0 ± 4.8%, n = 5; Figs. 6B, 7A; Table 2) and polysynapic (by 12.3 ± 6.2%, n = 6; Figs. 6A-C, 7B), and EPSPs elicited at C-fiber strength (by 46.6 ± 9.5%, n = 5; Figs. 6B, C, 7C; Table2) after inhibition was blocked, the KA depression was only significantly reduced, in comparison to that seen under normal perfusing conditions, in the SG cells receiving polysynaptic inputs ($P<0.01$). Besides reducing the EPSP amplitudes we found that KA reduced the area under the EPSPs (Fig. 6C, 7D). This result is consistent with anatomical (Hwang et al., 2001) and recent electrophysiological findings (Kerchner et al., 2001b; Lee et al., 2002) that presynaptic kainate receptors are present on GABA and glycinergic inhibitory interneurons in the spinal cord DH, where they regulate GABA/glycine release and likely contribute to the modulation of both inhibitory and excitatory synaptic transmission in the spinal cord DH. Thus, it can be concluded that whereas the KA-induced long-lasting depression of Aδ-fiber-evoked monosynaptic EPSP (Fig. 7A) and C-fiber-evoked EPSPs (Fig. 7C) occurs independently from long-term changes in synaptic inhibition, the KA-induced depression of the polysynaptic EPSP is dependent on the modulation of synaptic inhibition by the kainate receptors.

On the other hand, as illustrated in Fig. 8, a small population of SG cells receiving monosynaptic (1 out of 5 slices; Fig. 8A) or polysynaptic (3 out of 6 slices; Fig. 8B) inputs from Aδ- or C-fibers (1 out of 5 slices; Fig. 8C), showed transient potentiation, or biphasic effect consisting of initial depression followed by enhancement of EPSP amplitude by application of KA (3 μM, 2 min). This effect was never observed in SG cells perfused with the normal Krebs solution. The excitation frequently occurred within the first minute of the application of KA, or, occasionally several minutes after washout of KA. We assume that this excitatory effect was a result of a low concentration of KA during onset of application or upon washout, combined with a decreased inhibitory drive caused by bicuculline and strychnine.

Both GluR5 and GluR6 subunits contribute to the kainate receptor-induced long-lasting depression of excitatory synaptic transmission in the substantia gelatinosa

We have used kainate receptor mutant mice lacking GluR5 (Mülle et al., 2000) or GluR6 (Mülle et al., 1998) subunit to explore their contribution to receptors responsible for the depressant action of kainate receptors in the SG neurons. This action was postulated to arise from presynaptic GluR5-containing kainate receptors based on the action of the putative selective GluR5 agonist ATPA (Kerchner et al., 2001b), and the observation that GluR5 subunit is highly expressed in small-diameter DRG cells, on which they may act as autoreceptors (Agrawal and Evans, 1986; Lee et al., 1999; but see Stegenga and Kalb, 2001).

We compared the effects of kainate receptor activation on the peak amplitude of primary afferent Aδ- and/or C-fibers-evoked EPSPs in SG neurons from wild-type and kainate receptor mutant mice lacking GluR5 or GluR6 subunit. Conventional intracellular recordings from SG neurons in acute slice preparations were made both in the presence, and in the absence, of $GABA_A$ receptor- and glycine receptor-mediated synaptic inhibition. As shown in Fig. 9B, a dose-response curve showed a higher sensitivity to kainate for SG neurons recorded in wild-type mice as compared to GluR5-lacking mice. In a representative neuron from a mouse in which the GluR5 gene had been disrupted ($GluR5^{-/-}$ genotype; Mülle et al., 2000), bath application of 3 μM kainate hardly produced any depression of primary afferent fiber-evoked EPSPs (Fig. 9A). The concentration required to evoke a depression of EPSPs in this neuron was increased to 10 – 20 μM (Fig. 9A,B). The latter result indicates that the depressant effect of higher concentrations of kainate on EPSPs in GluR5 mutant mice may be mediated by activation of other receptors, but not of kainate receptors (Mulle et al., 1998, 2000; Contractor et al., 2000). The amplitude of Aδ- or C-fiber EPSPs was significantly decreased by 3 μM KA in wild-type mice but only 10-20 μM KA, for the similar degree of depression in GluR5 mutant mice (Fig. 9B). Taken together, these data show that at KA concentrations of up to 3 μM (to a few μM), only kainate receptors are activated by KA in the primary afferent fiber-SG neuron pathway and that these receptors contain the GluR5 subunit.

Summarized data from 25 SG neurons showing the time course of the KA-induced depression of the EPSPs of SG cells recorded in slices absent from GluR5 mutant mice are shown in Fig. 9C. In almost all slices (22 out of 25 slices; Fig. 9C) KA application caused initial slight depression, followed by full recovery. In slices obtained from GluR5 mutant mice, the KA-depression was accompanied by membrane depolarization (4.7 ± 0.7 mV, n = 25; Table 3). In addition, it should be noted that in 7 of 22 SG neurons, KA application caused a remarkable increase in the amplitude of the monosynaptic, polysynaptic, and C-fiber EPSPs (Fig. 10). This effect was never observed after

application of 3 μM KA to wild-type slices perfused with a normal Krebs solution, but was occassionally (5 of 16 cells) seen when bicuculline and strychnine were included in the perfusing solution to block $GABA_A$ and glycine receptor-mediated synaptic inhibition (Fig. 8).

To determine the identity of the subunit(s) comprising kainate receptor that depresses the Aδ-fiber-evoked monosynaptic or polysynaptic, and C-fiber-evoked excitatory transmission in the spinal SG neurons, we examined slices obtained from GluR5 or GluR6 mutant mouse in a normal Krebs solution or a solution containing 5 μM bicuculline and 2 μM strychnine (Fig. 11). In a normal Krebs medium, bath application of 3 μM KA for 2min reduced the amplitude of primary afferent Aδ-fiber-evoked monsynapic EPSPs to a similar degree in neurons from wild-type mice, and in mice in which the GluR5 (Figs. 12A, D and 17A; Table 2) or GluR6 (Figs. 15A and 17A; Table 2) subunits had been disrupted. In contrast, Aδ-afferent fiber-evoked polysynaptic and C-fiber-evoked EPSPs were significantly less depressed in amplitude/area by KA in slices from mice in which there was a null mutation either in GluR5 (Figs. 12, 17A; Table 2) or GluR6 (Figs. 15, 17A; Table 2) genes, than in the slices from wild-type mice. When bicuculline (10 μM) and strychnine (2 μM) were included in the perfusing solution to block $GABA_A$ and glycine receptors-mediated synaptic inhibition, KA (3 μM, 2 min) application to slices obtained from GluR5 mutant mice inhibited primary afferent Aδ-fiber monosynaptic (Figs. 13A, D, 17B; Table 2) and polysynaptic EPSPs (Figs. 13B, E, 17B; Table 2) to a degree similar to that observed in the slices perfused with a normal Krebs solution. However, in GluR5 mutant neurons, bath application of KA produced a depression of C-fiber-evoked EPSPs that was significantly smaller if compared with that recorded in slices of wild-type mice (Figs. 13C, F, 17B; Table 2). In contrast to the results obtained in GluR5 mutant mice, we found that in neurons from mice lacking the GluR6 subunit, the application of KA (3 μM, 2 min) had no depressant effect on monosynaptic (Figs. 16A, 17B; Table 2), polysynaptic (Figs. 16B, E, 17B), or C-fiber-evoked EPSPs (Fig. 16C, F, 17B; Table2).

Taken together, these data suggest that: 1) under normal physiological conditions the activation of both GluR5 and GluR6-containing receptors contributes to the depression of the excitatory synaptic transmission in the Aδ-fiber-evoked polysynaptic and C afferent fiber-mediated pathways in the SG region of adult mice; 2) In contrast, in the absence of synaptic inhibition mediated by $GABA_A$ and glycine receptors, GluR6 subunit is critically involved in inhibiting Aδ- and C-fiber-elicited primary afferent neurotransmission in the SG region. On a speculative note, our study envisages that polysynaptic Aδ- and C-fiber activated pathway, mice lacking GluR5 subunit may produce functional receptors, whereas the removal of GluR6 subunit might prevent either the production of receptors or their delivery to the presynaptic membrane.

Besides the small depressant effect of KA on monosynaptic EPSPs in GluR5 mutant mice, which is predominantly mediated by the AMPA receptors, bath application of KA (3 μM, 2 min) depressed or blocked completely (n = 1; Fig. 14) on the DAP5-sensitive component of EPSP which appeared following the perfusion of a slice with bicuculline and strychnine. This data indicate that, in contrast to the effect of KA on the AMPA receptor-mediated transmission, the NMDA receptor-mediated transmission in the mouse spinal DH may not be affected by the genetic deletion of GluR5 subunit in kainate receptors.

Activation of kainate receptors on mouse primary afferent neurons reduces mEPSC frequency and may modulate transmitter release

To provide further evidence for the functional kainate receptors on the adult mouse primary afferent neurons, and to determine their subunit composition, we have examined whether the application of KA modifies the frequency of action-potential independent miniature EPSCs (mEPSCs) recorded from the SG neurons in adult spinal slices of wild-type and GluR5 and GluR6 mutant mice. A previous report did find that KA increases frequency of action potential-independent mEPSCs recorded from capsaicin-sensitive SG neurons in young (P8) rat spinal cord slices (Lee et al., 1999). This finding supports a role for presynaptic kainate receptors in the regulation of synaptic transmission in the nociceptive pathway.

To test for the presence of presynaptic kainate receptors at adult mouse spinal SG excitatory synapses and for their possible role in the presynaptic kainate receptors-mediated depression of evoked EPSPs, we examined the effect of kainate receptor activation on frequency and amplitude of the spontaneous mEPSCs recorded by using whole-cell voltage-clamp technique. Recordings from SG neurons in wild-type spinal slices were made in the presence of 500 nM TTX, which blocked all voltage-gated Na^+ current, and 5 μM bicuculline plus 2 μM strychnine to eliminate inhibitory events. In some experiments, 50 μM DAPV was also used to block NMDA receptors. Neurons were voltage-clamped at –70 mV, and AMPAR-mediated mEPSCs appeared as inward currents at averaged background frequency of $10.8 \pm 5.6 s^{-1}$ (n = 10 slices from 7 mice). Upon application of KA (3 μM, 2 min), the frequency of mEPSCs during the exposure decreased to 64.8 ± 4.1% of the control value (n = 10, $P<0.01$; Fig. 18), suggesting that KA acted at a presynaptic locus. However, no significant change in mEPSC amplitude was observed during KA exposure (Fig. 18). Application of 50 μM GYKI 53655 or 10-20 μM CNQX eliminated mEPSCs, confirming that the postsynaptic events resulted from release of glutamate and predominant activation of AMPA receptor.

To determine the subunits comprising kainate receptors underlying the decrease in mEPSC frequency, we recorded mEPSCs in mutant mice. In contrast to neurons in wild-type mice, mEPSC frequencies in SG neurons from GluR5 or GluR6 mutant mice (GluR5-/-, 83.9 ± 6.8% of control, n = 7 slices from 4 mice, $P<0.05$ vs. wild-type; GluR6-/-, 85.9 ± 8.1% of control, n = 7 slices from 3 mice, $P<0.05$ vs. wild-type; Fig. 18) were significantly less reduced, with no significant change in mEPSC amplitude (Fig. 18). This result indicates that presynaptic kainate receptors containing GluR5 or GluR6 subunit can modulate action potential-independent glutamate release at excitatory synapses in the spinal DH. Surprisingly, a change in the holding current during KA application occurred in the SG cells from wild-type (-18.3 ± 6.5pA) and GluR5 mutant mice (-24.9 ± 6.1pA), but not in the cells from GluR6 mutant mice (-0.7 ± 1.7pA, $P<0.05$ vs. wild-type) (Table 3). This result is similar to that obtained with the intracellular recordings, where a significant reduction in membrane depolarization was observed in slices from the GluR6 mutant mice when compared to wild-type or GluR5 mutant mice (2.9 ± 0.7mV, n = 15 GluR6 mutant slices; $P<0.01$ vs. wild-type or GluR5-/-; Table 3). This finding indicates a possible presence of the GluR6 kainate receptor subunit on the postsynaptic membrane of the SG neurons, which is contrary to the previous anatomical data (Hwang et al., 2000) and the results of the *in situ* hybridization experiments in rats (Tolle, 1993).

Nanomolar concentration of KA strongly facilitates excitatory synaptic transmission in the SG

KA (30 nM, 2 min) produced a robust and long-lasting enhancement of the DR-evoked dual component (AMPA/NMDA)-mediated EPSPs (Figs. 5A, 19A). This effect, although observed in 1 out of 4 slices performed in the normal Krebs solution, was more frequently recorded in the presence of 5 μM bicuculline and 2 μM strychnine (n = 5 out of 5 slices from 5 mice). The mechanism underlying the facilitation of EPSPs at primary afferent-SG synapses has yet to be elucidated. It has been recently reported that activation of presynaptic kainate receptors by low doses of KA (50nM) can facilitate transmitter release from hippocampal mossy fiber synapses (Schmitz et al., 2001). This result indicates that at mossy fiber synapses this form of synaptic plasticity is mediated, at least in part, by the long-lasting activation of a kainate autoreceptors. It has been shown in the young (2-3-week-old) rat spinal DH that long-term potentiation of synaptic transmission, induced by high-frequency electrical stimulation, was critically dependent on the activation of NMDA receptors (Randić et al., 1993). To determine, therefore, if the induction of 30nM KA-induced long-lasting potentiation of synaptic responses is dependent upon NMDA receptors, the recordings were carried out in the presence of the NMDA receptor antagonist DAP5. We find that perfusion of the slices with the cocktail solution containing 50 μM DAP5 and bicuculline/strychnine blocked the long-lasting

potentiation of EPSPs induced by a low concentration of KA (30 nM, 2 min; Fig. 19A), indicating the NMDA-dependency of the effect. The next series of experiments was done using slices from the GluR5 or the GluR6 mutant mice to examine the possible involvement of the GluR5 or the GluR6 subunit in the KA (30nM)-induced long-lasting potentiation of EPSPs (Fig. 19B). In GluR5 mutant mice, bath-applied KA (30 nM, 2 min) evoked a short- or long-lasting potentiation in 6 out of 9 slices, with no change in the rest. However, none of the slices obtained from the GluR6 mutant mice showed any potentiation of EPSPs (no effect of KA in 5 out of 7 slices, and depression in the rest). The average time courses for the effects of KA (30 nM, 2 min) in nine GluR5 mutant SG cells, and seven GluR6 mutant cells, were illustrated in Fig. 19B. Interestingly, the long-lasting potentiation of EPSPs-induced by the low concentration of KA was significantly reduced by the absence of GluR5, and completely abolished in GluR6 mutants, suggesting that synaptic plasticity in the adult mouse spinal cord can be mediated by the GluR5 or GluR6 subunit-containing kainate receptors (Bortolotto, 1999; Gerber et al., 1999; Contractor et al., 2001).

The biphasic action of ATPA on primary afferent fiber-evoked EPSPs

As previously shown (Gerber et al., 1999; Kerchner et al., 2001b), and also in the present study, the activation of kainate receptor depresses primary afferent-evoked excitatory synaptic transmission in the rat and mouse SG region, the effect postulated, in a rat study, to arise from activation of presynaptic kainate receptors containing the GluR5 subunit (Kerchner et al., 2001b). In the present study, we tested this hypothesis by utilizing the putative GluR5 selective agonist (R,S)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid (ATPA; Clarke et al., 1997) and a GluR5 selective antagonist (3S,4aR,6S,8aR)-6-((4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline -3-carboxylic acid) (LY 382884; O'Neil et al., 1998), as well as gene-targeted mice deficient in the GluR5 subunit of the kainate receptor, to determine whether GluR5 subunit is involved in the depressant effect.

Here we show that bath application of (RS)-ATPA (1-3 μM, for 2 min) in a Krebs-bicarbonate medium causes predominantly a potentiation of EPSPs in 5 out of 7 cells (one cell depressed, and one showed no effect) receiving A and/or C fiber primary afferent input (Fig. 20A). This effect was accompanied by a depolarization of a membrane potential (3.3 ± 0.6 mV, n = 6). Summarizing the data obtained from all seven SG cells, the average time courses showed apparently slow-onset potentiation of EPSPs (Fig. 20A) with the maximum effect occurring between 9 and 15 min from the start of drug application. Therefore, we calculated the change in the EPSP amplitude-induced by

ATPA at 9-15 min window, and plotted in a scatter diagram to later compare with other genotypes (Fig. 20B).

When bicuculline (5 μM) and strychnine (2 μM) were included in the perfusing solution to block $GABA_A$ and glycine receptors, however, ATPA (1-3 μM for 2 min) reversibly depressed (62.6 ± 13.3 % of control, n = 3; data not shown) the A- and C-afferents-mediated EPSPs in 3 out of 4 SG cells tested, whereas one was potentiated. Interestingly, the ATPA-induced biphasic action (depression/potentiation) on EPSPs was not blocked by further addition of 50 μM of DAP5 to the cocktail solution (depression, n = 2; potentiation, n = 1; data not shown), indicating that the ATPA action is not NMDA-dependent.

In contrast to wild-type mice, SG neurons recorded in slices obtained from GluR5 mutant mice did more frequently show the depression (5 out of 9 slices; Fig. 20B), rather than the potentiation of EPSPs following the bath application of ATPA (1 or 3 μM, 2 min). The ATPA effect was accompanied by a depolarization of a membrane potential (3.25 ± 0.6mV, n = 4). The depressant effect of ATPA seen in the GluR5 mutants, was not blocked by bicuculline and strychnine (n = 4; data not shown), but it was absent in the slices obtained from GluR6 (n = 4) or GluR5/GluR6/KA2 (triple; n = 4) mutant mice (Fig. 20B). Interestingly, the resting membrane potential during KA application was not changed in GluR6 mutants (0.67 ± 1.2 mV, n = 3), but it was hyperpolarized in a triple mutant mice (-4.7 ± 2.9 mV, n = 3). These results show that: 1) the GluR5 subunit can contribute to a kainate receptor that regulates excitatory synaptic transmission in the SG region in a biphasic manner under normal conditions, and 2) the inhibitory tone is critical in determining the direction of the regulation of sensory transmission.

Taken together, our data indicate that ATPA, claimed as a GluR5 subunit selective agonist, has apparently potentiating effect on spinal synaptic transmission, which is probably mediated by GluR5-containing kainate receptors. In addition, the depressant effect of ATPA on EPSPs, or depolarizing effect on membrane potential may be a side effect deriving from the activation of other kainate receptor subunits, or AMPA receptors (Paternain et al., 2000; Clarke and Collingridge, 2002). It has been reported that in the absence of the blockade of AMPA receptors by GYKI 53655, concentrations of ATPA greater than 3 μM resulted in inward currents, presumably due to activation of AMPARs (Lauredsen et al., 1985; Clarke et al., 1997). Thus, our observations may reflect heterogeneity in the prevalence of GluR5 subunit among excitatory and inhibitory interneurons in the spinal SG or nonselective action on heteromeric receptors lacking GluR5 unit (Paternian et al., 2000).

Although we have shown that LY382884 reduced the KA-induced depression of EPSPs (Fig. 2), due to its non-selective depressant action on the basal synaptic transmission in the SG neurons, this

agent proved not to be useful in our pharmacological analysis of the involvement of GluR5 subunit in the action of ATPA in the SG region.

Kainate receptors are involved in long-term synaptic plasticity at primary afferent synapses in the mouse substantia gelatinosa

Involvement of kainate receptors in long-term synaptic plasticity has recently been indicated at hippocampal mossy fiber synapses (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001) and spinal primary afferent synapses (Gerber et al., 1999). However, it is still not clear which subunit of kainate receptors is involved in the long-term potentiation (LTP) of excitatory synaptic transmission. Bortolotto et al. (1999) showed that the induction of mossy fiber LTP, which is independent of NMDA receptor activation (Harris and Cotman, 1986), was occluded in the presence of the putative GluR5 subunit specific antagonist LY382884. However, Contractor et al. (2001) demonstrated that the mossy fiber LTP was significantly impaired in GluR6 mutant mice. Therefore, we tested the hypothesis that the GluR5 subunit may be involved in LTP in the spinal cord, and this result was reported in an abstract form (Gerber et al., 1999). As summarized in Fig. 21, twenty minutes after high-frequency tetanic stimulation (3 tetani of 100 pulses at 100Hz) of primary afferent fibers in dorsal roots to induce LTP, a significant increase in the peak amplitude of Aδ- and C-primary afferent fiber-evoked EPSPs was observed in wild-type mice (133.4 ± 12.6 % of the averaged baseline response, $n = 7$; $P<0.05$), but not in the GluR5 mutant mice (100.1 ± 8.7% of baseline, $n = 9$; $P>0.05$). However, the mean changes were significantly different between wild-type and GluR5 mutant mice ($P<0.05$; Fig. 21A). In addition to LTP, a subpopulation of cells showed long-term depression (LTD; Fig. 21B) when the same stimulation protocol, as for the induction of LTP, was used. Eighteen minutes after the induction of LTD, a significant depression of EPSPs was observed in wild-type mice (to 55.1 ± 13.3% of the averaged baseline response, $n = 7$; $P<0.01$), but not in GluR5 mutant mice (to 81.1 ± 10.4%, $n = 3$; $P>0.05$). However, the mean changes were significantly different between wild-type and the mutant mice ($P<0.05$). This result suggests that GluR5 kainate receptor subunit is involved in the synaptic plasticity in the mouse spinal cord DH (Gerber et al., 1999).

3. Discussion

Mice with disrupted KA receptor subunit genes offer the possibility to investigate the specific role of KA receptor subtypes in synaptic transmission. The present study revealed that KA receptors

comprised of GluR5 and/or GluR6 subunits have multiple and complex roles in regulating excitatory synaptic transmission in the substantia gelatinosa region of the spinal cord. Activation of KA receptors by exogenous agonist kainate can either suppress or facilitate primary afferent fiber Aδ-fiber- and/or C-fiber-mediated glutamatergic transmission in the adult mouse substantia gelatinosa of acutely isolated spinal cord slices. In difference to previous studies, which were based on pharmacological characterization of KA receptors, we used gene-targeted mice lacking either GluR5 or GluR6 KA receptor subunit to determine the identity of receptor subunits comprising KA receptors that play a role in modulation of primary afferent neurotransmission. Using this approach, we found that in the presence of synaptic inhibition, mediated by $GABA_A$ and glycine receptors, both GluR5 and GluR6 subunits are involved in inhibiting transmission at the primary afferent Aδ-fiber-activated polysynaptic pathways and C-fiber pathways. In the absence of synaptic inhibition, we report that KA receptors suppress excitatory transmission during intense stimulation, but with more mild levels of activation, they might be facilitatory. We determined that the GluR6 subunit is critically involved in inhibiting transmission at both primary afferent Aδ- and C-fiber monosynaptic pathways, whereas GluR5 plays a lesser role in inhibiting the C-fiber-activated pathway. Moreover, here we show that application of kainate at nanomolar concentrations revealed a long-lasting, NMDA receptor-dependent facilitation of primary afferent neurotransmission. This effect was abolished in GluR6-deficient mice, but only reduced in GluR5 mutants, suggesting that both GluR5 and GluR6 KA receptor subunits contribute to the KA receptor-mediated potentiation of excitatory transmission at synapses on SG neurons. These results suggest: 1) bidirectional regulation of excitatory synaptic transmission by KA receptors in the substantia gelatinosa; and 2) a role for kainate receptors in short- and long-term changes in synaptic strength. Biphasic effects of presynaptic kainate receptor activation on excitatory transmission have been demonstrated at hippocampal mossy fiber synapses with CA3 neurons (Kamiya and Ozawa, 2000; Schmitz et al., 2000, 2001; Contractor et al., 2000)

Kainate receptor-mediated depression of excitatory synaptic transmission

We have briefly reported that application of KA depresses AMPA receptor-mediated excitatory synaptic transmission in the SG region in slices obtained from adult mice (Gerber et al., 1999). It has been recently shown that KA and ATPA, a putative selective GluR5-kainate receptor agonist (Clarke et al., 1997; Hoo et al., 1999), suppressed NMDA receptor-mediated EPSCs in rat DH neurons evoked by stimulation of synaptically coupled DRG cells in DRG-DH neuron co-cultures (Kerchner et al., 2001b). Moreover, in recordings from young rat (P2-P21) DH neurons in spinal slices, KA suppressed NMDA receptor-mediated EPSCs, whereas ATPA reduced AMPA receptor-mediated

EPSCs evoked by dorsal root fiber stimulation (Kerchner et al., 2001b). Based on the observation of selectivity of ATPA for DRG cell KA receptors, this effect was postulated to arise from activation of presynaptic kainate receptors-containing GluR5 subunit (Kerchner et al., 2001b). This finding was in agreement with other evidence indicating that KA receptor expressing GluR5 subunit is present at high levels on small-diameter DRG neurons (Partin et al., 1993; Sato et al., 1993; Hoo et al., 1999; Hwang et al., 2001), but is much less prevalent in the spinal cord DH (Tölle et al., 1993; Hwang et al., 2001). However, the contribution of the GluR5, or other subtypes of KA receptor to the AMPA or NMDA receptor-mediated spinal sensory transmission and plasticity, has yet to be more directly demonstrated.

In this study we compared the effects of KA on excitatory transmission in monosynaptic and polysynaptic primary afferent Aδ- and C-fiber pathways in the SG region in wild-type and KA receptor GluR5 or GluR6 subunit gene-deficient mice, in order to determine which receptor subunits are involved in the kainate receptor depressant action. We found that in the presence of synaptic inhibition mediated by $GABA_A$ and glycine receptors, KA strongly suppressed polysynaptic EPSPs evoked by stimulation of primary afferent Aδ-fibers, and C-fiber-activated EPSPs (monosynaptic, polysynaptic), in neurons from wild-type mice. In contrast, the KA depressant effect on EPSP amplitudes recorded from SG neurons in slices from GluR5- and GluR6-deficient mice was significantly reduced, suggesting that both GluR5 and GluR6 receptor mechanism does account for, at least a part of, the KA depressant effect. However, in the absence of synaptic inhibition, we found that GluR6 subunit is predominantly involved in inhibiting transmission at both primary afferent Aδ- and C-fiber monosynaptic pathways, whereas GluR5 subunit plays a lesser role in suppressing the transmission in the C-fiber-activated pathways.

Our data with mice lacking GluR5 or GluR6 KA receptor subunit are at least consistent with the present evidence suggesting that GluR5 and GluR6 can exist as homomeric (Bettler et al., 1990; Sommer et al., 1992; Sato et al., 1993; Petralia et al., 1994; Swanson et al., 1996, 1998; Wilding and Huettner, 2001) and/or heteromeric receptor combinations (Bettler et al., 1990; Partin et al., 1993; Sahara et al., 1997) at primary afferent neurons. Furthermore, it is apparent that GluR6 expressed by DRG and/or SG interneurons, may have the major functional role in the regulation of transmission at both primary afferent Aδ- and C-fiber synapses in the SG region. This possibility does agree with what is known about the regional expression of individual subunits (Sato et al., 1993; Tölle et al., 1993; Petralia et al., 1994; Yung, 1998; Dai et al., 2002; Hwang et al., 2001, but see Stagenga and Kalb, 2001).

Because of the apparent divergence between our data from mice lacking GluR5 subunit and the

findings of pharmacological studies with ATPA in the rat spinal slices (Kerchner et al., 2001b), we examined the possibility for the ATPA-mediated depression of the primary afferent fiber-evoked EPSPs. We found that ATPA enhanced EPSPs-evoked by primary afferent stimulation in neurons from wild-type and GluR6-deficient mice; in contrast, suppression of EPSP amplitudes was recorded from neurons from GluR5 mutants. One potential explanation that may reconcile apparent discrepancy between the pharmacological and genetic observations is that ATPA activates heteromeric DH neuronal receptors-containing both GluR5 and GluR6 subunits. More recent studies (Bortolotto et al., 1999; Cui and Mayer, 1999; Paternain et al., 2000) have shown that heteromeric KA receptor subunit combinations formed by GluR5 plus GluR6 or GluR7 subunits retain sensitivity to ATPA. In addition, there is evidence that ATPA can activate heteromeric receptors formed by co-expression of the GluR6 and KA2 subunits in HEK 293 cells (Paternain et al., 2000).

It is of interest in relation to our data that in the CA3 region of the hippocampus, KA profoundly reduced EPSCs at mossy fiber and collateral synapses in neurons from wild-type and GluR5$^{-/-}$ mice, but had no effect on EPSCs in neurons from GluR6$^{-/-}$ mice (Contractor et al., 2000), the results that did not support pharmacological experiments with ATPA (Vignes et al., 1998) and LY 382884 (Bortolotto et al., 1999) suggesting a critical role for GluR5 receptors at mossy fiber and associational commissural synapses (Vignes et al., 1998; Bortolotto et al., 1999). In contrast, at perforant path synapses, KA receptor activation enhanced transmission, and the effect was abolished in both GluR5 and GluR6 knock-out mice (Contractor et al., 2000).

Presynaptic kainate receptors decrease action potential-independent glutamate release

KA receptors are expressed on DRG cell bodies (Huettner, 1990), on peripheral fibers and axon terminals (Ault and Hildebrand, 1993), and preferentially on central terminals of sensory neurons, on which they could act as true autoreceptors (Agrawal and Evans, 1986; Lee et al., 1999, 2002; Kerchner et al., 2001b; Hwang et al., 2001). Previous work has demonstrated strong depolarizing responses when KA was applied to dorsal roots (Agrawal and Evans, 1986). Evidence has been recently provided for the involvement of presynaptic kainate receptors in the KA-induced depression of primary afferent neurotransmission (Lee et al., 1999, 2002; Kerchner et al., 2001b). In the young rat (P8) spinal cord, Lee et al., (1999) showed that KA application increased the frequency of spontaneous TTX-insensitive postsynaptic currents (mEPSCs), although the synapses responsible for these currents (excitatory vs inhibitory; primary afferent synapses vs local synapses) were not identified. To provide further evidence for the functional KA receptors on the adult mouse primary afferent neurons, and to determine their subunit composition, we have examined the effects of KA

receptor activation on frequency and amplitude of mEPSCs recorded from the SG neurons in acutely isolated spinal slices of wild-type and GluR5- or GluR6-deficient mice. In contrast to the findings in the young rat spinal cord SG, we found that in a subset of the mouse SG neurons, mEPSC frequencies were reversibly decreased after activation of KA receptors; this decrease arose from activation of receptors incorporating both the GluR5 and GluR6 subunit. Although the excitatory synapses responsible for the mEPSCs are not identified (primary afferent vs local interneuronal synapses) with any degree of certainty, the observed decrease in frequency, and the dependence on GluR5 and GluR6 subunits supports the hypothesis that some, if not all, KA effects on mEPSC frequency occurred at primary afferent synapses, the result in agreement with presynaptic GluR5 and GluR6 localization in primary afferent terminals to the superficial laminae of the rat spinal cord (Ribeiro-da-Silva and Coimbra, 1982; Lee et al., 1999, 2002; Hwang et al., 2001). Our data suggest that KA receptor activation causes a decrease in mEPSC frequency, which could account for the depression of evoked EPSPs in adult mouse SG neurons.

The mechanism by which KA receptor activation modulates excitatory synaptic transmission, whether at primary afferent synapses or other central synapses, remains unclear (Frerking and Nicoll, 2000). Studies of the effects of KA receptor agonists on glutamate release from synaptosomes have provided contradictory results (Zhou et al., 1995; Chittajallu et al., 1996; Perkinton and Sihra, 1999). One hypothesis, that KA receptors regulate glutamate release by a mechanism involving direct depolarization of axons or axon terminals, is supported by the finding that in CA1 hippocampal neurons, KA induced a transient facilitation of evoked NMDAR-mediated EPSCs before a prolonged depression occurred (Chittajallu et al., 1996). More direct evidence that KA receptors mediate axonal depolarization comes from investigations of mossy fiber synapses demonstrating that presynaptic KA receptor-mediated depression of synaptic transmission was associated with increased mossy fiber excitability (Kamiya and Ozawa, 2000; Schmitz et al., 2000). This phenomenon was reproduced when synaptically released glutamate from mossy fibers, or associational-commissural fibers, was used instead of KA (Schmitz et al., 2000). A potential link between axonal depolarization and decrease of glutamate release has been proposed by Kamiya and Ozawa (1998, 2000), who showed reduced action potential-triggered Ca^{2+} influx into mossy fiber terminals with presynaptic KA receptor activation. The model proposed to explain these results is that KA receptors are present on the presynaptic terminal, which decrease release by a depolarization-induced inactivation of presynaptic calcium channels. Our data are at least consistent with such a model by providing direct evidence that presynaptic KA receptor activation suppresses primary afferent transmission by depolarization of presynaptic fibers. As an alternative to an ionotropic effect of KA on presynaptic

sites, some evidence supports a possible G-protein-mediated action of presynaptic KA receptor stimulation at CA3-CA1 synapses (Frerking et al., 2001).

Activation of kainate receptors potentiates excitatory synaptic transmission

We have recently reported that bath-applied low concentration of KA (30 nM, 2 min) produces a robust and long-lasting potentiation of AMPA/NMDA receptor-mediated excitatory synaptic responses in the spinal cord SG. The excitatory effect of KA on evoked synaptic responses was discovered recently in the Schaffer collateral-commissural synapses on hippocampal CA1 neurons (Chittajallu et al., 1996; 300 nM KA) and mossy fiber synapses on CA3 neurons (Schmitz et al., 2001). However, in all cases, the enhancement of the AMPA receptor-mediated EPSCs (Schmitz et al., 2001), synaptic field potential responses (Schmitz et al., 2001), and NMDAR-mediated EPSCs (Chittajallu et al., 1996; Schmitz et al., 2001) was reversible, indicating that KA receptors mediated a short-lasting plasticity. A series of detailed experiments done by Schmitz et al. (2001) showed that the enhancing effect of KA (50 nM) on synaptic responses is associated with a decrease in a paired-pulse facilitation, and no change in holding current or the responses to iontophoretically applied NMDA in the stratum lucidum of the hippocampus. Thus, they have excluded a possibility for its postsynaptic origin. Moreover, they showed that the enhancement of evoked synaptic responses could be mimicked by 4 mM K^+, and repetitive stimulation (25 or 100Hz)-releasing glutamate, suggesting that the facilitatory effect is exerted by a KA receptor-induced depolarization of presynapic terminal via ionotropic action. Although the mechanism underlying the KA-induced long-lasting potentiation of synaptic efficacy in our work is still unknown, it appears that it is the NMDA receptor-dependent process. More interestingly, the long-lasting enhancement of synaptic transmission was blocked in the GluR6 knockout mice, but only depressed in the GluR5 knockout mice, indicating that the NMDA receptor-dependent long-lasting potentiation induced by the low concentration of KA is mediated by KA receptors containing exclusively GluR6, or in addition to a smaller extent GluR5. In addition, our finding further suggests that there is a functional interaction between kainate and NMDA receptors (Ghetti and Heinemann, 2000).

Recently, in the spinal DH, Kerchner et al. (2001b) showed that low concentration (200 nM) of KA has an ability to enhance evoked inhibitory transmission between DH neurons. This result provides additional evidence that low concentration of KA may potentiate synaptic strength, although this happens at inhibitory synapses under a reduced neuronal excitability produced by raising the concentration of Ca^{2+} and Mg^{2+} from 2 mM to 6 mM. Therefore, our result obtained from adult mouse spinal slices demonstrates a different example of the long-lasting potentiation in the SG region, where

under the blockade of inhibitory transmission, low concentration of KA can enhance dorsal root-evoked synaptic transmission in a dose-dependent, the NMDA receptor-dependent, the KA receptor subunit-dependent, and long-lasting manner. Further, this result may explain the reduced magnitude of long-term potentiation induced by high-frequency stimulation in the GluR5 mutant mice (Fig. 21).

Summary and significance

In this study, we demonstrated that activation of kainate receptors composed of GluR5 and GluR6 subunits plays a role in the regulation of excitatory synaptic transmission in the adult mouse spinal DH neurons, either by a presynaptic or a postsynaptic mechanism. In contrast to previous studies (Kerchner et al., 2001), our data suggest that the GluR6 subunit is critically involved in suppression of the Aδ- and C-fiber-elicited primary afferent neurotransmission in the SG region. Although anatomical data from young (less than 3 week-old) rat have indicated the relative abundance of GluR5 subunit and paucity of the GluR6 subunit in the spinal neurons, information on the anatomical profile of kainate receptor subunits in the adult mouse spinal cord DH, is still not available. Thus, the developmental regulation of KA receptors and use of a different species may influence the expression of KA receptor subunits as well as the effect of kainate receptor activation in the spinal cord DH. We, here, summarize our idea about possible localization of kainate receptor GluR5 and GluR6 subunits, postulated on the basis of anatomical and electrophysiological data from rats and our data obtained in adult mouse spinal SG: 1) GluR6 subunit is expressed at Aδ primary afferent fiber terminals (Hwang et al., 2001; Partin et al., 1993; Sato et al., 1993); 2) both GluR5 and GluR6 subunits are expressed at C primary afferent fiber terminals (Dai et al., 2002; Hwang et al., 2001; Huettner, 1990; Kerchner et al., 2001b; Partin et al., 1993; Sato et al., 1993; Tolle et al., 1993; Yung, 1998); 3) GluR6 subunit is exclusively expressed on the somatic membrane of SG interneurons (Yung, 1998; but see also Tolle et al., 1993); 4) although these subunit composition of kainate receptors at the terminals of excitatory or inhibitory interneurons is presently not known, our data suggest that presynaptic terminals of excitatory interneurons may have both GluR5 and 6.

Evidence indicates that kainate receptors are associated with nociceptive pathways including primary afferents and DRG neurons (Agrawal and Evans, 1986; Huettner, 1990). Recent studies in humans suggest that the mixed AMPA/GluR5 antagonist, LY293558, can prevent capsaicin-induced hyperalgesia and allodynia with no effect on physiological nociception (Sang et al., 1998). Similar results are reported in animal studies where formalin-induced- (Simmons et al., 1998), but not acute physiological (Procter et al., 1998), nociceptive responses are reduced by GluR5 selective decahydroisoquinolines. In addition, it has been shown that a kainate receptor antagonist SYM 2081

prevents the transmission of nociceptive heat sensation (Li et al., 1999), and reduces allodynia and hyperalgesia which were induced in neuropathic pain models (Lauren et al., 2000; Sutton et al., 1999). These data indicate a functional role of kainate receptors in the development, transmission and maintenance of nociception.

Regulation of excitatory or inhibitory transmission in the DH is essential for proper processing of nociceptive and other sensory information. According to the gate control theory (Melzack and Wall, 1965), the inhibitory effect exerted by SG cells on first central transmission cells (probably, projection neurons) is increased by activity in large-diameter fibers and decreased by activity in small-diameter fibers. Thus, our findings, which provide a new infusion of evidence on subunit profiles of kainate receptors acting as excitatory or inhibitory regulator for the Aδ-fiber- or C-fiber-mediated excitatory transmission on SG neurons, further suggest that the process of nociceptive transmission in the spinal SG neurons can finely be tuned by the activation of kainate receptors with different subunit compositions. Therefore, building up information on synaptic or somatic location of kainate receptor subunits and their physiological functions in the spinal cord DH will provide us with clues to develop more specific therapeutic strategy for the treatment of pain.

Table 1. Passive and active membrane properties of SG neurons obtained from +/+, GluR5 mutant (-/-) and GluR6-/- mice

	+/+	GluR5-/-	GluR6-/-
Resting membrane potential (mV)	-74.9 ± 0.9 (89)	-74.5 ± 1.0 (59)	-78.0 ± 1.6 (31)
Input resistance (MΩ)	203.4 ± 32.8 (31)	184.2 ± 28.1 (21)	201.5 ± 50.0 (13)
Amono-EPSP$_{threshold}$ (V at 0.1 ms)	5.4 ± 0.7 (23)	5.2 ± 0.6 (18)	5.6 ± 0.4 (7)
Amono-EPSP$_{intensity}$ (V at 0.1 ms)	7.4 ± 0.6 (33)	6.0 ± 0.7 (21)	8.7 ± 1.0 (11)*
Amono-EPSP$_{amplitude}$ (mV)	10.2 ± 0.7 (33)	11.7 ± 0.9 (19)	10.3 ± 1.9 (9)
Amono-EPSP$_{CV}$ (m/s)	3.1 ± 0.2 (41)	3.0 ± 0.3 (25)	3.3 ± 0.4 (18)
C-EPSP$_{CV}$ (m/s)	0.6 ± 0.05 (27)	0.6 ± 0.1 (17)	0.7 ± 0.2 (7)

Values shown are the mean ± standard error of mean. The number in parentheses after a value gives the number of cells. Abbreviations: Amono, monosynatpic Aδ-fiber-evoked; C-EPSP, C fiber-evoked EPSPs; CV, conduction velocity. * indicate a significant difference between +/+ and GluR6-/- groups ($P<0.05$).

Table 2. Depression of primary afferent fiber-evoked EPSPs by bath-application of 3μM KA in different genotypes, synaptic potentials or perfusion media.

		Solution			
Genotype	Type of input	Krebs		B+S	
+/+	Am	69.3 ± 5.7 (8)		76.0 ± 4.8 (5)	
	Ap	45.8 ± 5.1 (5)**	vs. Am	87.7 ± 6.2 (6)**	vs. Krebs
	C	55.8 ± 5.0 (7)*	vs. Am	53.4 ± 9.5 (5)	
GluR5-/-	Am	79.9 ± 3.9 (10)		86.1 ± 2.8 (6)	
	Ap	80.1 ± 10.4 (7)*	vs. +/+	90.4 ± 5.6 (8)	
	C	75.8 ± 6.4 (9)*	vs. +/+	73.6 ± 6.3 (5)*	vs. +/+
GluR6-/-	Am	79.4 ± 4.1 (5)		105.6 ± 4.4 (6)**	vs. +/+
	Ap	87.3 ± 10.3 (5)**	vs. +/+	91.5 ± 5.9 (5)	
	C	82.2 ± 3.0 (7)**	vs. +/+	96.2 ± 2.8 (4)**	vs. +/+

Values expressed as percentage of control (mean ± standard error of mean) in the peak amplitude of EPSPs evoked by stimulation of primary afferent fibers. The number in parentheses after a value gives the number of cells. Abbreviations: B+S, Krebs solution containing 5μM bicuculline and 2μM strychnine; +/+, wild-type mice; GluR5-/-, GluR5 mutant mice; GluR6-/-, GluR6 mutant mice; Am, monosynaptic Aδ-fiber-evoked EPSPs; Ap, polysynaptic Aδ-fiber-evoked EPSPs; C, C-fiber-evoked EPSPs. Statistical significance of data is indicated by asterisks: *, $P<0.05$; **, $P<0.01$.

Table 3. KA -induced membrane depolarization and holding current changes in +/+, GluR5 mutant (-/-) and GluR6-/- mice

Membrane potential (mV)	+/+	GluR5-/-	GluR6-/-
Krebs	5.6 ± 0.7 (14)	4.7 ± 0.7 (25)	2.9 ± 0.7 (15)**
Bic + strych	4.6 ± 1.1 (11)	3.0 ± 0.4 (18)	3.1 ± 0.9 (11)
Holding current (pA)			
Bic + strych + TTX	-18.3 ± 6.5 (9)	-24.9 ± 6.1 (8)	-0.7 ± 1.7 (7)*

Values shown are the mean ± standard error of mean. The number in parentheses after a value gives the number of cells. Krebs, normal Krebs solution; Bic + strych, a solution containing 5 μM bicuculline + 2 μM strychnine; TTX, 0.5 μM tetrodotoxin. Statistical significance is indicated by asterisks: *$P<0.05$ and **$P<0.01$, vs. +/+.

Fig. 1. AMPA receptor-mediated excitatory postsynaptic potential.

A) A diagram shows experimental setup characterizing a mouse transverse spinal cord slice preparation with an attached dorsal root (5-10 mm), bipolar stimulating electrode (platinum wire) and recording electrode inside glass pipette. SG designates an area of 'substantia gelatinosa' which cells are mostly obtained from.

B) A repetitive stimulation at a frequency of 10 Hz (*right*) was used to determine synaptic connectivity. In a representative SG cell, EPSPs, evoked by electrical stimulation of the attached dorsal root, was identified as Aδ-fiber-evoked 'monosynaptic' (Amono-) EPSPs on the basis of constant latency and no failure. EPSP, recorded in another SG cell and determined as 'monosynaptic', was not affected by 50 μM APV, a competitive NMDA receptor antagonist, but completely blocked by 50 μM GYKI 53655, a selective AMPA receptor antagonist.

C) EPSP, which showed variable latencies and failures (*not shown*) following a repetitive stimulation at a frequency of 10 Hz (*right*), was determined as Aδ-fiber-evoked 'polysynaptic' (Apoly-) EPSPs. Apoly-EPSPs, recorded in another SG cell, were largely blocked by 50 μM APV.

D) The synaptic connectivity of C fiber-evoked (C-) EPSPs (stimulus intensity: 4.2 V/0.5 ms; conduction velocity, 0.53 m/sec) with constant latency and also a failure (*star*) at a frequency of 10 Hz (*right*) was not determined.

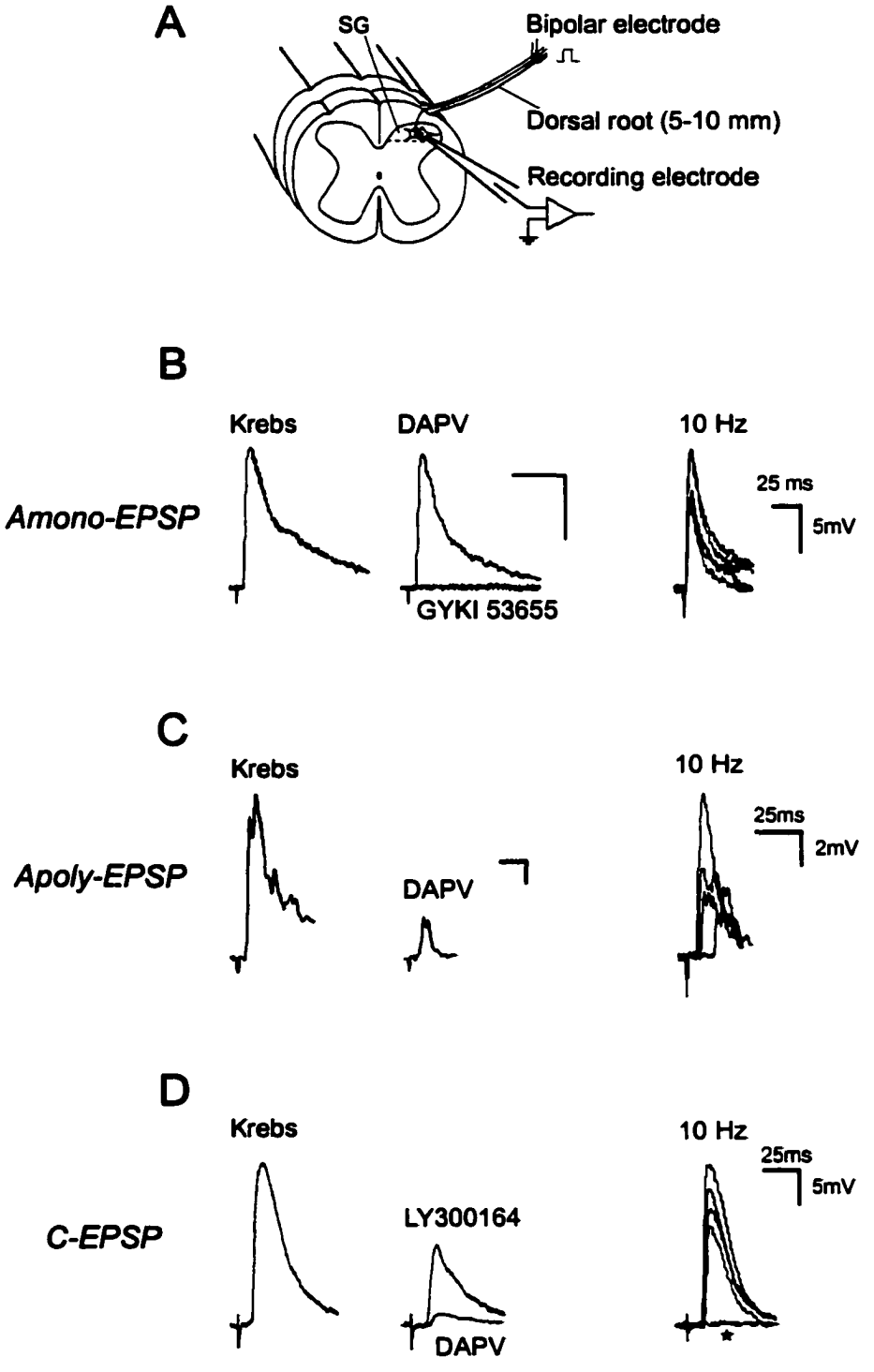

A
SG
Bipolar electrode
Dorsal root (5-10 mm)
Recording electrode
B
Krebs
DAPV
10 Hz
Amono-EPSP
25 ms
5mV
GYKI 53655
C
Krebs
10 Hz
Apoly-EPSP
25ms
2mV
DAPV
D
Krebs
10 Hz
25ms
5mV
C-EPSP
LY300164
DAPV

Fig. 2. Kainate receptor agonists suppress primary afferent neurotransmission in the mouse spinal dorsal horn.

A) A time course graph for nineteen slices obtained from eighteen wild-type mice shows long-lasting depression of EPSP peak amplitudes produced by bath application of 3μM KA for 2 min. Each point is mean ± s. e. m of baseline percentages from individual cells at a given time. Vm = -63 to -85 mV, 6-16-week-old mice.

B) The KA-induced depression of EPSPs was dose-dependent (n = 4 – 19 wild-type slices per concentration; $R^2 = 0.99$).

C) Domoate (0.3 or 1 μM), known as a more potent KA receptor agonist, reduced the peak amplitudes of EPSPs recorded from wild-type slices, in a dose-dependent manner. Time-courses at each concentration on the graph were pooled from three slices obtained from 3 mice. Vm = -65 to -73 mV, 9-week-old mice.

D) A summary histogram shows that the magnitude of the KA (3 μM, 2 min)-induced depression in normal Krebs solution is significantly reduced in the presence of a GluR5 subunit specific KA receptor antagonist, LY382884 (10 μM) (42.0 ± 3.9% and 18.1 ±5.0% inhibition in the absence or presence of LY382884, respectively; **P<0.01). Interestingly, LY382884 itself depressed the amplitudes of EPSPs by 22.8 ± 7.5% (P<0.05 vs. baseline). Data are presented as means ± s. e. m. Each number on the histogram designates the number of cells observed.

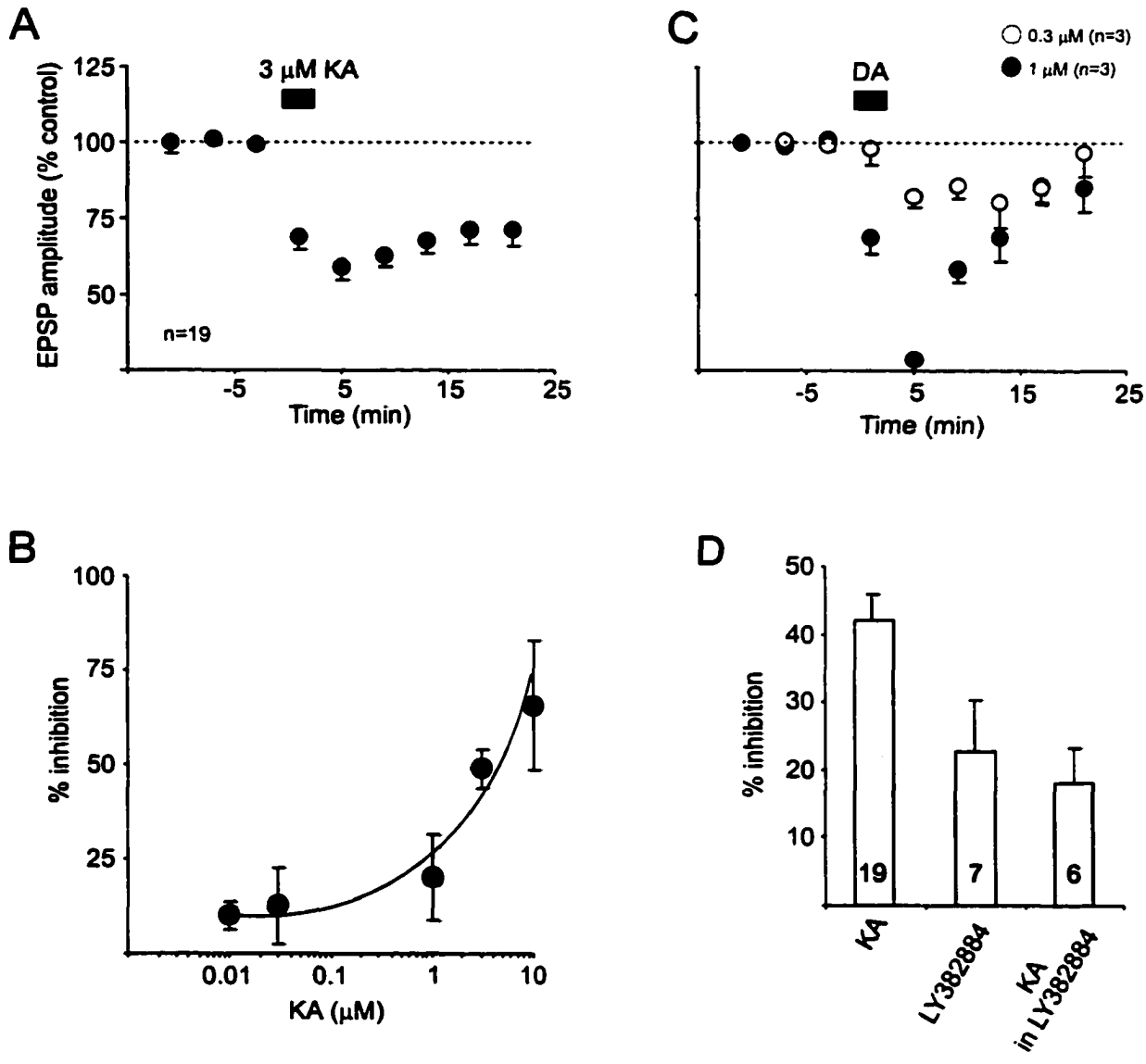

A
3 μM KA
EPSP amplitude (% control)
125
100
75
50
n=19
-5
5
15
25
Time (min)
C
0.3 μM (n=3)
1 μM (n=3)
DA
-5
5
15
25
Time (min)
B
100
75
50
25
% inhibition
0.01
0.1
1
10
KA (μM)
D
50
40
30
20
10
% inhibition
19
7
6
KA
LY382884
KA in LY382884

Fig. 3. Kainate receptor activation suppresses A- and C-primary afferent fibers-evoked excitatory synaptic potentials.

A-C) Three summary graphs showing the time-course of long-lasting depression of peak amplitude of EPSPs according to different types of synaptic inputs. *Amono*, A-primary afferent fiber-evoked monosynaptic EPSP; *Apoly*, A-primary afferent fiber-evoked polysynaptic EPSP; *C-EPSP*, C-primary afferent fiber-evoked EPSP. The peak amplitude of Apoly (*B*, n = 5 slices from 5 wild-type mice)- or C (*C*, n = 7 slices from 7 wild-type mice)-EPSPs was more effectively reduced by KA (3 μM for 2 min) than that of Amono-EPSPs (*A*, n = 8 slices from 8 wild-type mice). The corresponding time of sampled traces above graphs is indicated by the number. *Inset* shows the approximate location of tested SG cells. Each point is mean ± s.e.m at a given time. A, Vm = -63 to -87 mV, 7-13-week-old mice. B, Vm = -61 to -82 mV, 7-12-week-old mice. C, Vm = -64 to -73 mV, 7-14-week-old mice.

D) A summary histogram showing mean maximum effects of KA on Amono-, Apoly- or C-EPSPs. The inhibition of Apoly- and C-EPSPs by KA was significantly larger than that of Amono-EPSPs (Amono, 30.7 ± 5.7%, **P<0.01 vs. baseline; Apoly, 54.2 ± 5.1%, **P<0.01 vs. baseline, ##P<0.01 vs. Amono; C-EPSPs, 44.2 ± 5.0%, **P<0.01 vs. baseline, #P<0.05 vs. Amono).

E-F) Summary graphs showing the time-course of long-lasting depression of EPSPs in the area under the curve of Apoly and C.

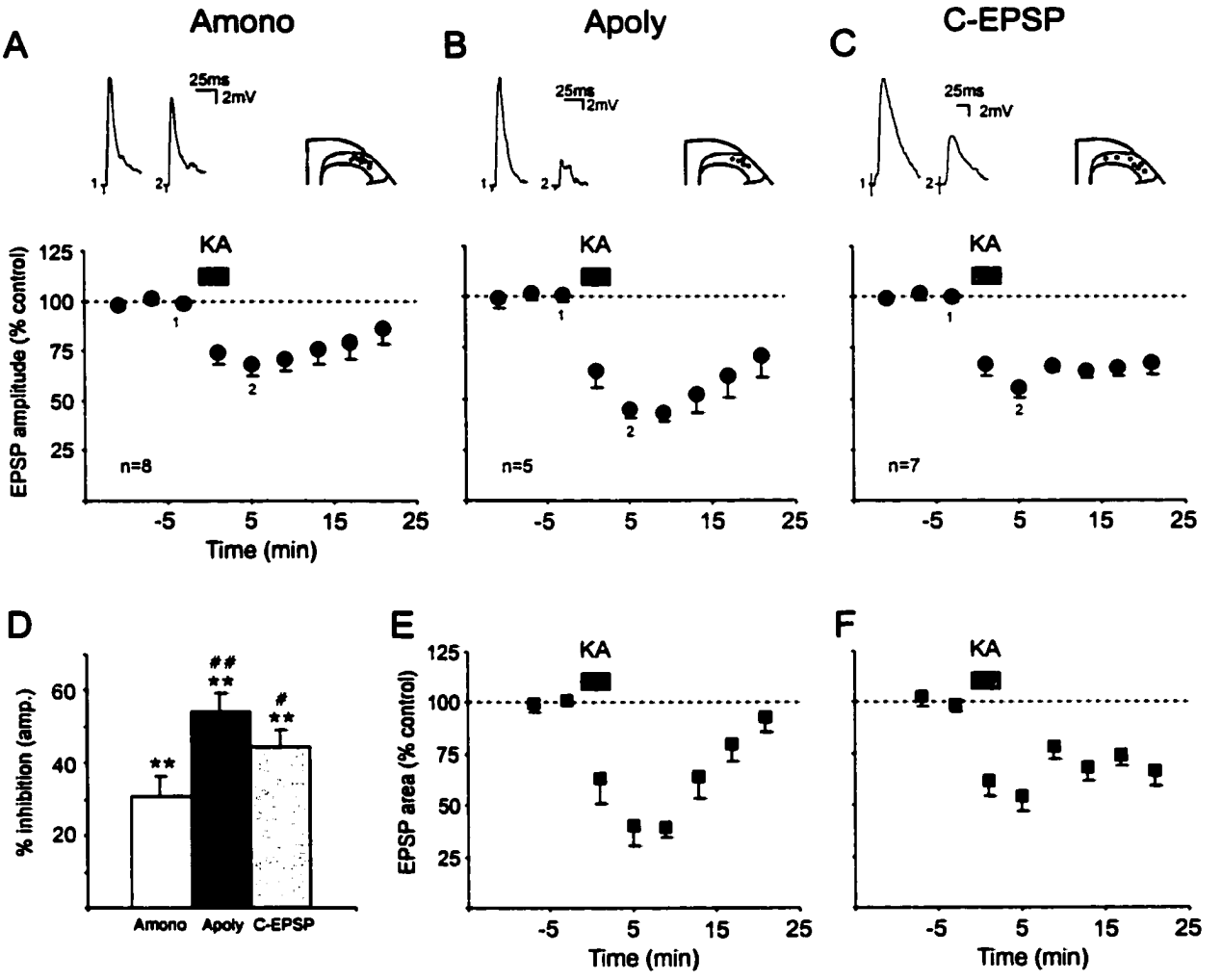

A
Amono
B
Apoly
C
C-EPSP
25ms
2mV
KA
EPSP amplitude (% control)
125
100
75
50
25
n=8
n=5
n=7
-5
5
15
25
Time (min)
D
% inhibition (amp.)
60
40
20
Amono
Apoly
C-EPSP
E
EPSP area (% control)
F

Fig. 4. KA increases the probability of synaptic failures and depress the AMPA- and NMDA-mediated amplitudes of the C-primary afferent fiber-evoked EPSPs.

The graph shows KA (3 μM, 2 min)-induced depression or failures on peak amplitudes of EPSPs evoked by C fiber stimulation (stimulus intensity, 4.2 V/0.5 ms; conduction velocity, 0.53 m/sec) in a SG neuron obtained from a wild-type mouse. The latter effect was not present in the presence of 5 μM bicuculline, a $GABA_A$ receptor antagonist, and 2 μM strychnine, a glycine receptor antagonist. Further, components of EPSP were isolated by the addition of LY300164, a selective AMPA receptor antagonist, and/or DAPV, a competitive NMDA receptor antagonist to the bath solution. The residual component of EPSP in the presence of 50 μM LY300164 and 50 μM DAPV was blocked by successive addition of 10 μM CNQX, an AMPA/KA receptor antagonist, indicating that all three types of ionotropic glutamate receptors, i.e. NMDA, AMPA and KA, mediate the EPSPs. Sample traces displayed above the graph are individual synaptic responses taken at the times indicated by the numbers. Bars indicate periods of application of each drug. Vm = -72mV, 11-week-old mouse.

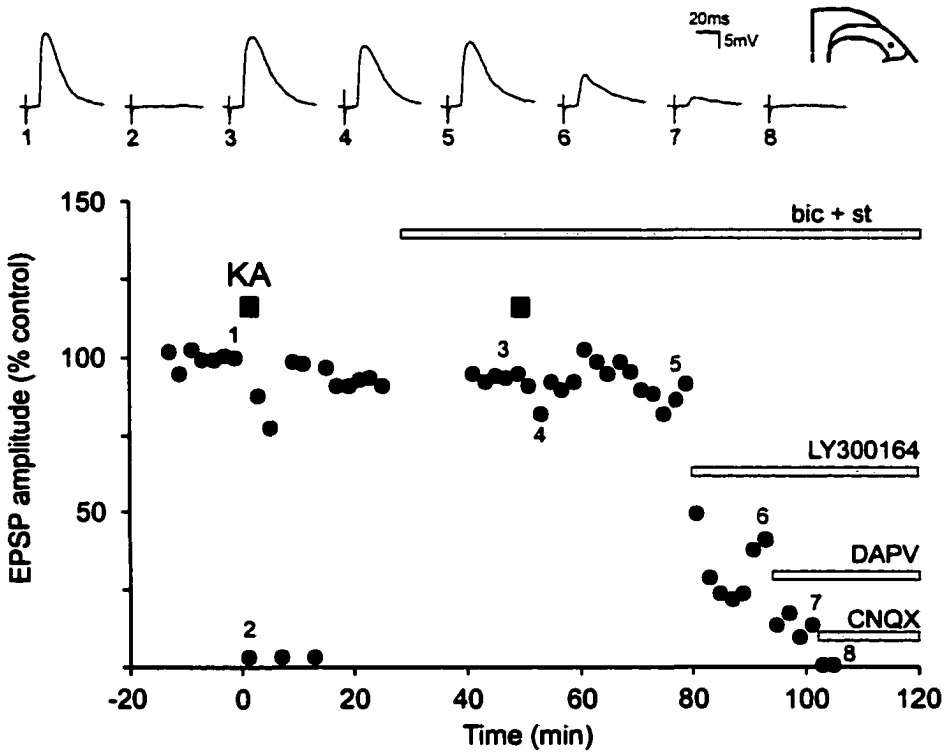

20ms
5mV
1
2
3
4
5
6
7
8
150
100
50
EPSP amplitude (% control)
KA
bic + st
LY300164
DAPV
CNQX
-20
0
20
40
60
80
100
120
Time (min)

Fig. 5. Concentration-dependency of the effects of KA on excitatory synaptic transmission in the substantia gelatinosa.

A) The effect of KA on the amplitude of EPSPs in the presence of 5 μM bicuculline and 2 μM strychnine was dose-dependent in wild-type mice. Apparently, 3 μM KA significantly suppressed the peak amplitude of EPSPs, whereas 0.03 μM KA significantly potentiated. However, no significant change was seen at the concentrations of 0.01 μM or 0.1 μM.

B) The graph shows the pooled data from sixteen wild-type slices (11 mice) in which KA (3 μM, 2 min) induced long-lasting depression in the presence of bicuculline and strychnine. Vm = -72 to -83mV, 12-15-week-old mice.

C) A summary histogram of the different effects of KA on primary afferent fibers-evoked EPSPs in different perfusion media. The depressant action of KA on EPSPs is significantly reduced by 5 μM bicuculline and 2 μM strychnine, a cocktail solution of bicuculline/strychnine with 10 μM CGP55845, a $GABA_B$ receptor antagonist, or further addition of 500 μM (*S*)-MCPG, a mGluR antagonist, to the cocktail. *P<0.05, **P<0.01. Each number on the histogram designates the number of slices observed.

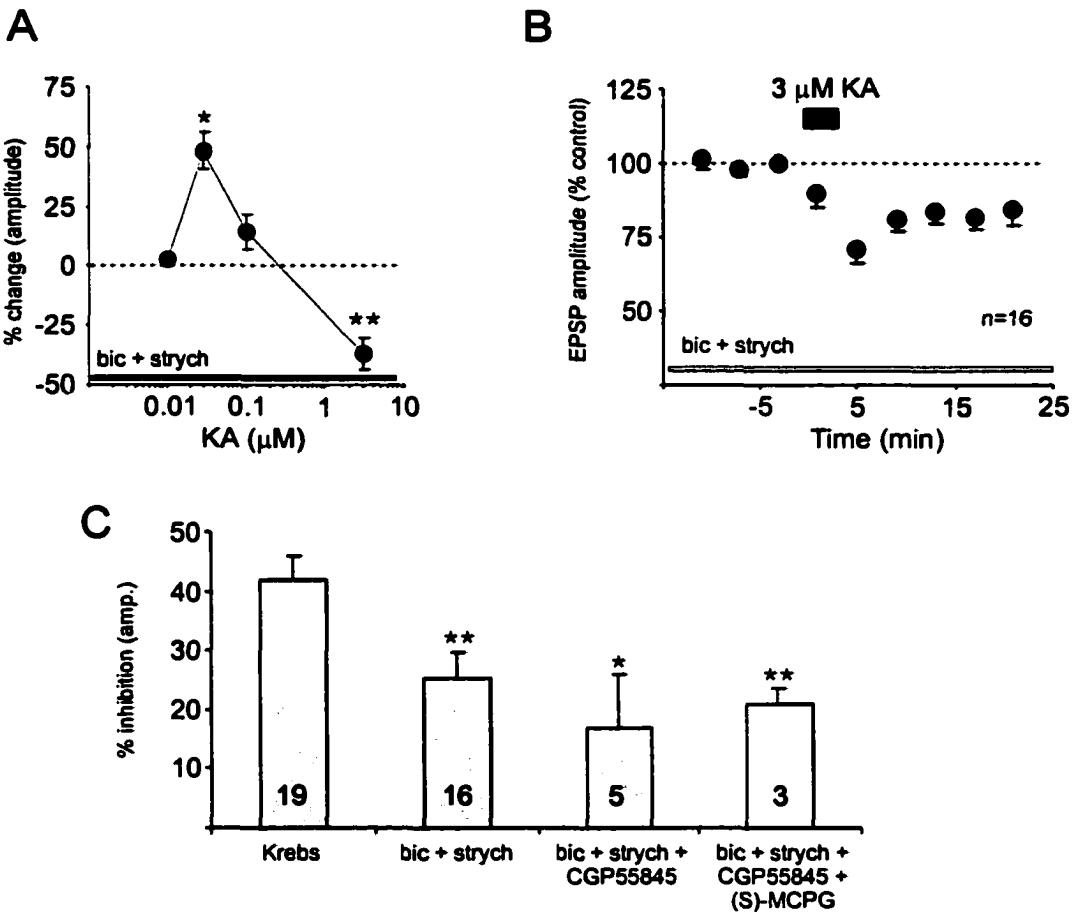

A
75
50
25
0
-25
-50
% change (amplitude)
*
**
bic + strych
0.01
0.1
1
10
KA (μM)
B
125
100
75
50
EPSP amplitude (% control)
3 μM KA
n=16
bic + strych
-5
5
15
25
Time (min)
C
50
40
30
20
10
% inhibition (amp.)
**
*
**
19
16
5
3
Krebs
bic + strych
bic + strych + CGP55845
bic + strych + CGP55845 + (S)-MCPG

Fig. 6. The depressant effect of KA on Aδ-fiber-evoked polysynaptic EPSPs is reduced in the blockades of synaptic inhibition.

A) KA (3 μM, 2 min)-induced depression of Aδ-fiber-evoked polysynaptic EPSPs amplitudes was reduced in the presence of 5 μM bicuculline and 2 μM strychnine (bic + strych). 50 μM DAPV blocked a large portion of the Apoly-EPSPs. Sample traces displayed above the graph are individual synaptic responses taken at the times indicated by the numbers. Bars indicate periods of application of each drug. Vm = -82 mV, 12-week-old wild-type mouse.

B-C) Histograms show the significant reduction of the 3 μM KA-induced depression of the Aδ-fiber evoked polysynaptic EPSPs (Apoly) by bicuculline and strychnine in amplitude (*B*) and area (*C*) (**P<0.01). However, no significant difference is found in Aδ-fiber monsynaptic EPSPs (Amono) or C-fiber EPSPs. Each number on the histogram designates the number of slices observed.

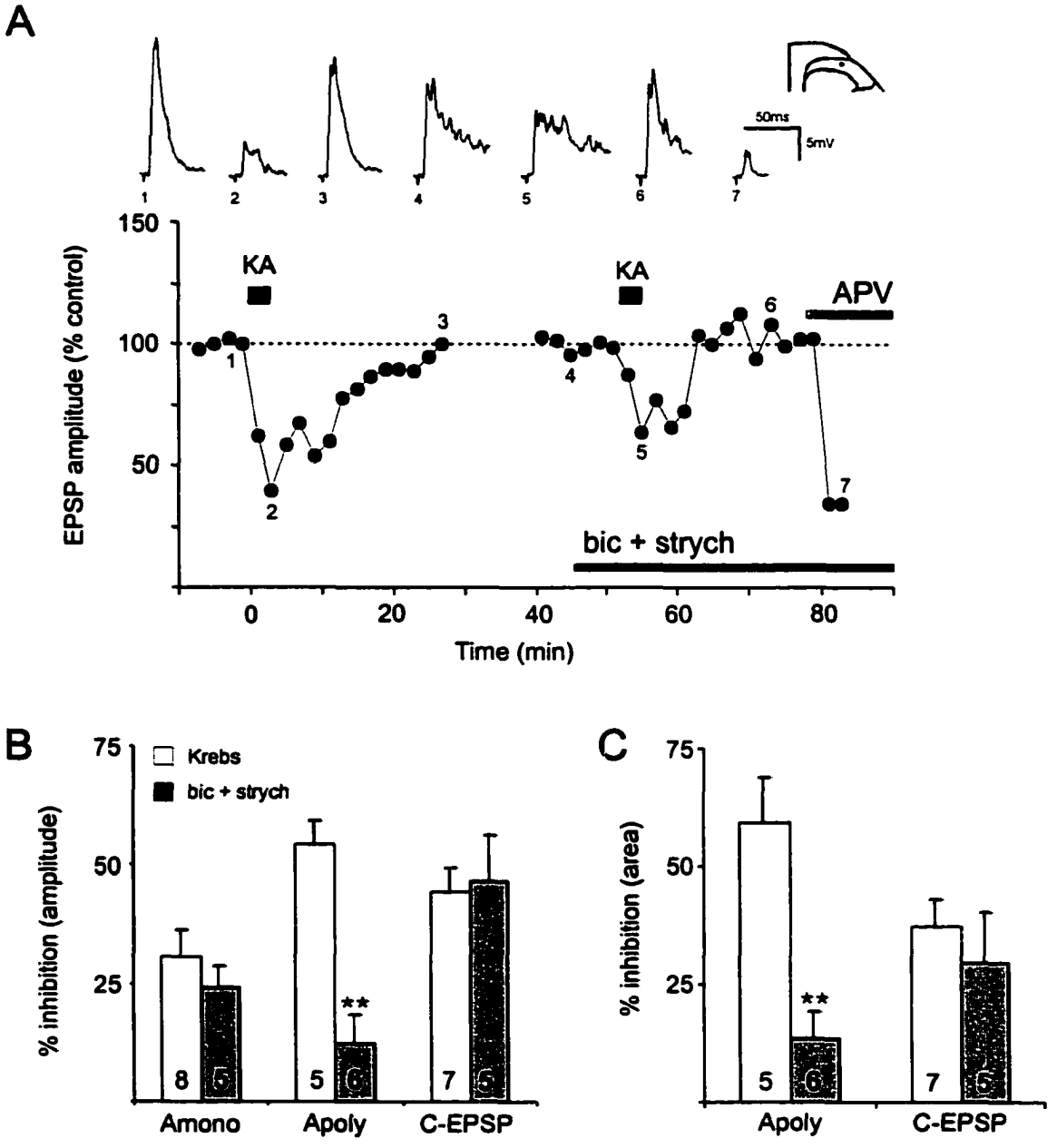

A
50ms
5mV
1
2
3
4
5
6
7
150
100
50
EPSP amplitude (% control)
KA
KA
APV
bic + strych
0
20
40
60
80
Time (min)
B
Krebs
bic + strych
75
50
25
% inhibition (amplitude)
8
5
5
6
7
5
Amono
Apoly
C-EPSP
**
C
75
50
25
% inhibition (area)
5
6
7
5
Apoly
C-EPSP
**

Fig. 7. The blockade of synaptic inhibition reduces the depressant effect of KA on A-primary afferent fiber-evoked polysynaptic EPSPs.

A-E) Summary graphs show the time-course of 3 μM KA-induced depression in peak amplitudes of Amono- (*A*), Apoly- (*B*) and C-EPSPs (*C*) and in area under the curve of Apoly- (*D*) and C-EPSPs (*E*) in Krebs solution or a solution containing 5 μM bicuculline and 2 μM strychnine (bic + strych) in wild-type mice. Each point was expressed as mean ± s.e.m. * ($P<0.05$) and ** ($P<0.01$) indicate significant difference between Krebs and bicuculline + strychnine solution at a given time. SG cells in bic + strych: *A*, Vm = -74 to -82 mV, 12-15-week-old mice; *B*, Vm = -73 to -83mV, 12-15-week-old mice; *C*, Vm = -72 to –80 mV, 12-14-week-old mice.

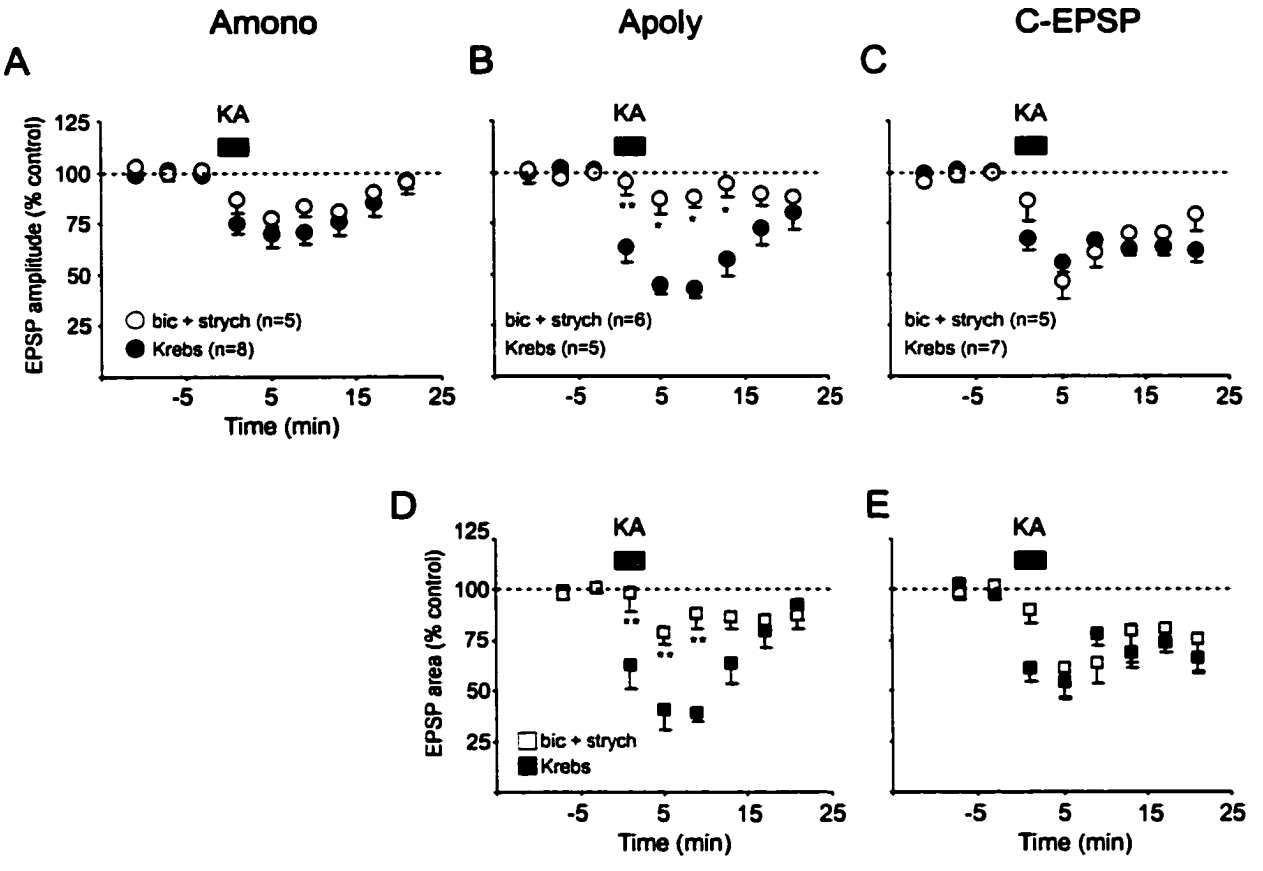

Amono
Apoly
C-EPSP
A
B
C
KA
EPSP amplitude (% control)
125
100
75
50
25
bic + strych (n=5)
Krebs (n=8)
bic + strych (n=6)
Krebs (n=5)
bic + strych (n=5)
Krebs (n=7)
-5
5
15
25
Time (min)
D
E
EPSP area (% control)
bic + strych
Krebs

Fig. 8. The blockade of inhibitory transmission occasionally changes the effect of KA from inhibitory to excitatory.

In three sampled wild-type SG cells with inputs from monosynaptic (*A*) and polysynapitc (*B*) Aδ-fibers, and C fibers (*C*), 3 μM KA (2 min), in the presence of 5 μM bicuculline and 2 μM strychnine, increased amplitudes of EPSPs. Interestingly, the effect of KA on C-input EPSPs was biphasic (both excitatory and inhibitory). A larger portion of monosynaptic A-input EPSPs was blocked by 50 μM GYKI 53655 (*3rd trace in A*), a selective AMPA receptor antagonist, whereas 50 μM DAPV (*3rd trace in B*) effectively blocked polysynaptic A-input EPSPs. The time (minute) following the start of KA application is indicated below the traces. *A*, Vm = -82 mV, 13-week-old mouse. *B*, Vm = -82mV, 14-week-old mouse. *C*, Vm = -80 mV, 13 week-old mouse.

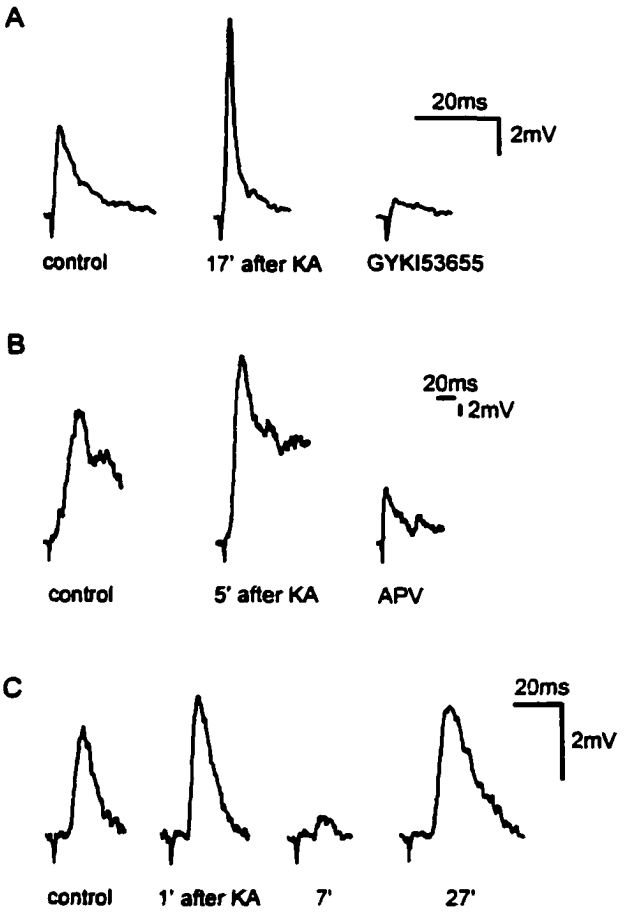
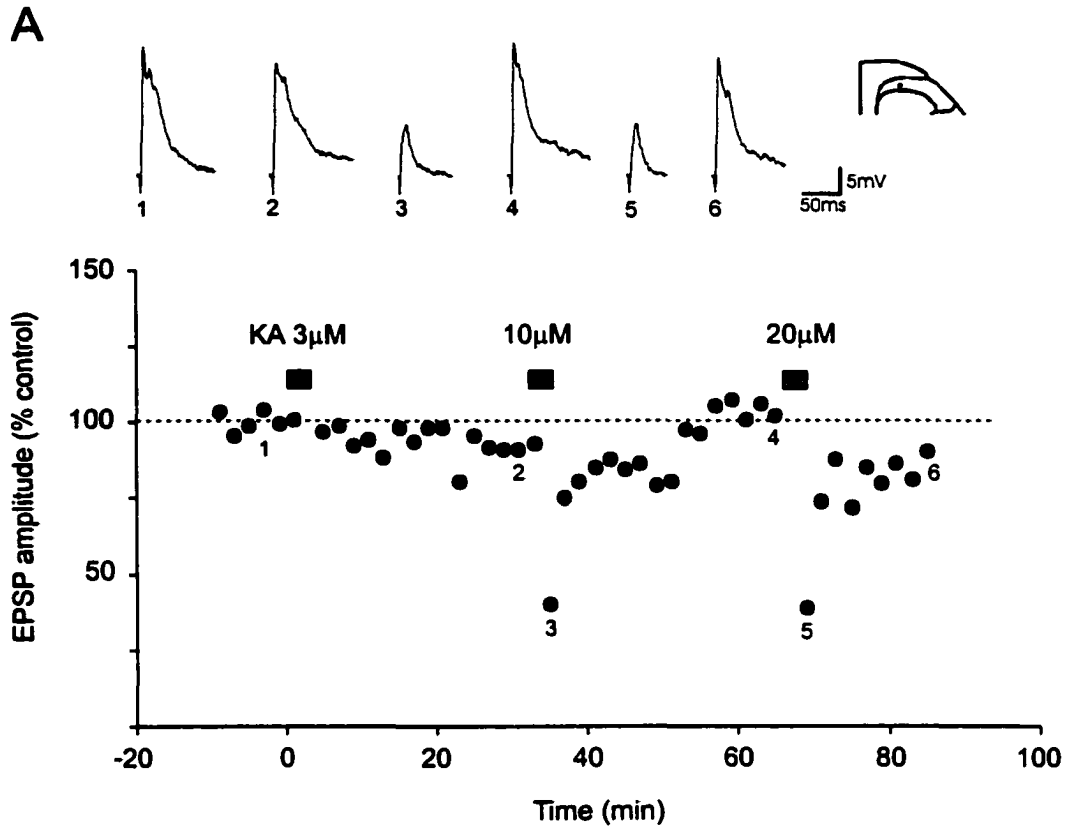

A
20ms
2mV
control
17' after KA
GYKI53655
B
20ms
2mV
control
5' after KA
APV
C
20ms
2mV
control
1' after KA
7'
27'

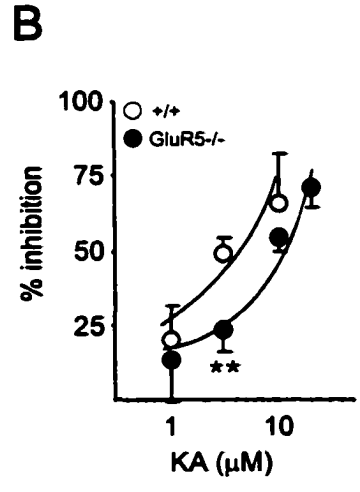
Fig. 9. Reduced depression of primary afferent neurotransmission in GluR5 mutant mice.

A) In a representative neuron obtained from a GluR5 mutant mouse, KA (3μM, 2min) exerted a decrease of the peak amplitude of Amono-EPSPs in a dose-dependent manner. Sample traces displayed above the graph are individual synaptic responses taken at the times indicated by numbers. A polysynaptic component can be seen in the decay phase of EPSPs. Vm = -79 mV, 14-week-old mouse.

B) Dose-response curves were made to summarize the effect of KA (3 μM) on EPSPs in GluR5 mutant (-/-) mice (*closed circles*; n = 3 – 25 slices per concentration; $R^2 = 0.94$), and also compared to the part of dose-response curve of wild-type mice shown in Fig. 2B (*open circles*). The curve was shifted to the right, and a statistical significance between wild-type and GluR5-/ mice was observed only at the concentration of 3 μM (**P<0.01).

C) A summary graph showing the time-course of 3 μM KA-induced depression of EPSPs for 25 slices obtained from 19 GluR5 mutant mice. After 15 min wash out of KA, amplitudes of EPSPs were recovered to 97.5 ± 5.1% of baseline in GluR5 mutant mice. However, at the same time, those in wild-type mice were 71.4 ± 4.8% of baseline (See fig. 2A). Vm = -63 to -85 mV, 9-16-week-old mice.


A
1
2
3
4
5
6
5mV
50ms
150
100
50
EPSP amplitude (% control)
KA 3μM
10μM
20μM
-20
0
20
40
60
80
100
Time (min)


B
100
75
50
25
% inhibition
+/+
GluR5-/-
**
1
10
KA (μM)

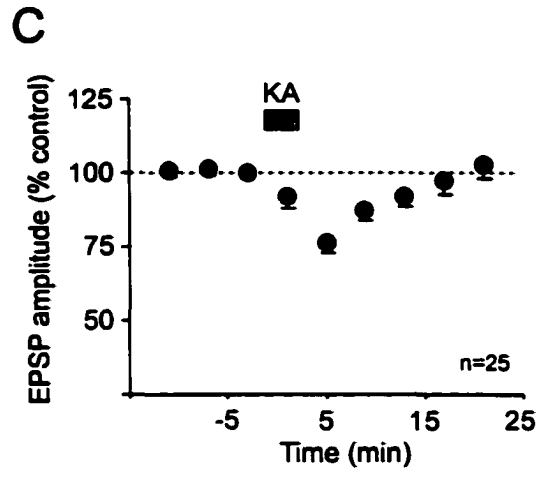

C
125
100
75
50
EPSP amplitude (% control)
KA
n=25
-5
5
15
25
Time (min)

Fig. 10. Activation of KA receptors exerts excitatory effects in subpopulation of GluR5 mutant (-/-) SG cells.

A-C) In three representative SG cells, with monosynaptic Aδ-fiber (*A*), polysynaptic Aδ-fiber (*B*) or C-fiber (*C*) input, obtained from GluR5 mutant mice, the peak amplitude of EPSPs recorded in Krebs solution was increased and occasionally reached to the threshold for firing action potentials by dorsal roots stimulation. Traces, at the corresponding time indicated by numbers, are shown above graphs. Some of overshoot part of action potentials in *A* and *C* was truncated by slash. The *stars* indicate EPSPs with action potential firing upon the stimulation, which hampers a correct measurement for the amplitude of EPSP. In B, a large portion of the recovered Apoly-EPSPs was inhibited by 50 μM DAPV, and the remaining component was completely blocked by the addition of 10 μM CNQX. A, Vm = -75 mV, 15-week-old mouse. B, Vm = -72 mV, 13-week-old mouse. C, Vm = -67 mV, 11-week-old mouse.

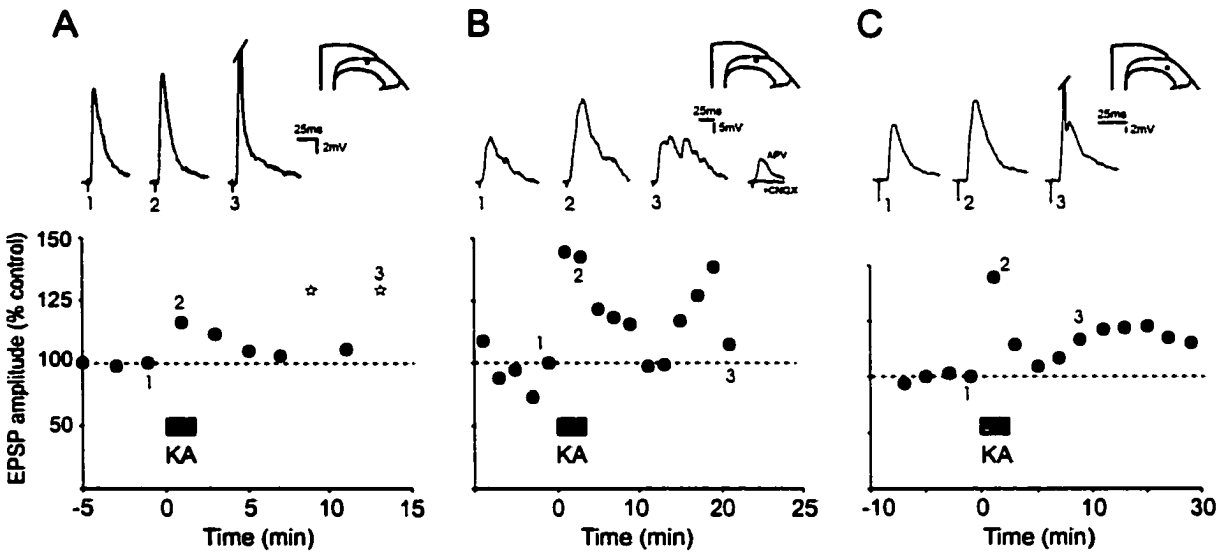

A
B
C
25ms
2mV
5mV
APV
CNQX
1
2
3
EPSP amplitude (% control)
150
125
100
50
KA
-5
0
5
10
15
20
25
-10
30
Time (min)

Fig. 11. Summary graphs showing each time-course of 3 μM KA-induced depression of EPSPs for all genotypes in the presence or the absence of 5 μM bicuculline and 2 μM strychnine. The degree of long-lasting depression by KA in the Krebs solution in wild-type mice was significantly reduced in GluR5 or GluR6 mutant mice. In the bicuculline and strychnine, the depression of EPSP amplitudes was significantly reduced in GluR5 mutant mice, but completely blocked in GluR6 mutant mice. Data are expressed as mean ± s.e.m. * ($P<0.05$) and **($P<0.01$) on the data represent significant difference against the value in wild-type mice at the same time and solution.

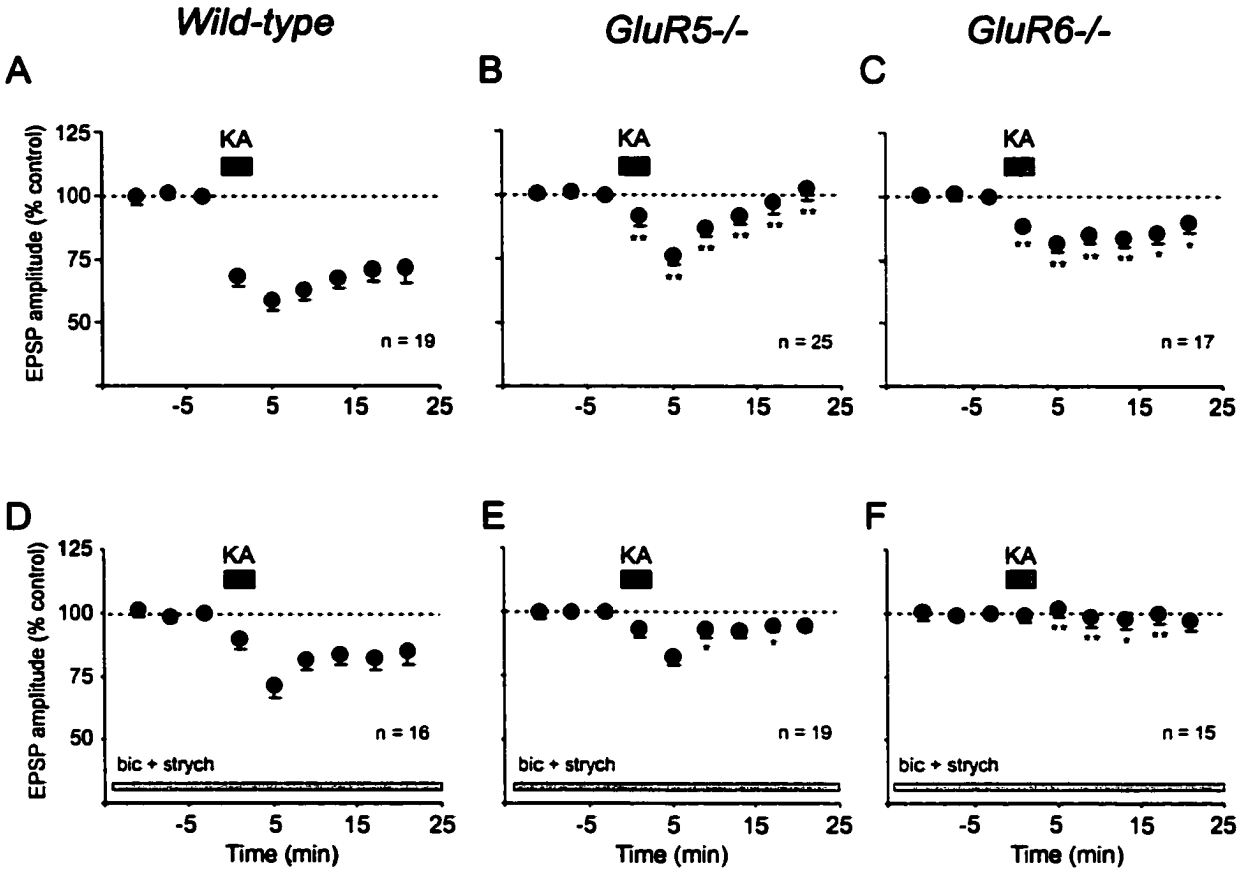

Wild-type
GluR5-/-
GluR6-/-
A
B
C
D
E
F
KA
EPSP amplitude (% control)
125
100
75
50
n = 19
n = 25
n = 17
n = 16
n = 19
n = 15
bic + strych
-5
5
15
25
Time (min)

Fig. 12. GluR5 subunit is involved in the long-lasting depression of synaptic transmission induced by the activation of kainate receptors.

A-F) Representative traces (*A-C*) recorded from wild-type (*upper traces*) and GluR5 mutant (-/-) (*lower traces*) slices are sampled for graphs summarizing for SG cells with inputs from monosynaptic Aδ-fiber (Amono, n = 10 slices from 9 GluR5-/- mice; *D*), polysynaptic Aδ-fiber (Apoly, n = 7 slices 7 GluR5-/- mice; *E*) and C-fiber (C-EPSP, n = 9 slices from 6 GluR5-/- mice). KA (3 μM, 2 min)-induced depression of EPSPs was significantly reduced in GluR5-/- SG cells with inputs from polysynaptic Aδ-fibers (*E*) and polysynaptic Aδ-fibers (*F*), but not in those from monosynaptic Aδ-fibers (*D*). The peak amplitude of monosynaptic EPSPs recorded from a GluR5-/- SG cell, was not affected by 50 μM DAPV but was almost completely blocked by the addition of 50 μM GYKI 53655 to DAPV. On the other hand, 50 μM DAPV alone decreased the peak amplitudes of Apoly-EPSPs by about 50%. The amplitudes of C-EPSPs (*C*, stimulus intensity, 20 V/0.5 ms; C.V., 0.45 m/s) from a GluR5-/- SG cell were also decreased by KA in a reversible manner. D, Vm = -75 to -88mV, 14-16-week-old GluR5-/- mice; E, Vm = -72 to -84 mV, 11-15-week-old GluR5-/- mice; F, Vm = -60 to -83 mV, 11-16-week-old GluR5-/- mice.

G) Diagrams show the approximate location of tested Amono-, Apoly- or C-input SG cells obtained from wild-type and GluR5-/- mice.

H-I) Summary graphs showing the time-course of long-lasting depression of EPSPs in the area under the curve of Apoly- (*H*) and C-EPSPs (*I*).

On summary graphs (D-F and H-I), asterisks indicate significant differences between wild-type and GluR5-/- at a given time (*$P<0.05$; **$P<0.01$), and data for wild-type mice are reproduced from Figure 2 for comparison. Each point is mean ± s.e.m. The corresponding times of sampled traces in A, B and C are indicated by numbers on the graphs (D-F).

Fig. 13. Kainate receptors containing GluR5 subunit modulate the C-primary afferent fiber-mediated excitatory neurotransmission.

A-F) Representative traces (*A-C*) recorded from wild-type (*upper traces*) and GluR5 mutant (-/-) (*lower traces*) slices in the presence of 5 μM bicuculine and 2 μM strychnine (bic + strych) are sampled for graphs summarizing for SG cells with inputs from monosynaptic Aδ-fibers (Amono, n = 6 slices from 6 GluR5-/- mice; *D*), polysynaptic Aδ-fibers (Apoly, n = 8 slices from 7 GluR5-/- mice; *E*) and C-fibers (C-EPSP, n = 5 slices from 4 GluR5-/- mice; *F*) inputs. A significant reduction of KA (3 μM, 2 min)-induced depression was observed only in C-EPSPs (F). Blockades of EPSPs by 50 μM DAPV and 50 μM GYKI53655 are shown in the sampled EPSPs recorded in GluR5-/- SG cells. Test stimulus intensities for C-EPSP traces in *C* are 15 V/0.5 msec (c. v., 0.35 m/s) and 30 V/0.5 ms (c.v., 0.54 m/s) for wild-type and GluR5-/- SG cells, respectively. D, Vm = -75 to - 88mV, 11-16-week-old GluR5-/- mice. E, Vm = -67 to -83 mV, 9-14-week-old GluR5-/- mice. F, Vm = -63 to -83 mV, 8-13-week-old GluR5-/- mice.

G) Diagrams show the approximate location of tested Amono-, Apoly- or C-input SG cells obtained from wild-type and GluR5-/- mice.

H-I) Summary graphs, showing the time-courses of changes of EPSPs in the area under the curve of Apoly- (*H*) and C-EPSP (*I*), also support the result from peak amplitude measurement.

On summary graphs (D-F and H-I), asterisks indicate significant difference between wild-type and GluR5-/- at a give time (*$P<0.05$. Each point is mean ± s.e.m at a given time. The numbers on the graphs (D-F) indicate the corresponding times of sampled traces in A-C.

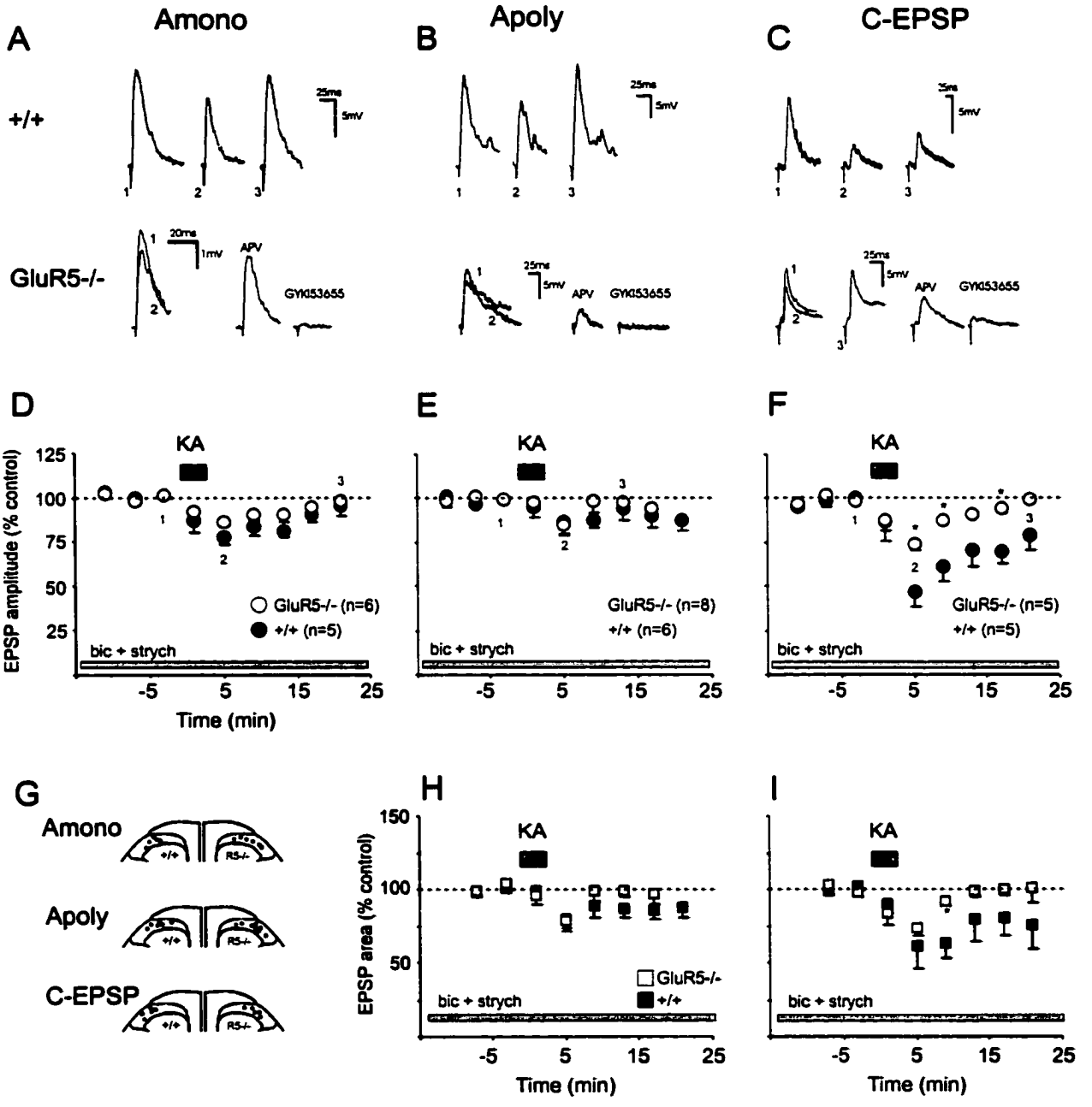

A
Amono
B
Apoly
C
C-EPSP
+/+
GluR5-/-
25ms
5mV
20ms
1mV
APV
GYKI53655
D
E
F
KA
EPSP amplitude (% control)
125
100
75
50
25
GluR5-/- (n=6)
+/+ (n=5)
GluR5-/- (n=8)
+/+ (n=6)
GluR5-/- (n=5)
+/+ (n=5)
bic + strych
-5
5
15
25
Time (min)
G
Amono
Apoly
C-EPSP
+/+
R5-/-
H
I
EPSP area (% control)
150
100
50
GluR5-/-
+/+

Fig. 14. Activation of kainate receptors differentially modulate AMPA receptor- or NMDA receptor-mediated synaptic transmission.

In a representative SG neuron, bath-applied 3 μM KA exerted small depression of monosynaptic EPSP amplitudes (*closed circle*) both in Krebs solution or in bicuculline (5 μM) and strychnine (2 μM)-containing (bic + strych) solution. However, late component of EPSPs, appeared following the inclusion of bicuculline and strychnine to the bath solution, was almost completely blocked by KA, which is prominent in the measurement for EPSP area under the trace curve (*open circle*). Further, after wash-out of KA, the recovered late component was completely blocked by 50 μM DAPV, indicating that it was NMDA receptor-mediated component. The small depression of monosynaptic component of EPSPs, which was observed in the Krebs or bic + strych solution, still existed under the condition of NMDA receptor blockade in a similar degree. Sample traces displayed above the graph are individual synaptic responses taken at the times indicated by numbers. Bars indicate periods of application of each drug. Vm = -75 mV, 16-week-old mouse.

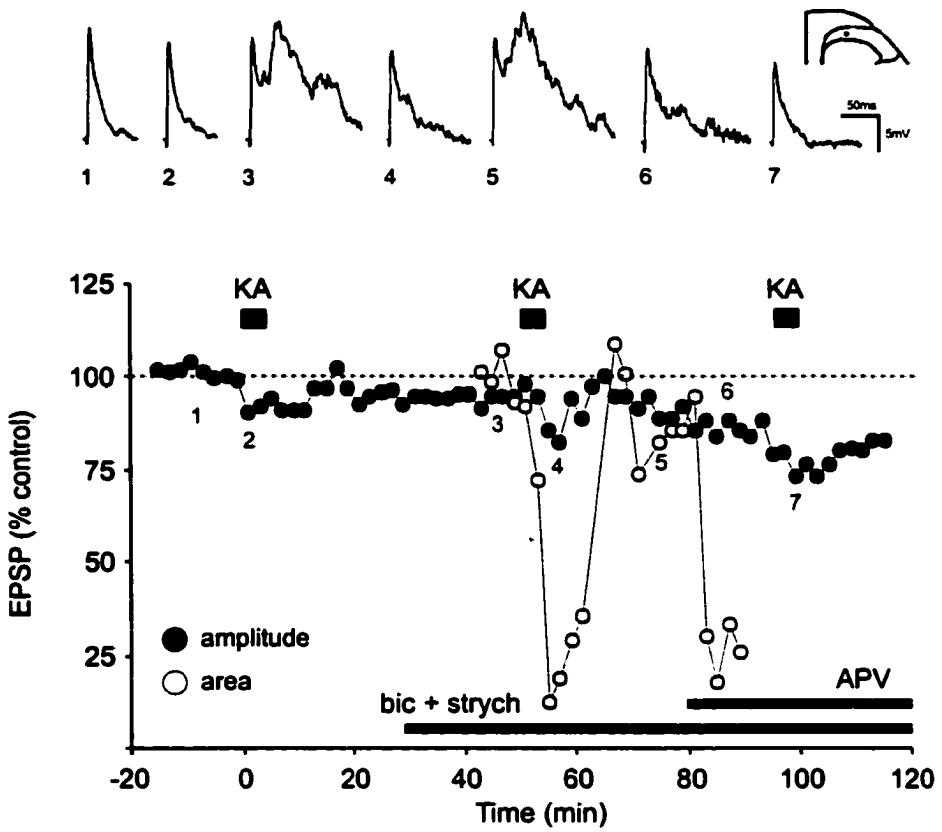

50ms
5mV
1
2
3
4
5
6
7
KA
KA
KA
125
100
75
50
25
EPSP (% control)
amplitude
area
bic + strych
APV
-20
0
20
40
60
80
100
120
Time (min)

Fig. 15. GluR6 kainate receptor subunit is also involved in the long-lasting depression of synaptic transmission induced by the activation of kainate receptors.

A-C) Graphs show the effects of KA on EPSP amplitudes in SG cells with monosynaptic (*A*, Amono; n = 5 slices from 5 GluR6 mutant (-/-) mice) and polysynapitc (*B*, Apoly; n = 5 slices from 5 GluR6-/- mice) Aδ-fiber, and C-fiber (*C*, C-EPSP; n = 7 slices from 6 GluR6-/- mice) inputs. Compared to wild-type mice, significant reduction in the magnitude of long-lasting depression of EPSPs was observed in Apoly- and C-EPSP groups, but not in Amono-EPSP group. The traces displayed above the graph are individual synaptic responses in GluR6-/- mice taken at the times indicated by numbers. *A*, Vm = -75 to -90 mV, 13-week-old GluR6-/- mice. *B*, Vm = -65 to -83 mV, 13-week-old GluR6-/- mice. *C*, Vm = -76 to -90 mV, 13-week-old GluR6-/- mice.

D) Diagrams show the approximate location of tested Amono-, Apoly- or C-input SG cells obtained from GluR6-/- mice.

E-F) Summary graphs, showing the time-courses of changes of EPSPs in the area under the curve of Apoly (*E*) and C-EPSP (*F*), also support the result from peak amplitude measurement.

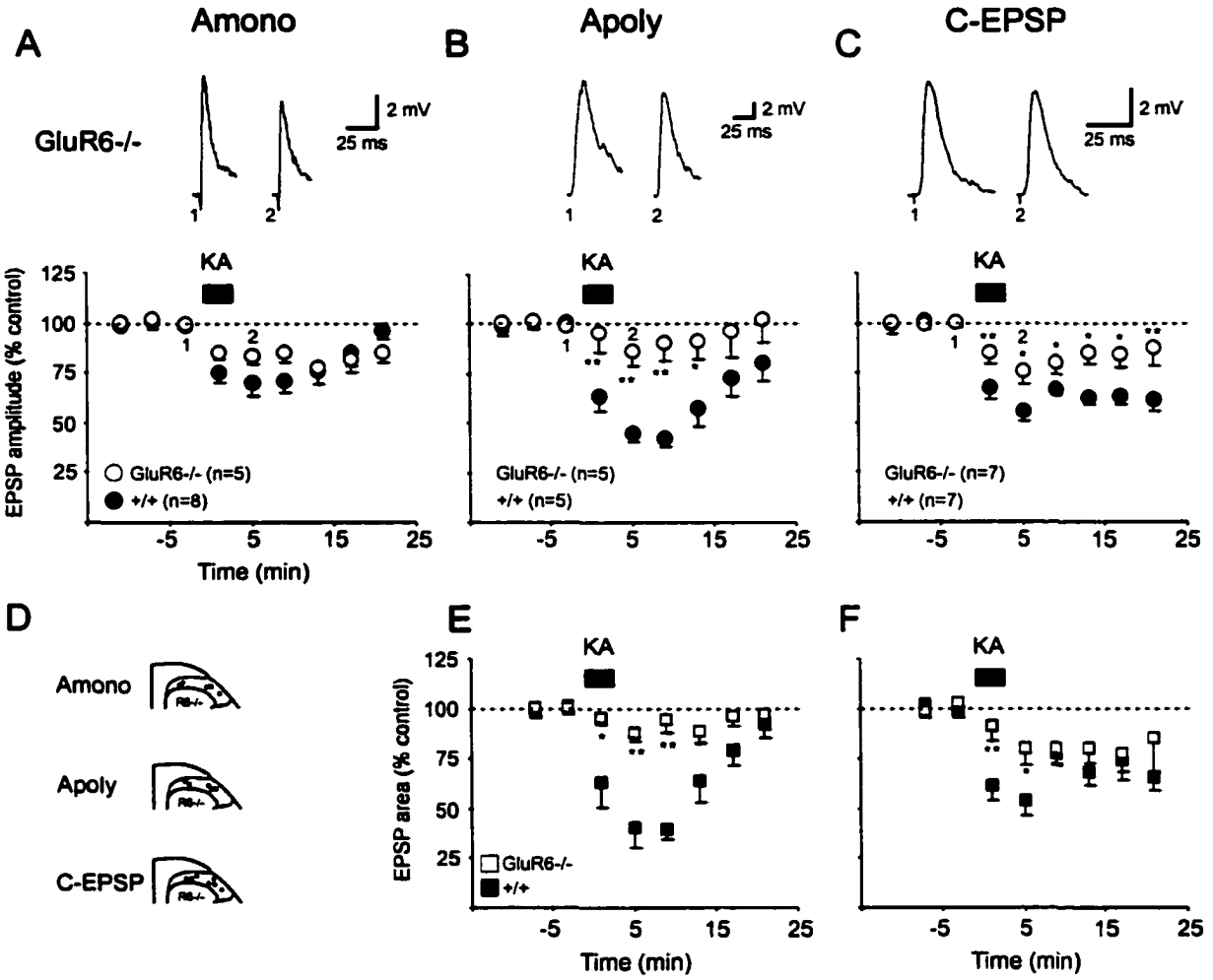

A
Amono
GluR6-/-
2 mV
25 ms
1
2
KA
EPSP amplitude (% control)
125
100
75
50
25
GluR6-/- (n=5)
+/+ (n=8)
-5
5
15
25
Time (min)
B
Apoly
2 mV
25 ms
KA
GluR6-/- (n=5)
+/+ (n=5)
C
C-EPSP
2 mV
25 ms
KA
GluR6-/- (n=7)
+/+ (n=7)
D
Amono
Apoly
C-EPSP
E
KA
EPSP area (% control)
GluR6-/-
+/+
Time (min)
F
KA
Time (min)

Fig. 16. Kainate receptors containing GluR6 subunit contribute to the A- and C-primary afferent fiber-depression of excitatory neurotransmission.

A-C) Graphs show the effects of KA on EPSP (Amono: n = 6 slices from 6 GluR6-/- mice, Apoly: n = 5 slices from 4 GluR5-/- mice, and C-EPSPs: n = 4 slices from 4 GluR6-/- mice) amplitudes recorded in the presence of 5 μM bicuculline and 2 μM strychnine (indicated by *bars*) in GluR6 mutant SG cells. Compared to the wild-type mice, a significant reduction in the magnitude of long-lasting depression of EPSPs was observed in Amono- and C-EPSP groups, but not in Apoly-EPSP group. The traces displayed above the graph are individual synaptic responses in GluR6-/- mice taken at the times indicated by numbers. *A*, Vm = -74 to -89 mV, 8-13-week-old GluR6-/- mice. *B*, Vm = -71 to -88 mV, 8-13-week-old GluR6-/- mice. *C*, Vm = -70 to -89 mV, 9-13 week-old GluR6-/- mice.

D) Diagrams show the approximate location of tested Amono-, Apoly- or C-input SG cells obtained from GluR6-/- mice.

E-F) Summary graphs, showing the time-courses of changes of EPSPs in the area under the curve of Apoly (*E*) and C-EPSP (*F*), also support the result from peak amplitude measurement.

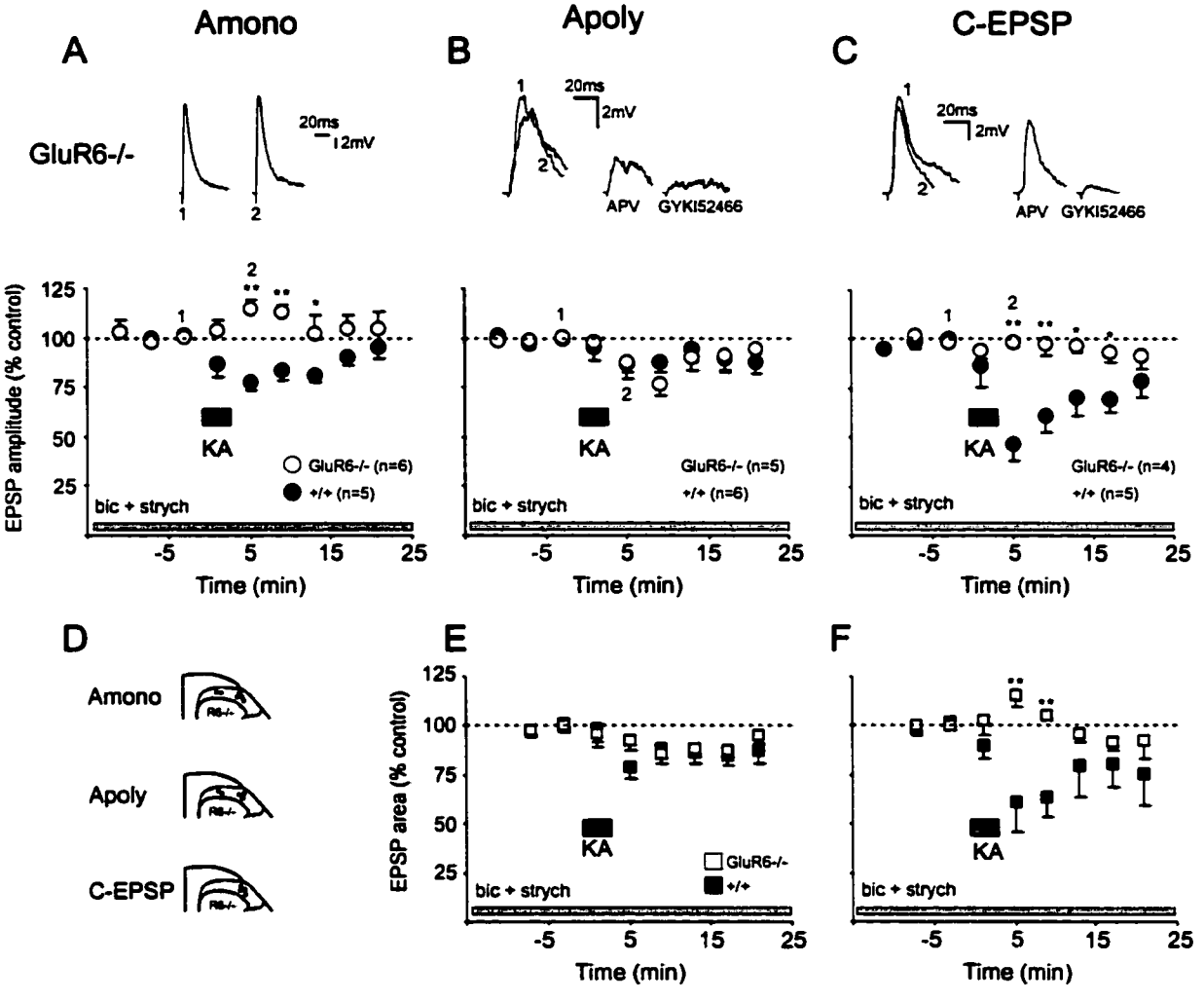

A
Amono
GluR6-/-
20ms
2mV
1
2
B
Apoly
20ms
2mV
APV
GYKI52466
C
C-EPSP
20ms
2mV
APV
GYKI52466
EPSP amplitude (% control)
125
100
75
50
25
KA
bic + strych
GluR6-/- (n=6)
+/+ (n=5)
GluR6-/- (n=5)
+/+ (n=6)
GluR6-/- (n=4)
+/+ (n=5)
-5
5
15
25
Time (min)
D
Amono
Apoly
C-EPSP
E
EPSP area (% control)
GluR6-/-
+/+
F

Fig. 17. Histograms summarize the effects of KA (3 μM, 2 min) on primary afferent fiber-evoked EPSPs in Krebs solution (*A*) and bicuculline and strychnine-containing (bic + strych) solution (*B*). Data, expressed as mean ± s. e. m. Statistical significance is indicated by * ($P<0.05$) and ** ($P<0.01$), compared to wild-type mice, and by ## ($P<0.01$) compared to GluR5-/- mice.

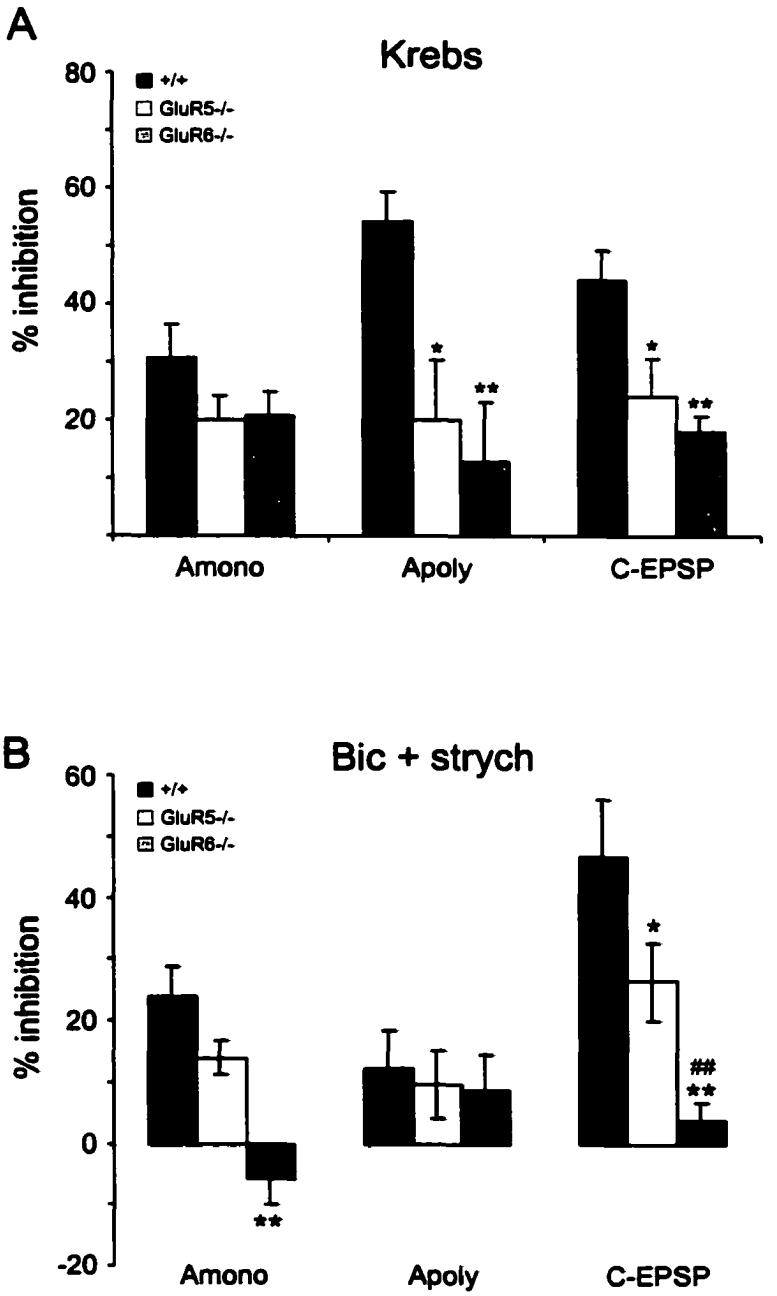

A
Krebs
+/+
GluR5-/-
GluR6-/-
% inhibition
80
60
40
20
Amono
Apoly
C-EPSP
B
Bic + strych
+/+
GluR5-/-
GluR6-/-
% inhibition
60
40
20
0
-20
Amono
Apoly
C-EPSP

Fig. 18. The KA-induced decrease in miniature EPSC frequency was mediated by the activation of GluR5- or GluR6-containing kainate receptors.

A) *Left*, Sample traces of miniature EPSC (mEPSC) recordings from a SG cell obtained from a wild-type slice. KA was bath-applied at a concentration of 3 μM for 2 min. *Middle*, *Right*, The cumulative probability histograms show that the inter-event intervals after the application of KA are significantly longer than that before, but amplitudes are not affected by KA. Vm = -67 mV, 7-week-old mouse.

B) *Left*, Sample traces of mEPSC recordings from a SG cell obtained from a GluR5 mutant (-/-) mouse. *Middle*, *Right*, The cumulative probability histograms show that the inter-event intervals after KA are significantly longer, but lesser degree to the wild-type cell, than that before KA, but amplitudes are unchanged. Vm = -65 mV, 9-week-old GluR5-/- mouse.

C) *Left*, Sample traces of mEPSC recordings from a SG cell obtained from a GluR6-/- mouse. *Middle*, *Right*, even slighter change between before and after KA application was observed in inter-event intervals and amplitudes cumulative distributions. Vm = -63 mV, 9-week-old GluR6-/- mouse.

D) Histograms summarize all experiments for KA-induced changes in the frequency (*left histogram*) or the amplitude (*right histogram*) of mEPSCs recorded from wild-type, GluR5-/- and GluR6-/- mouse slices. The decrease in the frequency of mEPSCs from wild-type slices (-35.2 ± 4.1%, n = 10 slices from 7 mice) was significantly reduced in GluR5-/- (-16.1 ± 6.8%, n = 7 slices from 5 mice; *$P<0.05$) and GluR6-/- (-14.1 ± 8.1%, n = 7 slices from 3 mice; *$P<0.05$) mice. No significant change or difference was observed in amplitude histogram among different genotypes. +/+, Vm = -58 to -73 mV, 7-week-old mice; GluR5-/-, Vm = -61 to -60 mV, 9-week-old mice; GluR6-/-, Vm = -59 to -67 mV, 9-week-old mice.

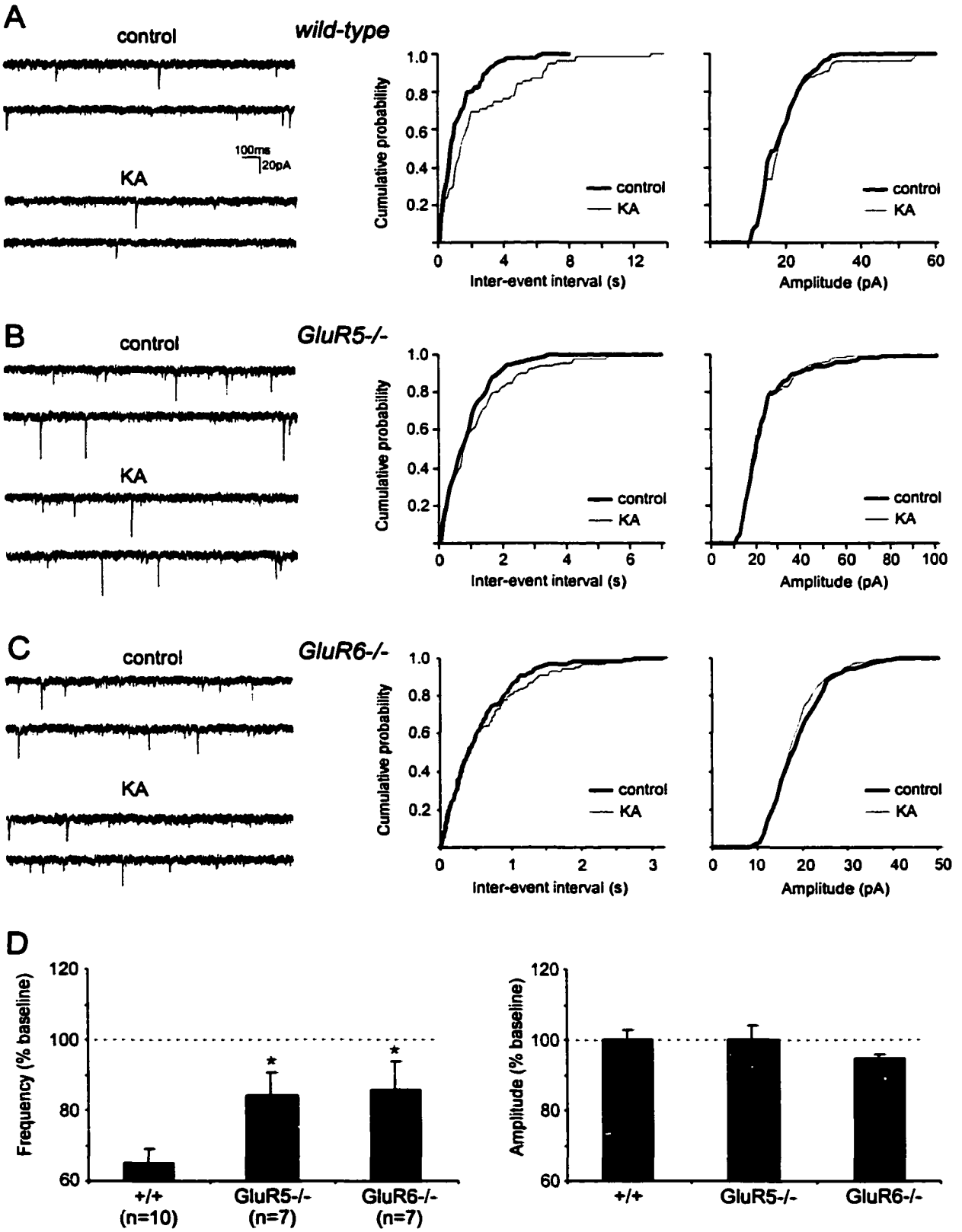

A
control
wild-type
100ms
20pA
KA
Cumulative probability
control
KA
Inter-event interval (s)
Amplitude (pA)
B
control
GluR5-/-
KA
C
control
GluR6-/-
KA
D
Frequency (% baseline)
Amplitude (% baseline)
+/+
(n=10)
GluR5-/-
(n=7)
GluR6-/-
(n=7)

Fig. 19. GluR5 and GluR6 subunits underlie NMDA receptor-dependent long-lasting potentiation of excitatory transmission induced by minimal activation of kainate receptors.

A) *Top*, Sampled traces recorded in the presence of 5 μM bicuculline and 2 μM strychnine (bic + strych) in a slice obtained from a wild-type (+/+) mouse are shown. The peak amplitude of Aδ-fiber-evoked monosynaptic EPSPs was increased by bath application of 30 nM KA (2min). *Middle*, In sampled traces recorded in the presence of bicuculline/strychnine and 50 μM DAPV, the peak amplitude of Aδ-fiber-evoked monosynaptic EPSP was not changed by 30 nM KA. *Bottom*, Summary graph for five wild-type slices in the absence of or five wild-type slices in the presence of 50 μM DAPV, a NMDA receptor blocker, shows that the 30nM KA-induced long-lasting potentiation of EPSPs was blocked by DAPV. The numbers indicate the corresponding time at which the sample traces are taken. Vm = -72 to -87 mV, 6-16-week-old mice.

B) A graph summarizing the effect of 30 nM KA (2 min) for nine GluR5 mutant (-/-) and seven GluR6 mutant (-/-) slices in the presence of 5 μM bicuculline and 2 μM strychnine is shown. The magnitude of long-lasting potentiation induced by 30 nM KA shown in *A* tends to decrease in the time-course graph of GluR5-/- mice. However, 30 nM KA-induced long-lasting potentiation was disappeared in GluR6-/-. GluR5-/-, Vm = -63 to -81 mV, 8-16-week-old mice; GluR6-/-, Vm = -72 to -89 mV, 8-13-week-old mice.

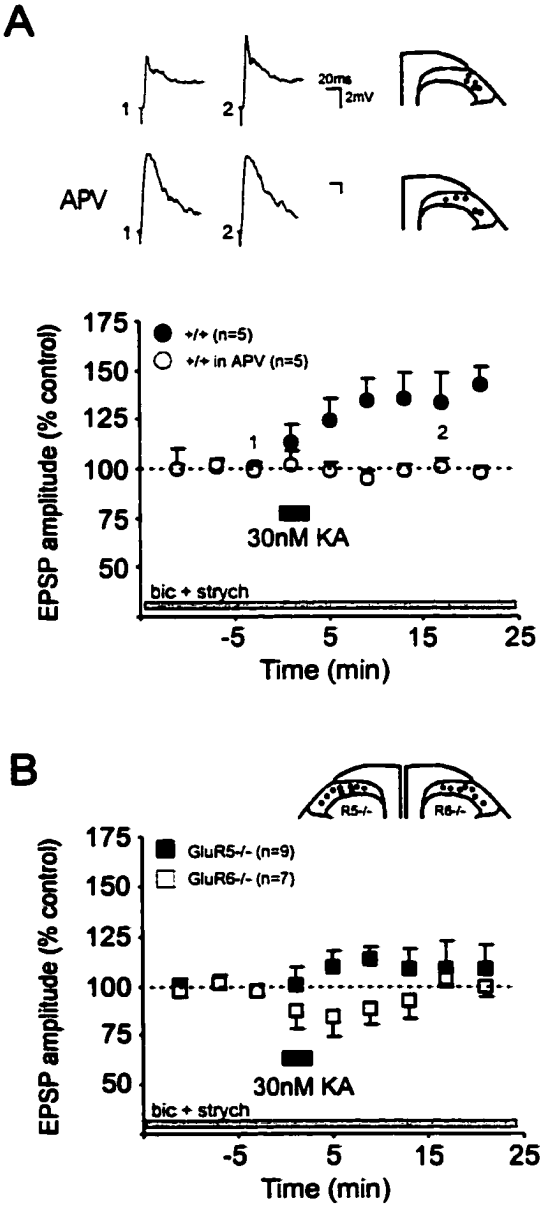

A
1
2
20ms
2mV
APV
1
2
EPSP amplitude (% control)
175
150
125
100
75
50
+/+ (n=5)
+/+ in APV (n=5)
1
2
30nM KA
bic + strych
-5
5
15
25
Time (min)
B
R5-/-
R6-/-
EPSP amplitude (% control)
175
150
125
100
75
50
GluR5-/- (n=9)
GluR6-/- (n=7)
30nM KA
bic + strych
-5
5
15
25
Time (min)

Fig. 20. The selective activation of GluR5-containing kainate receptors is excitatory on primary afferent neurotransmission.

A) In a pooled graph (n = 7 slices from 6 wild-type mice), bath-applied ATPA (1 μM, 2 min), a selective GluR5 specific agonist, potentiated EPSP peak amplitudes. Sample traces (*note*: on the basis of conduction velocity and test stimulus intensity, which are 0.53 m/sec and 4.2 V/0.5ms, respectively, these traces are considered as C-primary afferent fiber-evoked EPSPs) taken at a corresponding time indicated by numbers are shown above the graph. Vm = -65 to -85 mV, 8-12-week-old mice.

B) A scatter diagram summarizes the effect of ATPA in individual SG cells obtained from seven wild-type, nine GluR5 mutant (-/-), four GluR6-/- and four triple-/- (GluR5-/-/GluR6-/-/KA2-/-) slices. Each effect was calculated as percent change (positive values, potentiation; negative values, depression) of EPSP amplitudes at 9-15 min from the onset of ATPA because the peak potentiation of EPSPs by ATPA in the wild-type was usually observed at this time. Points with error bar (s.e.m.) are the means of the group. The mean percent change in GluR5-/- mice (-14.3 ± 6.9%) was significantly different from that in wild-type (32.0 ± 17.7%), GluR6-/- (35.5 ± 16.2%), or Triple-/- (3.2 ± 3.0%) mice ($P<0.05$).

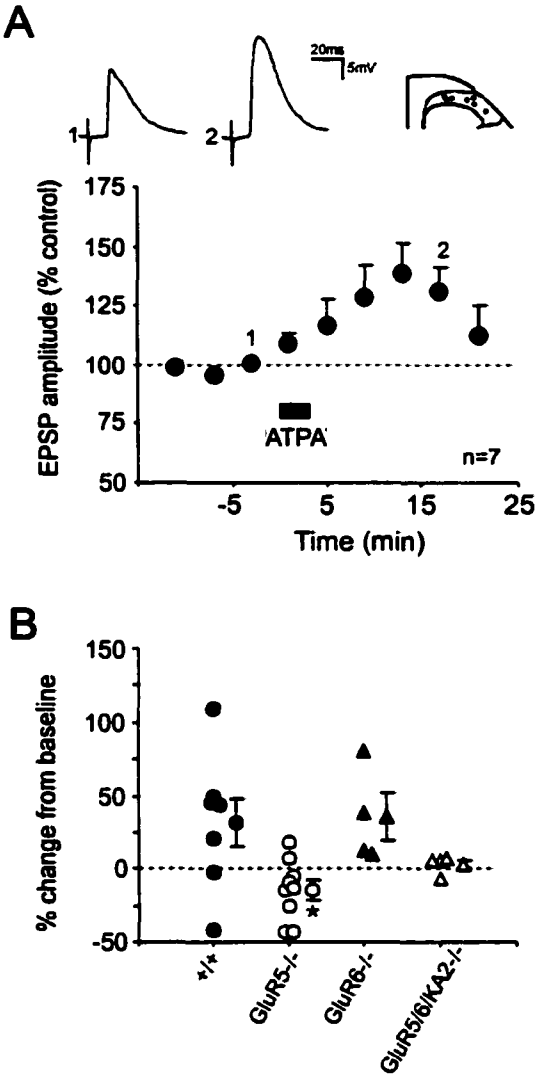
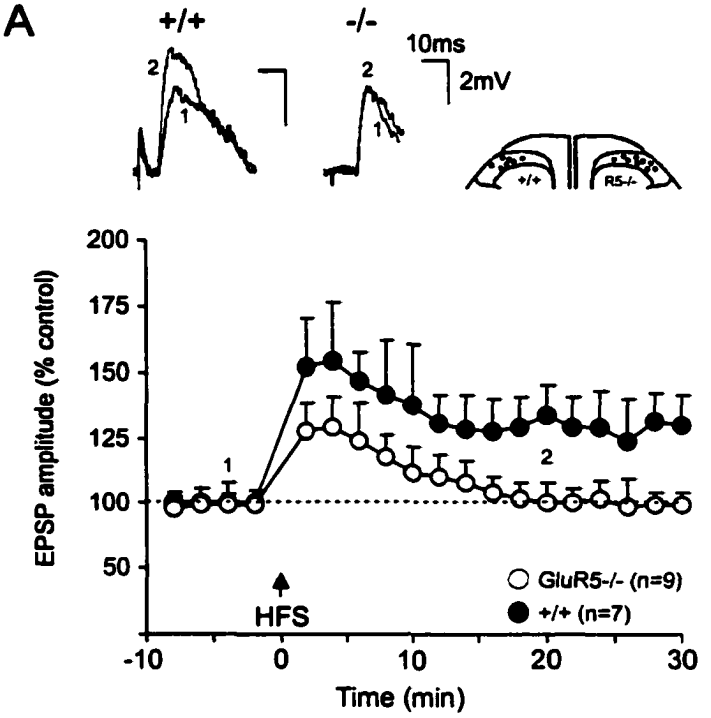
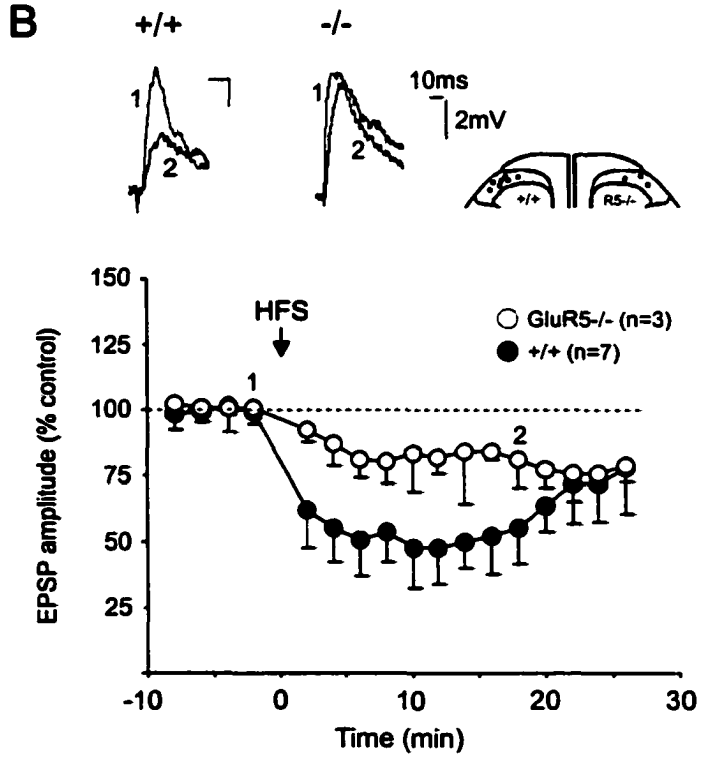

A
20ms
5mV
1
2
EPSP amplitude (% control)
175
150
125
100
75
50
ATPA
n=7
-5
5
15
25
Time (min)
B
% change from baseline
150
100
50
0
-50
+/+
GluR5-/-
GluR6-/-
GluR5/6/KA2-/-

Fig. 21. Impaired LTP and LTD in GluR5 mutant mice.

A) Long-term potentiation of A- and/or C-fiber-evoked EPSPs following high-frequency stimulation (HFS; 100Hz, 1 or 3 tetani) is reduced in GluR5 mutant (-/-) mice (+/+, 133.4 ± 12.0% of baseline at 20 min after HFS, n = 7 slices from 7 mice, $P<0.01$ vs. baseline, 7-week-old, Vm= -57 to -65 mV; GluR5-/-, 100.1 ± 8.7% at 20 min after HFS, n = 9 slices from 6 mice, 7-10-week-old, Vm= -61 to -67 mV, $P>0.05$ vs. baseline; $P<0.05$, between wild-type and GluR5-/-). Above the graph are displayed superimposed EPSPs recorded from neurons receiving C-fiber input prior to and 20 min after HFS in both +/+ and GluR5-/- mice.

B) Long-term depression of synaptic transmission induced by HFS (100Hz, 1 or 3 tetani) is reduced in GluR5-/- mice (+/+, 55.1 ± 13.3% of baseline at 18 min after HFS, $P<0.01$ vs. baseline, n = 7 slices from 7 mice, 7-12-week-old, Vm= -62 to -83 mV; GluR5 -/-, 81.1 ± 10.4% of baseline, $P>0.05$ vs. baseline, n = 3 slices from 3 mice, 44 to 49 d-old, -54 to -67 mV; $P<0.01$, between wild-type and GluR5-/-). Above the graph are displayed superimposed EPSPs recorded from neurons receiving Aδ-fiber input prior to and 18 min after HFS in both +/+ and GluR5-/- mice.


A
+/+
-/-
10ms
2mV
2
1
+/+
R5-/-
200
175
150
125
100
50
EPSP amplitude (% control)
HFS
GluR5-/- (n=9)
+/+ (n=7)
-10
0
10
20
30
Time (min)


B
+/+
-/-
10ms
2mV
1
2
+/+
R5-/-
150
125
100
75
50
25
EPSP amplitude (% control)
HFS
GluR5-/- (n=3)
+/+ (n=7)
-10
0
10
20
30
Time (min)

CHAPTER 5. GENERAL CONCLUSION

The spinal superficial dorsal horn (SDH), including substantia gelatinosa (SG), is an area where primary afferent fibers arising predominantly from skin, but also viscera and muscle, terminate and form first synaptic relay with dendrites of dorsal horn neurons. Glutamate is a major neurotransmitter at the primary afferent fiber-DH neuronal synapses, so that it makes glutamatergic synapses. It has been of great interest since the strength of glutamatergic synaptic transmission in the central nervous system, including the spinal cord, is not constant and can be modulated by the rate of activity in synaptic pathways. Therefore, experiments to elucidate the mechanism of physiological modulations at glutamatergic synapses have intensively been undertaken by neuroscientists.

During my PhD study, I used gene-targeted mice lacking GluR2 AMPA receptor subunit, or GluR5, GluR6 or GluR5/6/KA2 kainate receptor subunits to determine the functional role of these subunits comprising AMPA, or kainate receptors, in the adult mouse spinal cord SG region. By using conventional intracellular (current-clamp) recordings, and also whole-cell voltage-clamp recordings from *in vitro* mouse spinal cord slices, I have demonstrated the role of Ca2+-permeable AMPA receptors in the plasticity of sensory synaptic transmission in GluR2 mutant mice, and the modulatory role(s) of kainate receptors, composed of at least GluR5 or GluR6 subunits, on spinal excitatory synaptic transmission at Aδ- and/or C-primary afferent fiber-SG synapses. Here is a summary of the facts that I derived from my PhD work:

1) On the basis of the rectifying properties of AMPA receptor-mediated excitatory postsynaptic currents (EPSCs), the Ca^{2+} permeability through AMPA receptors expressed on the postsynaptic membrane of SG neurons, was efficiently increased due to the genetic deletion of GluR2 subunit.

2) The increased Ca^{2+}-permeability through AMPA receptors caused the enhancement of the high-frequency stimulation (100 Hz)-induced long-term potentiation (LTP) of excitatory postsynaptic potentials (EPSPs).

3) The enhanced LTP of synaptic transmission, caused by the deletion of GluR2 subunit, was NMDA receptor-independent and insensitive to the blockade of high-voltage activated L-type Ca^{2+} channels.

4) Bath application of kainate (3 μM, 2 min) significantly depressed the primary afferent fiber-evoked EPSPs recorded in the SG neurons obtained from wild-type mice.

5) Under physiological conditions, i.e., in the presence of synaptic inhibition, both GluR5 and GluR6 subunits contribute to the depressant action of kainate at the primary afferent C-fiber-activated monosynaptic and polysynaptic pathways, and Aδ-fiber-activated polysynaptic pathways.

6) In the absence of synaptic inhibition kainate produced a smaller depressant effect on C-fiber-evoked EPSPs in the SG neurons from mice lacking the GluR5 subunit in comparison to that seen in wild-type mice.

7) In contrast, in the absence of synaptic inhibition, the application of kainate had no effect on EPSPs recorded in SG neurons from mice in which the GluR6 gene had been disrupted at Aδ- and C-fiber synapses.

8) The application of KA (3 μM, 2 min) decreases the frequency of spontaneous miniature EPSCs (mEPSCs) at primary afferent synapses whereas their amplitude remained unchanged. This finding provides evidences that KA acted presynaptically to reduce evoked transmission.

9) In contrast to the KA effect in wild-type mice, the mEPSC frequencies in the SG neurons in slices from the GluR5 or GluR6 mutant mice were less reduced, and no significant change in mEPSC amplitude was observed.

10) After blocking inhibitory inputs, the application of kainate at a low concentration (30 nM, 2 min) revealed a long-lasting NMDA receptor-dependent potentiation of primary afferent neurotransmission.

11) In a normal Krebs-bicarbonate medium, bath application of (RS)-ATPA (1-3 μM, 2 min), a putative GluR5 agonist, causes both potentiation and depression of EPSPs in SG neurons obtained from wild-type mice.

12) In contrast to wild-type mice, the application of ATPA (1 or 3 μM, 2 min) causes

predominantly depression of EPSPs in SG neurons from GluR5 mutant mice.

13) The potentiating and the depressant effects of ATPA were not modified by bicuculline and strychnine, but the ATPA depressant effect was blocked by genetic deletion of GluR6 or GluR5/GluR6/KA2 subunits.

14) High-frequency tetanic stimulation (100 Hz) induced the long-term potentiation (LTP) of the Aδ- and/or C-primary afferent fiber-evoked EPSPs in wild-type mice, but not in the GluR5 mutant mice.

15) At a resting membrane potential the application of KA (3 μM, 2 min) caused a slow, dose-dependent and reversible depolarization in SG neurons of wild-type mice. This effect persisted in the presence of tetrodotoxin, a Na^+ channel blocker, indicating a direct postsynaptic action of kainate receptors on SG neurons.

16) The KA-induced depolarization persisted in the slices obtained from the GluR5 mutant mice, but it was not present in those from the GluR6 mutant mice.

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