Modulation of synaptic transmission from primary afferents to spinal substantia gelatinosa neurons by group III mGluRs in GAD65-EGFP transgenic mice

Lian Cui¹, Yoo Rim Kim¹, Hye Young Kim¹, Seok Chan Lee³, Hee-Sup Shin³, Gábor Szabó⁴, Ferenc Erdélyi⁴, Jun Kim¹, Sang Jeong Kim¹,²

¹Department of Physiology, Seoul National University College of Medicine, Seoul, Korea
²Department of Brain and Cognitive Sciences, Seoul National University, Seoul, Korea
³Center for Neural Science, Korea Institute of Science and Technology, Seoul, Korea
⁴Institute of Experimental Medicine, Department of Gene Technology and Developmental Neurobiology, Institute of Experimental Medicine, Budapest, Hungary

* Correspondence to
Sang Jeong Kim, MD, Ph.D, Department of Physiology, Department of Brain and Cognitive Sciences, Seoul National University College of Medicine, Yeongeon-dong, Jongro-gu, Seoul 110-799, Korea.
Tel: +82-2-740-8229; Fax: +82-2-763-9667; Email: sangjkim@snu.ac.kr

Key words: Group III mGluRs, GABAergic interneuron, primary afferent, spinal cord, pain

Running head: Modulation of spinal SG neurons by group III mGluRs
ABSTRACT

Group III metabotropic glutamate receptors (mGluRs) are involved in nociceptive transmission in the spinal cord. However, the cellular mechanism underlying the modulation of synaptic transmission from nociceptive primary afferents to dorsal horn neurons by group III mGluRs has yet to be explored. In this study, we used transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the GAD65 promoter to identify specific subpopulations of GABAergic inhibitory interneurons. By GABA immunolabeling, we confirmed the majority of GAD65-EGFP-expressing neurons were GABAergic. As GAD65-EGFP-expressing neurons have not been examined in detail before, we first investigated the physiological properties of GAD65-EGFP- and non-EGFP-expressing neurons in substantia gelatinosa (SG) of the spinal dorsal horn. Membrane properties, such as the resting membrane potential, membrane capacitance, action potential threshold, and action potential height, differed significantly between these two groups of neurons. Most EGFP-expressing neurons displayed a tonic firing pattern (73% of recorded neurons) and received monosynaptic Aδ and/or C primary afferent inputs (85% of recorded neurons). In contrast, we observed a delayed firing pattern in 53% of non-EGFP-expressing neurons. After identifying the physiological properties of EGFP-expressing neurons, we tested the effects of group III mGluRs on synaptic transmission pharmacologically. A group III mGluR agonist, L-AP4, attenuated Aδ fiber-evoked synaptic transmission, but did not affect C fiber-evoked synaptic transmission to EGFP-expressing neurons. Similar primary afferent-specific inhibition by L-AP4 was also observed in non-EGFP-expressing neurons. Moreover, Aδ fiber-evoked synaptic transmission was suppressed by a selective mGluR7 agonist, AMN082. These results suggest that modulation of the synaptic transmission from primary afferents to SG neurons by group III mGluR agonist is specific to the type of nociceptive primary afferents but not to the type of target neurons.
INTRODUCTION

Metabotropic glutamate receptors (mGluRs), which participate in nociceptive processing, are emerging as molecular targets for the treatment of pain (Dray 2003; Woolf and Salter 2000). A number of studies have shown that group I mGluRs activate the G\textsubscript{q/11} protein and, through the PLC pathway, increase synaptic transmission and neuronal excitability. On the other hand, group II and III mGluRs activate the G\textsubscript{i/o} protein, which reduces cAMP formation, thus decreasing synaptic transmission and neuronal excitability in various brain areas (Bushell et al. 1999; Conn and Pin 1997; Gereau and Conn 1995; Knopfel et al. 1995; Macek et al. 1996; Miller 1998; Neugebauer et al. 1997; Neugebauer et al. 2000; Schoepf et al. 1999; Schoppa and Westbrook 1997; Schrader and Tasker 1997). Hence, inactivation of group I mGluRs or activation of group II and III mGluRs may produce antinociceptive effects. In the spinal cord, two subtypes of group III mGluRs, mGluR4 and mGluR7, are expressed; in particular, mGluR7 is expressed on the nociceptive primary afferent terminals (Azkue et al. 2001; Kinoshita et al. 1998; Li et al. 1997; Ohishi et al. 1995). Recent studies have observed that intrathecal administration of a group III mGluR agonist inhibits hyperalgesia and allodynia in inflammatory and neuropathic pain (Chen and Pan 2005; Goudet et al. 2008). In addition, one recent study observed that group III mGluR activation had an antinociceptive effect on neuropathic pain in spinal slices (Zhang et al. 2009). However, the cell type-specific and primary afferent-dependent roles of group III mGluRs in the spinal dorsal horn have yet to be well defined.

The superficial dorsal horn (laminae I-II) of the spinal cord is a critical site for transmission and modulation of nociceptive information from the periphery to the brain (Light and Perl 1979). Superficial dorsal horn neurons receive A\textsubscript{\delta} and/or C primary afferent inputs, and they form a complicated intrinsic circuit. A\textsubscript{\delta} and C nociceptive primary afferents transmit qualitatively discriminable pain information. A\textsubscript{\delta} fibers mediate first, sharp pain and C fibers mediate second, burning pain (Torebjork and Hallin 1973). Several studies have found similar modulations on A\textsubscript{\delta} and C fiber-mediated synaptic transmission by various neuromodulators (Lao et al. 2004; Youn et al. 2008), while other studies have found different modulations (Ataka et al. 2000; Ikoma et al. 2007; Ito et al. 2000).

The superficial dorsal horn, consists of highly heterogeneous neurons (projection neurons, excitatory interneurons and inhibitory interneurons), and has a complex organization. Superficial dorsal horn neurons have been classified to several groups, based on their morphological and electrophysiological characteristics (Grudt...
and Perl 2002; Maxwell et al. 2007; Prescott and De Koninck 2002), and cell type-specific circuits between primary afferent inputs and spinal dorsal horn neurons have been reported (Lu and Perl 2005; 2003; Yasaka et al. 2007). Nevertheless, a detailed understanding of the intrinsic circuits in the superficial dorsal horn remains elusive as until now because it was difficult to identify whether intrinsic spinal neurons are excitatory or inhibitory. Recently, the development of transgenic mice has allowed researchers to identify GABAergic neurons associated with the GABA-synthesizing enzymes, glutamate decarboxylase (GAD) 65 or GAD67 (Heinke et al. 2004; Lopez-Bendito et al. 2004). Several lines of evidence have suggested that the loss of GABAergic inhibition plays an important role in inflammatory and neuropathic pain (Baba et al. 2003; Malan et al. 2002; Moore et al. 2002; Yaksh 1989). Thus, specifically studying GABAergic inhibitory interneurons may be useful to further understand how the synaptic transmission and intrinsic circuitry of the spinal cord contribute to the perception of pain.

In this study, we used transgenic mice, expressing EGFP under the control of the GAD65 promoter, to specifically label GAD 65-expressing inhibitory neurons. This has enabled us to compare the effects of group III mGluRs on excitatory synaptic transmission in EGFP-expressing neurons and non-EGFP-expressing neurons. Our data indicated that GAD65-EGFP-expressing inhibitory neurons have distinct physiological properties when compared to non-EGFP-expressing neurons. Activation of group III mGluRs attenuated the Aδ primaryafferent-mediated excitatory synaptic transmission in both neuron groups to a similar extent, but did not affect C primary afferent-mediated transmission.

METHODS

Animals

All experiments were undertaken using protocols approved by the Experimental Animal Care and Ethics Committee of Seoul National University. We used heterozygous C57BL/6J BAC transgenic mice, expressing EGFP under the control of the GAD65 promoter (Lopez-Bendito et al. 2004), provided by the Korea Institute of Science and Technology.

Electrophysiology and slice preparation

5-7 week-old transgenic mice were anaesthetized with 10% urethane (1.5 mg/kg IP). A laminectomy was
performed, and the lumbar segment was removed and placed in ice-cold solution, consisting of the following (in mM): NaCl, 95; KCl, 1.8; KH$_2$PO$_4$, 1.2; CaCl$_2$, 0.5; MgSO$_4$, 7; NaHCO$_3$, 26; glucose, 15; sucrose, 50, oxygenated with 95% O$_2$ and 5% CO$_2$, at a pH of 7.35-7.45 and an osmolarity of 310-320 mOsmol l$^{-1}$.

Transverse spinal cord slices (500-600 μm thickness) of the attached L4 or L5 dorsal roots were prepared using a Microm HM 650V vibratome (Microm, Germany) and they were incubated, at 34$^\circ$ C for 30 min.

The slice was transferred to the recording chamber and continuously superfused with recording solution, at a rate of 3-4 ml/min. The recording solution was consisted of the following (in mM): NaCl, 127; KCl, 1.8; KH$_2$PO$_4$, 1.2; CaCl$_2$, 2.4; MgSO$_4$, 1.3; NaHCO$_3$, 26; glucose, 15, oxygenated with 95% O$_2$ and 5% CO$_2$, at a pH of 7.35-7.45 and an osmolarity of 300-310 mOsmol $^{-1}$. Recordings were performed at 32$^\circ$ C. We identified the substantia gelatinosa (lamina II, SG) as a translucent band across the dorsal horn, visualized with an Olympus BX 50WI microscope (Olympus Optical, Tokyo, Japan). EGFP- and non-EGFP-expressing neurons in SG were distinguished by epifluorescence and recorded in the whole cell patch clamp configuration. Glass pipettes (3-5 MΩ) were filled with internal solution, consisting of the following (in mM): K gluconate, 120; KCl, 10; MgATP, 2; NaGTP, 0.5; HEPES, 20; EGTA, 0.5; phosphocreatine di(tris) salt, 10, at a pH of 7.29 and an osmolarity of 300 mOsmol $^{-1}$. In some experiments, a general G-protein inhibitor, GDP-beta-S (1 mM), was added into the internal solution to block the post-synaptic signaling. All data were acquired using an EPC-9 patch-clamp amplifier and Pulse software (HEKA, Germany), and the signals were filtered at 2 kHz. The resting membrane potential (RMP) was determined immediately after cell membrane rupture. Liquid junction potentials were not corrected. Firing patterns were tested based on that described by Heinke et al. (Heinke et al. 2004). To detect the voltage dependence of the firing pattern, we routinely applied depolarizing step pulses (ranging from 30 to 300 pA, at 30 pA increments and with a 1 s duration) from three different holding potential levels, as follows: between −50 and −65 mV (in most cases, −55 mV); between −65 and −75 mV (in most cases, −70 mV); and between −80 and −90 mV (in most cases, −85 mV). We classified the firing patterns according to how they appeared at the most negative holding potential. Membrane resistance ($R_m$) was calculated from the average of the steady-state voltage deflection in response to a 500 ms, 10 pA hyperpolarizing current step. Membrane capacitance (C) was calculated according to $C = \tau_m/R_m$, in which the membrane time constant ($\tau_m$) was calculated from the exponential curve fit of the response to the hyperpolarizing current step. The rheobase, the minimum current amplitude required to produce an action potential (AP), was estimated by applying 100 ms
current steps in 2 pA increments. We determined the voltage threshold at rheobase by measuring the membrane potential ($V_m$) at which $dV_m/dt$ equals 20 V/sec (Bekkers and Delaney 2001). We assessed the presence of A-type potassium current ($I_K$) and that of hyperpolarization-activated inward current ($I_h$) via voltage steps in the voltage clamp mode. The series resistance ($R_s$) was between 10 and 25 MΩ, and we discarded any experiments in which these values changed by more than 20% during the recordings.

To evoke synaptic currents, we applied 0.1 ms duration stimuli to the dorsal root through a suction electrode with a constant current stimulator (A360, WPI, USA), at 0.1 Hz. We defined evoked excitatory postsynaptic currents (EPSCs) as $A_\delta$ or C fiber-evoked EPSCs based on their stimulation thresholds ($A_\delta$, 0.05-0.30 mA; C, 0.8-2.0 mA) and estimated the conduction velocity (CV; $A_\delta$, 2-8 m s$^{-1}$; C < 2 m s$^{-1}$) as previously reported (Chen and Sandkühler 2000; Ruscheweyh and Sandkühler 2002). The evoked EPSCs were considered to be monosynaptic when the latency was unchanged by stimulation of $A_\delta$ fibers at 20 Hz, and when failures did not occur by stimulation of C fibers at 1 Hz. The mean amplitude of evoked EPSCs recorded prior to the agonist application served as controls. Significant changes from controls were assessed by measuring the amplitude of three or five consecutive responses at the end of the agonist application. Paired pulse ratio (PPR) was calculated by comparing the amplitude of continuously evoked EPSCs (EPSC2/EPSC1) with an interstimulus interval of 50 ms. Picrotoxin (100 μM) and strychnine (4 μM) were added into the recording solution to block inhibitory synaptic transmissions.

**Immunohistochemistry**

In order to prepare tissue for immunohistochemistry, 5-7 week-old transgenic mice were anaesthetized as before with 10% urethane, and transcardial perfusions were performed with 1 U/ml heparin in PBS, followed by a mixture of 1% paraformaldehyde (PFA) and 1% glutaraldehyde, at 37° C (Heinke et al. 2004; Todd and McKenzie 1989). Next, we performed laminectomies and incubated the lumbar spinal cord in 20-30% sucrose in PBS for 1-2 days at 4° C. Cryosectioning produced transverse sections of 6-10 μm thickness, which we pretreated with 1% sodium borohydrate in PBS, for 30 min, and then washed in PBS, for 60 min. The sections were incubated overnight with the primary antibody, rabbit anti-GABA (Sigma, A2052, 1:5000) in PBS containing 5% normal goat serum at room temperature. As a control for the GABA binding by the antibody, sections were incubated in identical fashion, but omitting the primary antibody. Sections were then rinsed for 30 min and incubated with PBS containing the secondary antibody, Alexa Fluor 594-conjugated goat anti-rabbit
IgG (1:400, Molecular Probes, Eugene, Oregon, USA), and 4',6-diamidino-2-phenylindole (DAPI; 1 μg/ml, Molecular Probes, Eugene, Oregon, USA) for 1-2 h at room temperature. Sections were rinsed and mounted with Gel/Mount (Biomeda, Burlingame, CA, USA) and imaged using confocal microscopy (Olympus IX 81, Japan).

Statistics

Data are expressed as mean ± SEM. “N” indicates the number of neurons tested. Student’s paired or unpaired t-tests and χ² tests were used for statistical data comparisons, with P < 0.05 considered significant.

Drugs

All drugs were bath applied, using a gravity perfusion system. Fast solution changes were ensured by placement of the perfusion tip close to the cells being recorded from. Picrotoxin and strychnine were purchased from Sigma (St. Louis, MO, USA), and L(+)-2-amino-4-phosphonobutyric acid (L-AP4), (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), N-phenyl-7-(hydroxylimino)cyclopropa[h]-chromen-1a-carboxamide (PHCCC), N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), and tetrodotoxin (TTX) were from Tocris (Bristol, BS11 9XJ, UK).

RESULTS

Distribution of the EGFP-expressing neurons in the spinal cord of GAD65-EGFP transgenic mice

We used GAD65-EGFP transgenic mice to identify the GABAergic inhibitory interneurons via EGFP expression. GAD65-EGFP-expressing neurons were highly distributed in laminae I-III and moderately distributed in laminae IV-VI and X (Figure 1A), in agreement with a recent report (Labrakakis et al. 2009). This expression pattern is similar to that of GAD67-EGFP-expressing neurons (Heinke et al. 2004). To confirm that the GAD65-EGFP-labeled neurons produce GABA, we performed immunohistochemistry in the glutaraldehyde-fixed tissues. In the spinal cord transverse slices, ~79% of the EGFP-labeled neurons were GABA-immunopositive in SG (Figure 1B, C, and D; 80%, 74%, and 82% of counted neurons in three animals, 404-432 of EGFP-expressing neurons in SG were counted per animal). Conversely, ~60% of the GABA-
immunopositive neurons were EGFP-labeled (57%, 58%, and 65% in the three studied animals, 549-613 of GABA-immunoreactive neurons in SG were counted per animal). This suggests that most of the EGFP-expressing neurons were GABAergic inhibitory interneurons, and the EGFP-expressing neurons represented approximately 60% of the GABAergic neurons in SG of these transgenic mice.

Physiological properties of the EGFP-expressing neurons in SG of the spinal cord

Applying rectangular step current injections to EGFP-expressing neurons in SG revealed six firing patterns (Heinke et al. 2004; Prescott and De Koninck 2002; Ruscheweyh et al. 2004; Ruscheweyh and Sandkuhler 2002). Tonic firing neurons generated action potentials at regular intervals throughout the current pulse (Figure 2A). Delayed firing neurons showed a delayed onset of spike in response to the current injection (Figure 2B). Gap firing neurons exhibited a long first interspike interval, followed by tonic firing. Injecting currents at just above the threshold in these neurons produced a pronounced delay in the generation of the first action potential (Figure 2C). Initial bursting neurons displayed one or few action potentials at the beginning of current injection, even when strongly depolarized (Figure 2D). Bursting firing neurons were characterized by a short burst of two to four action potentials, riding on a slow depolarizing wave, at the onset of a depolarizing current pulse. Upon stronger current injections, this bursting firing was followed by tonic firing (Figure 2E). Phasic firing neurons displayed irregular action potentials during current injection (Figure 2F). 73% of recorded EGFP-expressing neurons (49/67) displayed tonic firing, and the remaining neurons displayed delayed firing, gap firing, initial bursting, bursting firing, and phasic firing patterns (Figure 2G). In comparison, 53% of the recorded non-EGFP-expressing neurons (34/64) exhibited delayed firing, and the remaining neurons showed tonic firing, gap firing, initial bursting, bursting firing, and phasic firing patterns (Figure 2H). These firing patterns differed from those of EGFP-expressing neurons in GAD67-EGFP transgenic mice, where initial bursting (adaptation generating short burst of spikes) was the most frequent firing pattern. This fits the presumption that Na⁺ channels are more highly expressed in GAD65-expressing neurons than in GAD67-expressing neurons (Melnick et al. 2004).

Next, we examined whether there were differences in the passive and active membrane properties of EGFP- and non-EGFP-expressing neurons (Table 1). We found that EGFP-expressing neurons exhibited more hyperpolarized resting membrane potentials than non-EGFP-expressing neurons. Membrane capacitances of EGFP-expressing neurons were larger than those of non-EGFP-expressing neurons. EGFP-expressing neurons...
displayed more negative voltage thresholds and smaller rheobase for action potential generation than non-EGFP-expressing neurons. Also, action potential heights in EGFP-expressing neurons were higher than in non-EGFP-expressing neurons. Finally, in voltage clamp mode, a larger proportion of non-EGFP-expressing neurons exhibited voltage-dependent, rapidly activating and inactivating outward K_A currents than EGFP-expressing neurons (Figure 3A, B, C1, and C2). Large K_A currents in the non-EGFP-expressing neurons may cause more depolarized voltage threshold for action potential initiation, leading to a delayed firing onset (Grudt and Perl 2002; Melnick 2009; Ruscheweyh and Sandkuhler 2002; Yoshimura and Jessell 1989). However, the two groups of neurons displayed hyperpolarization-activated currents in similar proportions (Figure 3D).

Synaptic transmission from the primary afferents to the spinal dorsal horn neurons are primarily mediated by glutamate, one of the important excitatory neurotransmitters. In the transverse spinal cord slices, dorsal root stimulation induced various patterns of EPSCs in the EGFP-expressing neurons of SG (Figure 4). 84.7 % of recorded EGFP-expressing neurons received monosynaptic A_δ or C primary afferent inputs, whereas 35.3 % of recorded non-EGFP-expressing neurons received monosynaptic inputs (table 2). In SG, most EGFP-expressing inhibitory neurons received direct nociceptive inputs from primary afferents, and may contribute to the modulation of excitability in the intrinsic circuit.

Effects of group III mGluRs on excitatory synaptic transmission in EGFP- and non-EGFP-expressing neurons

To demonstrate the action of group III mGluRs on synaptic transmission, we examined the effects of a selective group III mGluR agonist, L-AP4, on the dorsal root-evoked EPSCs in GAD65-EGFP transgenic mice. Recordings were made, as before, from EGFP- and non-EGFP-expressing neurons, located in SG of spinal cord slices. Monosynaptic EPSCs were chosen for our investigation. In the EGFP-expressing neurons, L-AP4 (20 μM) application transiently reduced the amplitude of the A_δ fiber-evoked EPSCs (to 58.6 ± 7.9 % of control, \( P < 0.05, n = 11 \), Figure 5A), and the paired pulse ratio (PPR) was increased concomitantly (to 163.9 ± 28.8 % of control, \( P < 0.05 \)). In one exceptional neuron, the depression of A_δ fiber-evoked EPSCs amplitude by L-AP4 was persisted more than 30 min after washout (data not shown). Similarly, L-AP4 reversibly attenuated the amplitude of A_δ fiber-evoked EPSCs in the non-EGFP-expressing neurons (to 59.4 ± 6.3 % of control, \( P < 0.05, n = 9 \), Figure 5B), and the PPR was also increased slightly (to 136.5 ± 23.0 % of control, \( P > 0.05 \)). During L-AP4 application, L-AP4 did not produce significant postsynaptic currents in these two groups of neurons (data
not shown), indicating the effects of L-AP4 are through a presynaptic mechanism. In contrast to Aδ fiber-evoked EPSCs, the amplitude of C fiber-evoked EPSCs was unaffected by L-AP4 in either EGFP-expressing (to 91.0 ± 4.4 % of control, $P > 0.05$, $n = 6$, Figure 5C) or non-EGFP-expressing neurons (to 95.8 ± 4.8 % of control, $P > 0.05$, $n = 5$, Figure 5D), except for in one EGFP-expressing neurons, which potentiated transiently by L-AP4 (data not shown). The effects of L-AP4 on both Aδ and C fiber-evoked EPSCs were not significantly different in both groups of neurons ($P > 0.05$; Figure 5E), which suggests that this modulation is specific to the type of primary afferents but not to the type of target neurons.

Pre-application of the group III mGluR antagonist, CPPG, abolished the effects of L-AP4, confirming that this mechanism requires group III mGluRs (Figure 6A and B). To further investigate if the mechanism of group III mGluR mediated depression is expressed presynaptically or postsynaptically, we applied a general G-protein inhibitor, GDP-beta-S (1 mM) via the patch pipette. In these recordings, L-AP4 still depressed the amplitude of the Aδ fiber-evoked EPSCs (to 40.7 ± 6.7 % of control $n=5$), and these depression were not significantly different with recordings where control K-gluconate internal solution was used ($p > 0.05$, Figure 6A and B).

These results are consistent with a pre-synaptic expression mechanism.

**Effects of group III mGluR subtype agonists on excitatory synaptic transmission in SG neurons**

In the spinal cord, two subtypes of group III mGluRs are expressed, mGluR4 and mGluR7 (Azkue et al. 2001; Kinoshita et al. 1998; Li et al. 1997; Ohishi et al. 1995). To determine which group III mGluR subtypes are involved in L-AP4 mediated inhibition, we employed group III mGluR subtype-selective agonists. Since L-AP4 had no target cell specific action, we examined the effects of subtype-specific agonists on Aδ fiber-mediated transmission, in both EGFP- and non-EGFP expressing neurons. The specific mGluR4 allosteric potentiator, PHCCC was applied, which amplifies the effect of L-AP4 in brain slices (Abitbol et al. 2008; Jones et al. 2008; Marino et al. 2003a; Marino et al. 2003b; Valenti et al. 2005). We found that 1 µM of L-AP4 produce a small but discernable inhibition of Aδ fiber-evoked EPSCs amplitude (Figure 7A), and selected this concentration for testing the effect of PHCCC. However, PHCCC failed to potentiate the L-AP4 induced inhibition (L-AP4 1µM, to 85.5 ± 5.5 % of control, $n = 12$; 1 µM L-AP4 + 30 µM PHCCC, to 89.1 ± 3.6 % of control, $n = 6$; $P > 0.05$; Figure 7B). Then, we tested the effect of the specific mGluR7 allosteric agonist, AMN082, which produced a transient and reversible inhibition of Aδ fiber-evoked EPSCs amplitude in a few neurons (to 90.8 ± 4 % of
control, \( P < 0.05, n = 16, \) Figure 7C and D). This indicated that inhibition by L-AP4 may have at least partially
involved the activation of mGluR7.

DISCUSSION

In this study, we investigated the physiological properties of EGFP-expressing GABAergic neurons in SG of the
spinal cord, using GAD65-EGFP transgenic mice. EGFP-expressing neurons exhibited significantly different
characteristics, compared to non-EGFP-expressing neurons, in terms of firing patterns, membrane properties,
and primary afferent input properties. Activation of the group III mGluRs by L-AP4, transiently diminished the
amplitude of \( \alpha \delta \) fiber-evoked EPSCs, but did not induce notable changes in the amplitude of C fiber-evoked
EPSCs. In addition, the inhibitory effects of L-AP4 did not differ significantly between EGFP- and non-EGFP-
expressing neurons. This indicates that the modulation of group III mGluRs is not dependent on the type of
interneurons in SG, but it is dependent on the type of primary afferent inputs to the target neurons.

Physiological properties of GAD65-EGFP-expressing neurons

In these GAD65-EGFP transgenic mice, firing patterns between EGFP-expressing and non-EGFP-expressing
neurons were moderately different. Approximately 73% of the EGFP-expressing neurons in SG displayed a
tonic firing pattern, whereas, non-EGFP-expressing neurons more frequently displayed a delayed firing pattern,
indicating that these two groups of neurons have different input-output properties. Most of the non-EGFP-
expressing neurons possess \( K_\alpha \) channels, resulting in raised action potential thresholds and delayed firing (Grudt
and Perl 2002; Melnick 2009; Ruscheweyh and Sandkuhler 2002; Yoshimura and Jessell 1989). Recent studies
have investigated the relationship between firing properties and the putative functional role of dorsal horn
neurons. These studies suggest that tonic firing neurons act as inhibitory neurons, whereas delayed firing
neurons act as excitatory neurons (Balasubramanyan et al. 2006; Lu et al. 2007; Lu et al. 2009). This is
consistent with our observations that most EGFP-expressing GABAergic neurons displayed a tonic firing
pattern and a major portion of the non-EGFP-expressing neurons (presumably excitatory) exhibited a delayed
firing pattern. One previous study described how intrinsic membrane properties correlated with particular types
of cutaneous sensory afferent inputs (Lopez-Garcia and King 1994). Therefore, GAD65-EGFP transgenic mice
may provide a convenient and reliable tool for understanding how these functionally different dorsal horn
neurons relate to distinctive primary afferent inputs and contribute to the transmission of nociceptive information in the spinal dorsal horn network.

Immunohistochemical studies have demonstrated that the GABA-synthesizing enzyme isoforms, GAD65 and GAD67, appear colocalized in the spinal dorsal horn neurons and that the two enzymes' immunoreactive intensities differed in some neurons (Erlander and Tobin 1991; Mackie et al. 2003). In this study, a small portion (~5%) of the EGFP-expressing neurons exhibited an initial bursting firing pattern, and this firing pattern was reported in most of the GAD67-EGFP-expressing neurons (Heinke et al. 2004). This finding indicates that a subpopulation of GAD65-EGFP-expressing neurons may overlap with the subpopulation of GAD67-EGFP-expressing neurons, but we cannot exclude the possibility that substantial subpopulation may not overlap.

Heinke et al. (2004) demonstrated that, in GAD67-EGFP transgenic mice, about 35% of GABAergic neurons were identified by EGFP fluorescence in SG. In contrast, our study detected about 60% of GABAergic neurons as GAD65-EGFP-expressing neurons. Additionally, the firing patterns of these GAD65-EGFP-expressing neurons were distinct from those of GAD67-EGFP-expressing neurons. GAD65 and GAD67 are the products of different genes, they are predicted to have different functions (Erlander and Tobin 1991). Interestingly, although no study has yet established the exact mechanisms of disinhibition-induced hyperalgesia and allodynia, Moore et al. showed that, in the neuropathic pain model, the number of GAD65-expressing neurons decreased in the spinal cord, but the number of GAD67-expressing neurons were unaffected (Moore et al. 2002). Consequently, GAD65-GABAergic neurons may play an important role in neuropathic and inflammatory pain, and their role may be distinct from that of the GAD67-GABAergic neurons.

**Effects of L-AP4 on synaptic transmission**

In the spinal cord, thinly myelinated Aδ fibers mediate sharp and well-localized pain. In contrast, unmyelinated C fibers modulate dull and less localized pain. Our results show that group III mGluRs have differential effects on primary Aδ and C fiber-mediated synaptic transmissions. The group III mGluR agonist, L-AP4, significantly inhibited Aδ fiber-evoked EPSCs, but did not affect C fiber-evoked EPSCs. An increment of PPR accompanied this inhibition, and postsynaptic application of a general G-protein inhibitor did not abolish it, suggesting that this effect has a presynaptic mechanism. Many neuromodulators, such as baclofen, opioid, serotonin, norepinephrine, anandamide, and nociceptin, have previously shown these primary afferent-dependent
In vivo studies have revealed that the activation of group III mGluRs inhibits hyperalgesia and allodynia in inflammatory and neuropathic pain models but does not induce any changes under normal conditions (Chen and Pan 2005; Goudet et al. 2008). Our present study shows comparable inhibitory effects by group III mGluRs in EGFP- and non-EGFP-expressing neurons in vitro. This equal action, by group III mGluRs and upon both (presumed) inhibitory and excitatory neurons, may account for the lack of net effect by group III mGluRs under normal conditions. Therefore, further studies are needed to investigate the effects of group III mGluRs on inhibitory and excitatory neurons under conditions of pathological pain.

The subtypes involved in the group III mGluRs-induced presynaptic inhibition remain unclear. Electron microscopy data show that group III mGluR subtypes, mGluR4 and mGluR7, are expressed in the spinal dorsal horn. In particular, mGluR7 is expressed on the nociceptive primary afferent terminals (Azkue et al. 2001; Kinoshita et al. 1998; Li et al. 1997; Ohishi et al. 1995; Okamoto et al. 1994). In addition, previous studies have shown that intrathecal administration of PHCCC or AMN082 inhibits hyperalgesia and allodynia (Goudet et al. 2008; Osikowicz et al. 2008). In this study, we examined the effects of AMN082 and PHCCC on synaptic transmission from primary afferent to SG neurons, to dissect out each subtype of group III mGluR in the spinal cord. AMN082, an allosteric agonist of mGluR7, transiently depressed the amplitude of A\(\delta\) fiber-evoked EPSCs, although the extent of this depression was less than that produced by L-AP4. De Rover et al. reported that AMN082 reduces glutamate release. However, the extent of the inhibition was less than that produced by L-AP4, which corresponds with our findings (de Rover et al. 2008). Moreover, PHCCC, an mGluR4 allosteric potentiator, did not significantly potentiate the L-AP4 induced inhibition. Thus, mGluR7, but not mGluR4, may be involved in inhibition by L-AP4 in synaptic transmission from primary afferent to SG neurons.

Therefore, we suggest that activation of group III mGluRs suppresses A\(\delta\) fiber-mediated excitatory synaptic transmission to the spinal cord but does not affect C fiber-mediated transmission. Furthermore, no target neuron-specific role was exhibited in the GAD65-EGFP transgenic mice. We expect that there may be a primary afferent-specific modulation of group III mGluRs in the spinal neural circuits for the management of pain.

ACKNOWLEDGEMENTS

We would like to express our gratitude to Ji Young Kim, Sung Won Hur and Tom Sanderson for their excellent
assistance in editing the manuscript. Thanks to Dr. Yun Hwa Hong for her kind help with the immunostaining.

This work was supported by the Center of Excellence Program of the Korea Institute of Science and Technology (2E21510-10-005), the Korea Research Foundation (2009-0080939) and WCU program (R32-10142).
REFERENCES

Abitbol K, Acher F, and Daniel H. Depression of excitatory transmission at PF-PC synapse by group III metabotropic glutamate receptors is provided exclusively by mGluR4 in the rodent cerebellar cortex. *J Neurochem* 105: 2069-2079, 2008.


de Rover M, Meyf FJ, and Ramakers GM. Presynaptic metabotropic glutamate receptors regulate glutamatergic input to dopamine neurons in the ventral tegmental area. *Neuroscience* 154: 1318-1323, 2008.


Jones PJ, Xiang Z, and Conn PJ. Metabotropic glutamate receptors mGluR4 and mGluR8 regulate transmission in the lateral olfactory tract-piriform cortex synapse. *Neuropharmacology* 55: 440-446, 2008.


Knopfel T, Kuhn R, and Allgeier H. Metabotropic glutamate receptors: novel targets for drug development. *J...
452 Macek TA, Winder DG, Gereau RWt, Ladd CO, and Conn PJ. Differential involvement of group II and group III mGluRs as autoreceptors at lateral and medial perforant path synapses. *J Neurophysiol* 76: 3798-3806, 1996.
454 Malan TP, Mata HP, and Porreca F. Spinal GABA(A) and GABA(B) receptor pharmacology in a rat model of neuropathic pain. *Anesthesiology* 96: 1161-1167, 2002.
463 Neugebauer V, Zinebi F, Russell R, Gallagher JP, and Shinnick-Gallagher P. Cocaine and kindling alter the sensitivity of group II and III metabotropic glutamate receptors in the central amygdala. *J Neurophysiol* 84:


Legends for Figures

Figure 1. Distribution of EGFP-expressing neurons and immunolocalization of GABA-immunopositive neurons in GAD65-EGFP transgenic mice

A: Photomicrograph of a transverse spinal cord slice shows that the EGFP-expressing neurons are highly distributed in laminae I-III, and moderately distributed in laminae IV-VI and X. The white box in the superficial lamina indicates the area magnified in B, C, and D. B: EGFP-expressing neurons, shown at a higher magnification. C: Immunostaining for GABA. D: Colocalization of EGFP-expressing neurons and GABA-immunopositive neurons.

Figure 2. Firing patterns of EGFP- and non-EGFP-expressing neurons in SG of GAD65-EGFP transgenic mice

Firing patterns were determined via rectangular step-current injections in the current clamp mode (holding potential of current injection, −85 mV). The EGFP-expressing neurons displayed a tonic firing pattern (A), delayed firing pattern (B), gap firing pattern (C), initial bursting firing pattern (D), bursting firing pattern (E), and phasic firing pattern (F). The bottom traces indicate injected currents. G: In the EGFP-expressing neurons, 73% displayed a tonic firing pattern, and 4%, 12%, 5%, 3%, and 3% of neurons exhibited a delayed firing pattern, gap firing pattern, initial bursting firing pattern, bursting firing pattern, and phasic firing pattern, respectively. H: In non-EGFP-expressing neurons, 53% of neurons showed a delayed firing pattern, and 17%, 17%, 8%, 2%, and 3% of neurons showed a tonic firing pattern, gap firing pattern, initial bursting firing pattern, bursting firing pattern, and phasic firing pattern, respectively.

Figure 3. A-type potassium (K_A) currents and hyperpolarization-activated currents in EGFP- and non-EGFP-expressing neurons.

A: This representative EGFP-expressing neuron, displaying a tonic firing pattern, did not show any current in 200 ms of depolarizing voltage steps (from −80 mV to −40 mV). Lower trace: voltage step protocol. B: An example of a non-EGFP-expressing neuron, displaying a delayed firing pattern and exhibiting voltage-dependent, rapidly activating and inactivating outward currents. Lower trace: voltage step protocol. C1-C2: Properties of A-type potassium (K_A) current in delayed firing neurons. Steady-state inactivation currents were
generated in response to voltage steps from increasingly depolarized holding potentials (from −100 to −40 mV, in 2 mV increments). Activation currents were generated by stepping the membrane potential (from −80 mV to −38 mV, in 2 mV increments) from a constant holding potential. Lower trace: voltage step protocol. The amplitude of the $K_A$ currents were determined by subtracting the steady-state current at the end of a 200 ms voltage pulse from the maximal current evoked by the voltage step. Activation (filled circles) and inactivation (open circles) of the $K_A$ current was normalized to the value recorded in a voltage step from −100 mV to −40 mV. Recovery of the $K_A$ current from inactivation was obtained by first stepping the membrane potential to 0 mV, then applying a hyperpolarizing voltage prepulse to −90 mV of varying duration (2 ms to 100 ms, 2 ms increments), and stepping back to 0 mV (n = 6). Lower trace: voltage protocol. D: Properties of hyperpolarization-activated currents in the EGFP- and non-EGFP-expressing neurons. Voltage steps (from −50 mV to −130 mV, in 10 mV decrements, 1 s duration) were applied in voltage clamp recording, and current versus voltage plots were taken from the beginning and end of each voltage step (n = 16). Holding potential: −70 mV. TTX (1 μM) was present during the voltage clamp recording.

Figure 4. Primary afferent inputs to the EGFP-expressing neurons in SG

The EGFP-expressing neurons received various primary afferent input patterns, with superimposition of 20 or 10 traces of evoked EPSCs induced by dorsal root stimulation, at 20 Hz or 1 Hz, respectively. A: Monosynaptic $A_\delta$ fiber-evoked EPSCs (CV = 2.9 m/s). B: Polysynaptic $A_\delta$ fiber-evoked EPSCs (CV = 2.1 m/s). C: Monosynaptic C fiber-evoked EPSCs (CV = 0.3 m/s). D: Polysynaptic C fiber-evoked EPSCs (CV = 0.1 m/s). E: Monosynaptic $A_\delta$ (CV = 3.2 m/s) and monosynaptic C fiber-evoked EPSCs (CV = 0.3 m/s). F: Polysynaptic $A_\delta$ (CV = 2.7 m/s) and monosynaptic C fiber-evoked EPSCs (CV = 0.3 m/s). All scale bars are 100 pA and 5 ms.

Figure 5. Effects of a group III mGluRs agonist on excitatory synaptic transmission to EGFP- and non-EGFP-expressing neurons

A: In EGFP-expressing neurons, a 10-min application of a selective group III mGluR agonist, L-AP4, at 20 μM depressed the amplitude of $A_\delta$ fiber-evoked EPSCs. The time course shows the depression effect is transient (to 58.6 ± 7.9 % of control, $P < 0.05$, n = 11). Paired pulse ratio (PPR) also increased ($P < 0.05$; gray trace, control;
black trace, L-AP4). B: In non-EGFP-expressing neurons, the amplitude of Aδ fiber-evoked EPSCs was also attenuated by L-AP4 (to 59.4 ± 6.3 % of control, \( P < 0.001, n = 9 \)). C: Application of L-AP4 did not induce significant changes in the amplitude of C fiber-evoked EPSCs (to 91.0 ± 4.4 % of control, \( P > 0.05, n = 6 \)). D: In the non-EGFP-expressing neurons, similar to the EGFP-expressing neurons, showed no significant changes by L-AP4 in the amplitude of C fiber-evoked EPSCs (to 95.8 ± 4.8 % of control, \( P > 0.05, n = 5 \)). E: The effects of L-AP4 did not differ significantly between the EGFP- and non-EGFP-expressing neurons in the amplitude of Aδ and C fiber-evoked EPSCs (\( P > 0.05 \)).

Figure 6. The effects of a group III mGluR agonist on Aδ fiber-induced excitatory synaptic transmission to EGFP- and non-EGFP-expressing neurons were blocked by an antagonist but were unaffected by a postsynaptic G-protein inhibitor.

A: In the Aδ fiber-evoked EPSCs, the inhibition effects of a group III agonist, L-AP4, at 20 \( \mu \)M for 5 min, were blocked by a 5 min pretreatment with an antagonist, CPPG, at 100 \( \mu \)M. B: The bar graph shows the summary (CPPG, 89.3 ± 3.7 % of control, CPPG + L-AP4, 84.1 ± 4.9 % of control, \( P > 0.05, n = 6 \)). C: The amplitude of Aδ fiber-evoked EPSCs, recorded with the internal solution containing a G-protein inhibitor, GDP-beta-S (1 mM), was transiently depressed by application of L-AP4 (20 \( \mu \)M). D: The inhibitory effects of L-AP4 did not differ significantly between the control (K-gluconate) internal solution and the GDP-beta-S-containing internal solution (K-gluconate, to 58.6 ± 7.9 % of control, \( n = 11 \); K-gluconate + GDP-beta-S, to 40.7 ± 6.7% of control, \( n = 5, P > 0.05 \)).

Figure 7. The effects of group III mGluR subtypes on Aδ fiber-induced excitatory synaptic transmission to EGFP- and non-EGFP-expressing neurons

A: Dose-response relationship for L-AP4 inhibition of the amplitude of Aδ fiber-evoked EPSCs in EGFP- and non-EGFP-expressing neurons. The concentration that produced a half-maximal inhibition of Aδ fiber-evoked EPSCs was 1.1 \( \mu \)M (\( n = 4 \)-12 cells for each concentration). B: The bar graph shows the inhibition by L-AP4 was not significantly potentiated in the presence of the mGluR4-specific positive allosteric modulator, PHCCC (L-AP4 1\( \mu \)M, to 85.5 ± 5.5 % of control, \( n = 12 \); 1 \( \mu \)M L-AP4 + 30 \( \mu \)M PHCCC, to 89.1 ± 3.6 % of control, \( n = 6 \); \( P > 0.05 \)). C: An example showing the time course of the Aδ fiber-evoked EPSCs amplitude mediated by the
mGluR7 specific allosteric agonist, AMN082 (10 μM), and L-AP4 (20 μM). The transient and reversible suppression effect of AMN082 (to 38.0% of control) was less than that seen with L-AP4 (to 12.5% of control). The first trace shows superimposed monosynaptic Aδ fiber-evoked EPSCs elicited by stimulation of the dorsal root at 20 Hz; the latency was constant. The remaining traces show before and during AMN082 and L-AP4 applications (arrows indicate monosynaptic Aδ fiber-evoked EPSCs). D: AMN082 induced pronounced suppression of Aδ fiber-evoked EPSCs in a few neurons (90.8 ± 4% of control, $P < 0.05$, n = 16).
Figure 2

A  Tonic firing       B  Delayed firing       C  Gap firing       D  Initial bursting       E  Bursting       F  Phasic firing

EGFP-expressing neurons (n=67)

Non-EGFP-expressing neurons (n=64)

G

H

Proportion of firing patterns (%)
Figure 3

A  EGFP-expressing non-EGFP-expressing

B  non-EGFP-expressing

C1  C2

D

Normalized current

Relative current

Voltage (mV)

Prepulse duration (ms)

Current (pA)

Voltage (mV)

Begin  End

-300
Figure 4

A  Monosynaptic A\(\delta\)  
   20 Hz

B  Polysynaptic A\(\delta\)  
   20 Hz

C  Monosynaptic C  
   1 Hz

D  Polysynaptic C  
   1 Hz

E  Monosynaptic A\(\delta\)  
   \& Monosynaptic C  
   20 Hz

F  Polysynaptic A\(\delta\)  
   \& Monosynaptic C  
   20 Hz
Figure 5

A δ fiber-evoked EPSC
EGFP-expressing

Control  L-AP4

50 pA 20 ms

L-AP4

eEPSC amplitude (%)

Time (min)

PPR (%)

Control  L-AP4

B δ fiber-evoked EPSC
Non-EGFP-expressing

Control  L-AP4

50 pA 20 ms

L-AP4

eEPSC amplitude (%)

Time (min)

PPR (%)

Control  L-AP4

C fiber-evoked EPSC
EGFP-expressing

200 pA 20 ms

L-AP4

eEPSC amplitude (%)

Time (min)

D fiber-evoked EPSC
Non-EGFP-expressing

200 pA 20 ms

L-AP4

eEPSC amplitude (%)

Time (min)

E

eEPSC amplitude (%)

EGFP Non-EGFP  EGFP Non-EGFP

Aδ  C

n.s.  n.s.

*  *  

n.s.
Figure 6

A. Graph showing the eEPSC amplitude (%) over time (min) with the application of L-AP4 and CPPG.

B. Bar graph comparing eEPSC amplitude (%) in Control, CPPG, and CPPG + L-AP4 treatments. The bars are labeled with n.s. indicating no significant difference.

C. Graph showing the eEPSC amplitude (%) over time (min) with the application of GDP-beta-S internal and L-AP4.

D. Bar graph comparing eEPSC amplitude (%) in K-gluconate and K-gluconate + GDP-beta-s treatments.
### TABLE 1. Membrane properties of EGFP- and non-EGFP-expressing neurons in SG

<table>
<thead>
<tr>
<th></th>
<th>EGFP-expressing Neurons (n=55)</th>
<th>Non-EGFP-expressing Neurons (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential (mV)</td>
<td>-61±1*</td>
<td>-56±1</td>
</tr>
<tr>
<td>Membrane resistance (MΩ)</td>
<td>805±47</td>
<td>711±44</td>
</tr>
<tr>
<td>Membrane capacitance (pF)</td>
<td>61±4*</td>
<td>39±3</td>
</tr>
<tr>
<td>Action potential threshold (mV)</td>
<td>-36±1*</td>
<td>-30±1</td>
</tr>
<tr>
<td>Action potential threshold – RMP (mV)</td>
<td>25±1</td>
<td>26±1</td>
</tr>
<tr>
<td>Action potential width at base (ms)</td>
<td>1.3±0.1</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Action potential height from base (mV)</td>
<td>79±1*</td>
<td>61±1</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>39±5*</td>
<td>134±12</td>
</tr>
<tr>
<td>KA current (%)</td>
<td>34.5% (19/55)*</td>
<td>70.7% (29/41)</td>
</tr>
<tr>
<td>Hyperpolarization-activated current (%)</td>
<td>45.5% (25/55)</td>
<td>36.6% (15/41)</td>
</tr>
</tbody>
</table>

Significance of difference between EGFP- and non-EGFP-expressing neurons is indicated by * (P <0.05).
**TABLE 2. Frequency of the primary afferent inputs to EGFP- and non-EGFP-expressing neurons in SG**

<table>
<thead>
<tr>
<th>Type of primary afferent input</th>
<th>EGFP-expressing neurons (n=190)</th>
<th>Non-EGFP-expressing neurons (n=139)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monosynaptic Aδ</td>
<td>80 (42.1 %) *</td>
<td>34 (27.3 %)</td>
</tr>
<tr>
<td>Polysynaptic Aδ</td>
<td>51 (26.8 %)</td>
<td>41 (38.1 %)</td>
</tr>
<tr>
<td>Monosynaptic C</td>
<td>81 (42.6 %) *</td>
<td>15 (19.4 %)</td>
</tr>
<tr>
<td>Polysynaptic C</td>
<td>23 (12.1 %) *</td>
<td>30 (23.0 %)</td>
</tr>
<tr>
<td>Convergent Aδ and C</td>
<td>40 (21.1 %)</td>
<td>19 (13.7 %)</td>
</tr>
<tr>
<td>Total monosynaptic</td>
<td>161 (84.7 %) *</td>
<td>49 (35.3 %)</td>
</tr>
<tr>
<td>Total polysynaptic</td>
<td>74 (38.9 %) *</td>
<td>71 (51.1 %)</td>
</tr>
</tbody>
</table>

Statistical significance of difference is indicated by * (P < 0.05).