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Activation of group I mGlu receptors contributes to facilitation of NMDA receptor membrane current in spinal dorsal horn neurons after hind paw inflammation in rats

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Abstract

The interaction between the group I metabotropic glutamate (mGlu) receptors and *N*-methyl-D-aspartate (NMDA) receptors plays a critical role in spinal hyperexcitability and hyperalgesia. The cellular mechanisms underlying this interaction remain unknown. Utilizing an *ex vivo* spinal slice preparation from young adult rats, we investigated the group I mGlu receptor modulation of NMDA receptor-mediated current in superficial dorsal horn neurons by patch clamp recording after complete Freund's adjuvant (CFA)-induced hind paw inflammation. We show that NMDA receptor-mediated dorsal root stimulation-evoked EPSC (eEPSC) and NMDA-induced current was enhanced in the inflamed rats, compared to naïve rats and this effect was attenuated by AIDA (1 mM), a group I mGlu receptor antagonist. There were also increases in the frequency and amplitude of miniature excitatory postsynaptic currents in the presence of tetrodotoxin, suggesting enhanced presynaptic glutamate release probability and postsynaptic membrane responsiveness in inflamed rats. DHPG (10 μ M), a selective group I mGlu receptor agonist, further facilitated NMDA receptor-mediated eEPSC and NMDA-induced current in inflamed rats. The DHPG-produced facilitation of NMDA-induced current was blocked by intracellular dialysis of GDP-beta-S (1 mM), a G protein antagonist, and BAPTA (15 mM), an intracellular calcium chelating agent; and by pretreatment with U73,122 (10 μ M), a PLC inhibitor, or 2-APB (100 μ M), an IP₃-receptor antagonist. These findings support the hypothesis that signal transduction coupling between group I mGlu receptors and NMDA receptors underlies the activation of NMDA receptors in spinal hyperexcitability and hyperalgesia.

Keywords

N-methyl-D-aspartate receptors; Metabotropic glutamate receptors; Spinal cord slice; Electrophysiology; Pain; Hyperexcitability

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1. Introduction

Metabotropic glutamate (mGlu) receptors, a family of G protein-linked receptors, are classified into group I (mGlu1,5 receptors), II (mGlu2,3 receptors), and III (mGlu4,6,7,8 receptors) based on their signal transduction pathways, pharmacology profiles, and sequence homology (Conn and Pin, 1997). While groups II and III mGlu receptors are dominantly located presynaptically, group I mGlu receptors are expressed primarily on postsynaptic membranes, although a presynaptic localization has also been observed (Jia et al., 1999; Neugebauer 2002; Shigemoto et al., 1997). Group I mGlu receptors are distributed in the superficial spinal dorsal horn (Berthele et al., 1999; Guo et al., 2004), and implicated in a variety of pain conditions (Adwanikar et al., 2004; Guo et al., 2004; Neugebauer, 2002; Scotland and Coderre, 2007). It has been well established that the *N*-methyl-D-aspartate (NMDA) receptor, a subtype of ionotropic glutamate receptors, plays an important role in synaptic plasticity, dorsal horn hyperexcitability and hyperalgesia (Dubner and Ruda, 1992; Guo and Huang, 2001; Ren et al., 1992; Woolf and Thompson, 1991).

Studies suggest that interactions between the mGlu and NMDA receptors underlie the mechanism of modulation of neuronal plasticity (Alagarsamy et al., 2001; Guo et al., 2004; Millan, 1999). In CNS neurons, NMDA receptors are linked to mGlu receptors in the postsynaptic membrane (Guo et al., 2004; Naisbitt et al., 1999). Activation of mGlu receptors enhances the effects of ionotropic glutamate receptor function (Budai and Larson, 1998; Neugebauer, 2002; Stanfa and Dickenson, 1998). Previous studies have shown that mGlu receptor agonists enhance NMDA-mediated increase in intracellular calcium concentration and NMDA-induced currents in dorsal horn neurons (Bleakman et al., 1992; Bond and Lodge, 1995; Cerne and Randić, 1992). The response to mGlu receptor agonists is potentiated in the spinal cord in hyperalgesic but not naive animals, and this effect is reversed by an NMDA receptor antagonist (Boxall et al., 1998). Intrathecal administration of the mGlu1,5 receptor agonist (S)-3,5-DHPG in mice produced hyperalgesia that was attenuated by NMDA receptor antagonists and silencing of the NR2B subunit of the NMDA receptor by RNA interference (Gabra et al., 2007). We have shown that a group I mGlu receptor-NMDA receptor coupling cascade leads to phosphorylation of the NMDA receptor and initiation of spinal dorsal horn hyperexcitability and behavioral hyperalgesia after inflammation (Guo et al., 2004).

Despite converging evidence, the intracellular signaling mechanisms underlying the interaction between mGlu and NMDA receptors in inflammatory pain are still largely unknown. In the present study we performed *ex vivo* patch clamp recordings from spinal dorsal horn neurons in young adult rats to directly assess the intracellular mechanisms involved in the modulation of NMDA receptor-mediated current by inflammation and group I mGlu receptor activation. The results show that NMDA receptor-mediated membrane current was enhanced in complete Freund's adjuvant (CFA)-inflamed rats and this effect was attenuated by antagonism of group I mGlu receptors. DHPG, a selective agonist of group I mGlu receptors, further facilitated NMDA receptor current in inflamed rats and the mechanisms underlying this facilitation involved postsynaptic intracellular G protein, the PLC pathway and IP₃-sensitive Ca²⁺ release.

2. Materials and methods

2.1. Inflammation pain model

The method was described elsewhere (Guo et al., 2002, 2004). Briefly, young adult male Sprague Dawley rats of 28–35-day-old were used in all experiments. Inflammation was induced by injecting CFA (50 µl, 25 µg of *Mycobacterium tuberculosis*; Sigma, St. Louis, USA) suspended in an oil-saline (1:1) emulsion into one hind paw. The CFA treatment

protocol reliably led to inflammation of the injected hind paw and resulted in significantly decreased paw withdrawal latencies to a noxious thermal stimulus and decreased thresholds to mechanical stimuli compared to that in control animals (Guo et al., 2002, 2004; Guo and Huang, 2001; Iadarola et al., 1988). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland Dental School.

2.2. Slice preparation

Transverse slices of 300–600- μ m thickness were cut from the L4–5 spinal level in all rats. The methods used to prepare spinal cord slices and to analyze synaptic transmission were similar to those described elsewhere (Yang et al., 2000, 2001). Briefly, 24 h after CFA or saline injection, rats were anesthetized with pentobarbital sodium solution (50 mg/kg i.p.), and a laminectomy was performed to extract lumbosacral spinal cord segments. The spinal cord was quickly immersed in ice-cold high-sucrose solution containing (in mM): 50 sucrose, 95 NaCl, 3.6 KCl, 0.5 CaCl_2 , 1.2 NaH_2PO_4 , 6 MgCl_2 , 26 NaHCO_3 , and 12 glucose, bubbled with mixed gas of 95% O_2 –5% CO_2 . Dorsal and ventral roots were carefully cut except in some slices where associated dorsal roots were kept for dorsal root stimulation. The cutting solution for thick (600- μ m) dorsal root-attached slices was modified to (in mM): 206 sucrose, 2.8 KCl, 1.0 CaCl_2 , 1 MgCl_2 , 2 MgSO_4 , 1.25 NaH_2PO_4 , 26 NaHCO_3 , 10 Glucose, and 0.4 ascorbic acid. The pia-arachnoid membrane was removed. The spinal cord trunk was mounted on a microslicer (Integraslice 7550, Campden Instruments, UK). The slices were then quickly transferred to a mixed gas-bubbled external solution containing (in mM): 126 NaCl, 26 NaHCO_3 , 10 glucose, 2.5 KCl, 2 CaCl_2 , 1.2 MgCl_2 , and 1.25 NaH_2PO_4 in an incubation beaker at 35°C–36°C for 1 h recovery before recordings.

2.3. Whole-cell Patch clamp recordings

After incubation, a slice was transferred to a 0.8-ml recording chamber and quickly perfused (10–15 ml/min) with external solution. Recordings were made at room temperature for thin slices (300 μ m) and 36 $\pm$ 1°C for thick slices (600 μ m). Superficial dorsal horn neurons were visualized using infrared Nomarski optics on an upright microscope (E600FN, Nikon, Japan). Blind whole cell patch-clamp recordings were performed in experiments involving dorsal root stimulation. Whole-cell voltage-clamp recordings were performed with series resistance compensation using an EPC 10 amplifier (Heka, Germany) with the above mentioned external solution (pH 7.3, 295–305 mOsm). The holding potential was –70 mV to maintain physiological conditions. To reveal NMDA receptor-mediated EPSC (small outward synaptic current) by dorsal root stimulation, the cell was depolarized to +40mV. To exclude Mg^{2+} -dependent block of NMDA-gated channels (Chen and Huang, 1992; Mayer et al., 1984), Na^+ was substituted for Mg^{2+} when recording NMDA-induced current unless otherwise indicated. In both control and CFA groups, recordings were performed from dorsal horn ipsilateral to CFA or saline injection. In dorsal root stimulation experiments, recording pipettes were filled with an internal solution containing (in mM) 110 Cs_2SO_4 , 0.5 CaCl_2 , 2 MgCl_2 , 5 tetraethylammonium, 5 EGTA, 2 $\text{Na}_2\text{-ATP}$, 0.5 $\text{Na}_2\text{-GTP}$, and 5 HEPES, and had a resistance of 8–12 M Ω . In other experiments, recording pipettes were filled with an internal solution containing (in mM) 130 K-gluconate, 20 KCl, 2 MgCl_2 , 0.5 EGTA, 2 $\text{Na}_2\text{-ATP}$, 0.5 $\text{Na}_2\text{-GTP}$, and 10 HEPES, and had a resistance of 5–8 M Ω .

To examine postsynaptic-intracellular signal pathways, an alternating two-pipette recording protocol was used, which allows changing the pipette solution while recording from the same cell (Sekizawa and Boham, 2006). For the first part of the two-pipette protocol, conventional whole-cell recording was conducted with the 1st pipette containing normal solution. After completing the 1st recording, the recording pipette was removed and the neuron under study was viewed under infrared differential interference contrast. Only those neurons with basic sharp images and without obvious swelling (by visual check) were

selected for the 2nd pipette recording. A total of 43/133 (32%) checked neurons met these criteria. Under the two recording conditions, pipette resistance, resting membrane potential and NMDA-induced current were all similar (fluctuation < 15%). In the second pipette, Na-GTP was replaced by 1 mM of the nonhydrolyzable guanosine-5-O-(2-thiodiphosphate) analog, GDP-beta-S, to determine the involvement of G-proteins (Yang et al., 2000). To examine the role of intracellular Ca_{2+} , 15 mM BAPTA was included in the second pipette. All internal solutions were adjusted to 290 mOsm, pH 7.2 with KOH.

Drugs were applied by switching the perfusion solution to one containing the drug at a known concentration using a three-way tap. The signals were digitized at 50 kHz, and stored on a personal computer using Pulse V8.76 data acquisition program (Heka). The resting membrane potential was measured immediately after establishing the whole-cell configuration. Input resistance of each neuron was measured from the reaction to hyperpolarizing voltage steps (100 ms, from -70 to 80 mV). NMDA induced a reversible slow inward (excitatory) membrane current in all neurons tested (Rusin et al., 1993). The amplitude of the membrane current was measured from the baseline at the onset of the drug to the peak of the response. Percentage of facilitation of the NMDA-induced current was defined as the following: % facilitation = $(\text{Peak}_{\text{NMDA plus drug}} - \text{Peak}_{\text{NMDA}}) \times 100 / \text{Peak}_{\text{NMDA}}$. Some neurons showed rundown of NMDA current during the recordings (Garraway et al., 2003). Neurons with rundown of NMDA current larger than 20% during recording were not used for further testing.

To produce synaptic activation, dorsal rootlets were stimulated with suction stimulating electrodes (0.4 ms pulse width, 0.06–1 mA, 0.2–20 Hz). The length of dorsal root between the site of stimulation and dorsal horn was 0.5–1.5 cm, which was used to estimate conduction velocity of nerve fibers (Yang et al., 2001). In all voltage-clamp recordings involving application of NMDA, 0.5–1 μM tetrodotoxin was routinely included in the bath solution to block action potential-dependent synaptic transmission. The glycine receptor antagonist strychnine (1 μM) and the GABA_A receptor antagonist bicuculline (10 μM) were also included in the external solution. The AMPA/KA receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 10 μM) was also applied in some experiments to verify NMDA-induced current.

2.4. Drugs and data analysis

DHPG [(S)-3,5-dihydroxyphenylglycine] was purchased from Tocris, USA. AIDA (1-Amino-2,3-dihydro-1H-indene-1,5-dicarboxylic acid), 2-APB (2-Aninoethoxydiphenylborate), AP-5 [(±)-2-amino-5-phosphonopentanoic acid], BAPTA [1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid], baclofen, NMDA, CNQX, tetrodotoxin, GDP-beta-S, U-73,122, and U-73,343 were purchased from Sigma, USA. All drugs, except intracellular chemicals (GDP-beta-S and BAPTA) and tetrodotoxin, were first dissolved in dimethyl sulfoxide (DMSO) at 1000 times of the final concentration as stock solution, respectively, and then diluted to the final concentration in external solution immediately before use. Tetrodotoxin was firstly dissolved in distilled water at 1000 times the final concentration. In control experiments, drugs dissolved in DMSO *per se* did not have any effects on either synaptic transmission or postsynaptic membrane (data not shown, see Yang et al., 2000).

Data were analyzed with SigmaPlot 10.0 (Systat, USA) or Axograph 4.0 (Axon Instruments, USA). The results are expressed as mean $\pm$ S.E.M., and statistical significance was set at $P < 0.05$ using ANOVA with *post hoc* comparisons, Student's *t*-test or Kolmogorov-Smirnov (K-S) test (Yang et al., 2004).

3. Results

All neurons were recorded from laminae I/II of the superficial dorsal horn. Only neurons with resting membrane potentials more negative than -50 mV and exhibiting glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs) were included in the analysis.

3.1. CFA inflammation induces an enhanced NMDA receptor responses in spinal dorsal horn neurons

In the dorsal root attached preparation, electrical stimulation at suprathreshold intensity evoked inward membrane current, or eEPSC, in dorsal horn neurons (holding potential = -70 mV). The evoked responses were stimulation intensity-dependent as shown by graded electrical stimulation (Fig. 1A). eEPSC was abolished when tetrodotoxin (1 μ M) was added to the bath. NMDA receptor mediated synaptic current, or eEPSC, was obtained by depolarizing the cell to $+40$ mV and verified. As shown in Fig. 1B, dorsal root stimulation evoked current in the presence of CNQX (10 μ M), strychnine (1 μ M), and bicuculline (10 μ M), but was abolished when AP-5 (0.02 – 1 mM) was added to bath. The NMDA receptor-mediated eEPSC was also stimulation intensity-dependent (Fig. 1B). In rats with CFA-induced hind paw inflammation, the evoked NMDA receptor current was significantly enhanced with 300 – 600 μ A stimulation when compared to control rats ($P < 0.01$, $n = 8$) (Fig. 1B).

Similar results were obtained with the NMDA receptor agonist. As illustrated in Fig. 2A, in voltage-clamp recording mode (Holding potential = -70 mV), bath application of NMDA (15 μ M, 30 s) induced a transient and stable slow inward membrane current, and this depolarization was reversible and reproducible. In naive group neurons in “normal” external solution that contained 1.2 mM Mg^{2+} , the amplitude of the NMDA-induced inward slow membrane current was small (10 ± 2 pA, $n = 14$) (Fig. 2A). When magnesium was removed from the bathing solution to exclude the Mg^{2+} -dependent block of NMDA channels (Chen and Huang, 1992; Mayer et al., 1984), the amplitude of the NMDA current expectedly increased to 53 ± 17 pA ($n = 4$, $P < 0.05$, unpaired t -test). The average amplitude of the membrane current in the CFA group was 24 ± 5 pA in 1.2 mM Mg^{2+} solution and 128 ± 17 pA in 0 Mg^{2+} solution ($n = 12$), significantly higher than that of the naive group ($P < 0.01$) (Fig. 2A). Pretreatment with a selective NMDA receptor antagonist, AP-5 (0.02 – 1 mM, 8 min), blocked the NMDA-induced inward current, confirming that it was mediated by the NMDA receptor (Fig. 2B).

The NMDA responses were concentration-dependent. Fig. 2C shows representative dose-dependent NMDA responses of a neuron in a CFA-inflamed rat. The graph in Fig. 2C (right) summarizes neuronal responses to NMDA in the control and CFA groups ($n = 5$ for each group). The amplitude of NMDA-induced current was significantly increased in the CFA group at 1 – 500 μ M ($P < 0.01$ – 0.05 , ANOVA).

The increased NMDA-induced current prompted us to assess the presynaptic glutamate release by comparing the frequency and amplitude of miniature EPSCs (mEPSCs) recorded in the presence of tetrodotoxin (0.5 μ M), Mg^{2+} (1.2 mM), strychnine (1 μ M), and bicuculline (10 μ M) at a holding potential of -70 mV (Yang et al., 2000). As shown in Fig. 3A, B, both average frequency and amplitude of mEPSCs were increased in the CFA group, from 2.0 ± 0.2 Hz and 18.2 ± 2.5 pA to 3.6 ± 0.5 Hz and 31.0 ± 4.1 pA, in naive ($n = 6$) and CFA ($n = 7$) groups, respectively ($P < 0.05$) (K-S test, Yang et al., 2004). These results suggest that CFA inflammation enhanced presynaptic glutamate release probability and postsynaptic membrane responsiveness. However, when the cells' passive membrane properties were compared between the CFA and control groups, neither resting membrane potential nor input resistance show significant differences (Fig. 3C). The average resting

membrane potential and input resistance were -60.6 ± 0.9 mV and 554 ± 41 M Ω in the naive group ($n = 10$), and -58.5 ± 0.8 mV and 504 ± 34 M Ω in the CFA group ($n = 12$), respectively ($P = 0.10$ for resting membrane potential; $P = 0.35$ for input resistance; Fig. 3C). These results suggest that factors other than passive membrane properties contribute to the increased membrane responsiveness after inflammation.

3.2. Group I mGlu receptor antagonist produces selective reduction of NMDA-induced current in inflamed rats

We have previously shown that group I, but not groups II or III mGlu receptors interacted with NMDA receptors after inflammation to facilitate NR2B subunit phosphorylation and behavioral hyperalgesia (Guo et al., 2004). Both mGlu1 and mGlu5 receptor subtypes contributed to inflammation-induced NR2B phosphorylation and hyperalgesia (Guo et al., 2004). Thus, we next examined the role of group I mGlu receptors in NMDA receptor currents in CFA-inflamed rats and compared the results with that of the naive controls. NMDA receptor mediated eEPSC was induced by dorsal root stimulation (300 μ A) in the presence of CNQX (10 μ M), strychnine (1 μ M), and bicuculline (10 μ M) under holding potential of +40 mV. As shown in Fig. 4A, in the CFA rat, the amplitude of NMDA receptor-mediated synaptic current was reduced in the presence of 1 mM AIDA (8 min pretreatment), a group I mGlu receptor antagonist. On the contrary, AIDA did not have an effect on the amplitude of eEPSC in the naive control group. Fig. 4B summarizes the results from the two groups. The results from individual neurons show that NMDA receptor-mediated eEPSC were reduced in all six neurons in the CFA group in the presence of AIDA (Fig. 4B left). The amplitude of eEPSC was significantly decreased in the CFA group when compared to the pre-AIDA value ($n = 6$, $P < 0.01$, paired t -test). In the naive control group, however, the amplitude of NMDA current was not affected by AIDA ($n = 6$).

The amplitude of NMDA (10 μ M)-induced inward current was also reduced in the presence of 1 mM AIDA (8 min pretreatment) in the CFA rat (Holding potential = -70 mV) (Fig. 5A). AIDA had little effect on the amplitude of NMDA current of neurons in the naive control group. Fig. 5B summarizes the results from the two groups: the average NMDA current in the CFA group was decreased from 73 ± 12 pA to 53 ± 11 pA, which was $72 \pm 8\%$ of the pre-AIDA value ($n = 5$, $P < 0.01$, paired t -test). In the naive control group, however, the amplitude of NMDA current was 29 ± 4 pA (without AIDA) and 28 ± 4 pA (in the presence of AIDA), which was $98 \pm 2\%$ of pre-AIDA value ($n = 6$, $P = 0.40$, paired t -test). These results suggest that group I mGlu receptors are activated after inflammation, supported by potentiation of action potential-independent mEPSCs in the CFA group (Fig. 3), and contribute to CFA-induced enhancement of NMDA receptor current.

3.3. Postsynaptic activation of group I mGlu receptors facilitates NMDA receptor current

We then examined whether direct activation of group I mGlu receptors could facilitate the effect of inflammation on NMDA receptor channel function. At the holding potential of -70 mV, bath application of a selective group I mGlu receptor agonist DHPG alone did not have an effect on membrane current at 1 μ M concentration. At 100 μ M concentration, DHPG produced a slow inward current with an increase in the frequency of mEPSCs, suggesting an increased presynaptic glutamate release. In 2 of 6 cells tested, bath application of DHPG at 10 μ M only produced a slight increase in the frequency of mEPSCs, as well as a small depolarization membrane current (5 pA and 8 pA) (data not shown, see Park et al., 2004). We subsequently applied DHPG at 10 μ M concentration to study its effect on NMDA receptor current.

In slices from rats with CFA-induced hind paw inflammation, DHPG significantly facilitated the dorsal root stimulation (300 μ A)-evoked NMDA receptor-mediated synaptic

current when compared to control ($P < 0.01$, $n = 8$) (Holding potential = +40 mV) (Fig. 6). DHPG also produced a slight increase in stimulation-evoked NMDA receptor synaptic current in naïve rats recorded under the same conditions ($P < 0.05$, $n = 9$) (Fig. 6B). Comparing the absolute increases in NMDA receptor-mediated eEPSC (Fig. 6B) indicates that DHPG produced a significantly larger increase in neurons from inflamed rats than naïve rats ($P < 0.01$).

Bath application of DHPG also facilitated NMDA-induced current. Fig. 7A illustrates a representative chart recording of the NMDA responses of dorsal horn neurons from a CFA-inflamed rat before, during and after treatment of DHPG (Holding potential = -70 mV). Pretreatment with DHPG (10 μ M, 8 min) led to an increase in the amplitude of NMDA (10 μ M)-induced membrane current (in Mg^{2+} -free solution). In the presence of DHPG, the average amplitude of NMDA-induced current was $129 \pm 8\%$ of the baseline ($n = 6$, $P < 0.01$, paired t -test). In the presence of AIDA (1 mM, 8 min pretreatment), the DHPG facilitation was significantly decreased to $105 \pm 3\%$ of the baseline ($n = 6$, $P < 0.05$) (not shown). These results indicate that the DHPG-produced enhancement of NMDA-induced current was mediated by group I mGlu receptor activation. DHPG also effectively facilitated NMDA-induced current to $131 \pm 11\%$ of control ($n = 6$, $P = 0.02$, paired t -test) in neurons from naïve rats. Since the NMDA receptor response in the absence of DHPG was significantly larger in neurons from inflamed rats, when compared to naïve rats (Figs. 1,2), the similar percent increases in NMDA-induced current during the DHPG treatment in the CFA and naïve groups do not necessarily indicate the same levels of facilitation. Comparing the absolute increases in peak membrane inward current (Fig. 7A, right) revealed that DHPG produced a significantly larger increase in NMDA-induced current in neurons from inflamed rats (19 ± 5 pA, $n = 6$) than naïve rats (8 ± 5 pA, $n = 6$) ($P < 0.05$). This result is consistent with that from dorsal root stimulation (Fig. 6B).

We further examined whether DHPG-produced facilitation of NMDA responses involved postsynaptic mGlu receptors by blocking intracellular G-proteins since mGlu receptors signal through G-protein-dependent pathways (Anwyl 1999; Neugebauer 2002). An alternating two-pipette recording protocol (Sekizawa and Boham, 2006) from the same cell was used with the first pipette containing normal solution and the second pipette including GDP-beta-S (1 mM), a non-hydrolyzable GDP analog and G-protein antagonist. The first electrode was used to confirm that the clamped neuron was a DHPG-facilitated cell. The second electrode was utilized to change intracellular components because the whole-cell configuration permits rapid intracellular dialysis of the clamped neurons with the pipette containing GDP-beta-S, as shown previously (see Methods in Yang et al., 2000).

Neurons showing similar NMDA current amplitude ($< \pm 15\%$ change) with the second electrode as compared to that of the NMDA response with the first recording electrode were used to test the effect of intracellular GDP-beta-S ($n = 5$). The recording conditions did not change between the two pipettes: the second pipette resistance ($102 \pm 4\%$), resting membrane potential ($102 \pm 3\%$), series resistance ($106 \pm 8\%$), and baseline current ($114 \pm 6\%$) were not significantly different from that of the initial pipette (Holding potential = -70 mV) ($P > 0.05$, $n = 5$). In these 5 neurons, NMDA-induced inward current was facilitated by DHPG pretreatment ($142 \pm 13\%$ of control, $P < 0.05$, paired t -test) with the first recording pipette. After 10~20 min of configuration establishment, the DHPG-produced facilitation of NMDA-induced current was assessed again with the GDP-beta-S containing pipette. As illustrated in Fig. 7B, the NMDA-induced slow current was not sensitive to DHPG after intracellular GDP-beta-S dialysis. In 5 neurons, the NMDA current was $105 \pm 1\%$ of baseline (paired t -test, $P > 0.05$). The effects of DHPG on NMDA-induced current were significantly different between the two recording conditions ($P < 0.05$). In the naïve group, DHPG facilitation of NMDA current was also prevented by intracellular GDP-beta-S

dialysis, from $158 \pm 14\%$ to $108 \pm 4\%$ ($n = 8$, $P < 0.05$). When recording with a control second pipette that contained a normal solution, DHPG produced a similar facilitation of NMDA-induced current ($n = 5$) (not shown). These observations indicate that the DHPG facilitation of NMDA-induced current was blocked by intracellular G protein inactivation, suggesting involvement of postsynaptic mGlu receptors.

3.4. Effect of PLC inhibitor on DHPG-produced facilitation

Group I mGlu receptors are coupled through Gq/11 proteins to activate PLC (Conn and Pin, 1997). We investigated whether the DHPG facilitation of the NMDA ($10 \mu\text{M}$)-induced current involved the intracellular PLC pathway. In CFA-inflamed rats, DHPG ($10 \mu\text{M}$) facilitated NMDA-induced current to $146 \pm 14\%$ of baseline ($n = 5$, $P < 0.05$) (Holding potential = -70 mV) (Fig. 8A, D). On the same neurons, pretreatment of U73,122 ($10 \mu\text{M}$), a membrane permeable PLC inhibitor, blocked DHPG action on NMDA current facilitation to $107 \pm 6\%$ of baseline (Fig. 8B, D). In slices pretreated with U73,343 ($10 \mu\text{M}$), an inactive enantiomer of U73,122, DHPG facilitated NMDA current ($151 \pm 11\%$ of baseline, $n = 4$) (Fig. 8C, D). Similar results were observed in the naive group ($n = 5$) (Fig. 8D).

3.5. DHPG facilitation of NMDA current depends on intracellular Ca^{2+}

NMDA receptor activation induces an increase of intracellular Ca^{2+} concentration either in normal neurons (MacDermott et al., 1986) or neurons under pain conditions (Isaev et al., 2000). Recent data support the view that activation of group I mGlu receptors induces a Ca^{2+} -gradient in the spinal dorsal horn (Heinke and Sandkühler, 2007). Our previous study indicates that group I mGlu receptor-NMDA receptor coupling involves IP_3 -sensitive intracellular Ca^{2+} release (Guo et al., 2004). We further studied the role of intracellular calcium in the DHPG facilitation of NMDA ($10 \mu\text{M}$)-induced current in dorsal horn neurons. As shown in Fig. 9A and C, in the CFA group, the DHPG pretreatment facilitated NMDA current ($120 \pm 3\%$ of baseline, $n = 5$, $P = 0.01$) (Holding potential = -70 mV). When the recording pipette was switched to include BAPTA (15 mM), an intracellular calcium chelator, the facilitation of NMDA current by DHPG was significantly reduced to $110 \pm 2\%$ of baseline ($n = 5$, $P < 0.05$ vs. DHPG-facilitation without BAPTA), suggesting that an increase in cytosolic Ca^{2+} was required for DHPG-produced facilitation. A similar effect was observed in the naive control group ($n = 5$) (Fig. 9C).

The IP_3 receptor-sensitive calcium release is involved in intracellular Ca^{2+} mobilization. When slices from CFA-inflamed rats were pretreated with an IP_3 -receptor antagonist, 2-APB ($100 \mu\text{M}$) (Maruyama et al., 1997), the DHPG facilitation of NMDA current was significantly reduced from $151 \pm 17\%$ to $108 \pm 3\%$ of baseline ($n = 4$, $P < 0.05$) (Fig. 9B, C). In the naive group, 2-APB decreased the DHPG-produced facilitation from $140 \pm 11\%$ to $111 \pm 5\%$ of baseline ($n = 4$, $P < 0.05$) (Fig. 9C).

4. Discussion

Utilizing an *ex vivo* spinal slice preparation from young adult rats, the present study addressed cellular mechanisms of group I mGlu receptor-NMDA receptor interaction after tissue injury. We show that NMDA receptor-mediated eEPSC and NMDA-induced current were significantly increased in the CFA-inflamed rats and this effect was attenuated with antagonism of group I mGlu receptors. There were also increases in the frequency and amplitude of action potential-independent mEPSCs in inflamed rats, suggesting enhanced presynaptic glutamate release. DHPG, a selective agonist of group I mGlu receptors, further facilitated NMDA receptor-mediated eEPSC and NMDA-induced current in dorsal horn neurons after inflammation and the mechanisms underlying this facilitation involved postsynaptic intracellular G protein, PLC pathway and IP_3 -sensitive Ca^{2+} release. Thus,

activation of group I mGlu receptors, likely maintained by presynaptic input, primes NMDA receptors for further enhanced excitability and contributes to central nociceptive sensitization.

4.1. NMDA receptor current is enhanced after inflammation

Functional changes in NMDA receptors are associated with plasticity of the nervous system, which has been extensively implicated in central sensitization and enhanced pain after peripheral stimulation (Dubner and Ruda, 1992; Ren et al., 1992; Woolf and Thompson, 1991). Our results indicate that NMDA receptors in the superficial spinal dorsal horn are more responsive after inflammation, as shown by dose-dependent increases in NMDA receptor-mediated eEPSC and NMDA-induced current. This observation is consistent with the previous report on dissociated dorsal horn neurons from CFA-inflamed rats (Guo and Huang, 2001). It is also consistent with inflammation-induced increase in NR2B subunit tyrosine phosphorylation (Guo et al., 2004), which has been shown to up-regulate NMDA receptor channel function in dorsal horn neurons (Wang and Salter, 1994). Multiple factors may contribute to inflammation-induced functional plasticity of the NMDA receptor, which include reduced Mg^{2+} blockage (Guo and Huang, 2001; Zhang et al., 1996), greater calcium influx (Heinke and Sandkühler, 2007; Isaev et al., 2000), intracellular Ca^{2+} release (Guo et al., 2004), and changing channel kinetics associated with NMDA receptor phosphorylation (Guo et al., 2004; Yu et al., 1997).

We also observed an increase in the frequency and mean amplitude of spontaneous mEPSCs near the resting membrane potential in dorsal horn neurons after inflammation. Miniature EPSCs of spinal neurons may be mediated by action potential-independent spontaneous release of glutamate from presynaptic terminals (Hori and Endo, 1992). Significantly enhanced primary afferent input after injury may facilitate presynaptic glutamate release and contribute to potentiation of mEPSC (Baba et al., 1999). Dorsal horn mEPSCs are believed to be primarily mediated by AMPA receptors. Spontaneous mEPSCs in dorsal horn neurons are not affected by deletion of the NR1 subunit of the NMDA receptor in the spinal cord (South et al., 2003) and can be abolished by AMPA/KA receptor antagonists (Park et al., 2004). In the present study, including AP-5 in the bath had no effect on mEPSCs and CNQX could completely block them (data not shown). The increased mEPSCs help to build up increased responsiveness of NMDA receptors. While we show that group I mGlu receptors facilitate NMDA receptor current, apparently, the increased presynaptic glutamate release and related enhanced mEPSC involving AMPA receptors is an additional contributor to enhanced activation of NMDA receptors after inflammation.

4.2. DHPG-produced facilitation of NMDA receptor current

Our results show that the antagonism of group I mGlu receptors selectively attenuated the NMDA receptor current in CFA-inflamed, but not naive rats. These results suggest that group I mGlu receptors were activated after inflammation and interacted with NMDA receptors. The increased presynaptic glutamate release as suggested by enhanced mEPSCs in dorsal horn neurons from inflamed rats supports this hypothesis. Consistently, bath application of the group I mGlu receptor agonist DHPG produced an enhancement of NMDA receptor-mediated eEPSC and NMDA-induced current. Although DHPG itself had little effect on membrane current, it potentiated NMDA-mediated current in dorsal horn neurons. It is notable that DHPG was able to further increase synaptic NMDA receptor current in dorsal horn neurons of CFA-inflamed rats even when the NMDA current was already enhanced after inflammation. These results suggest that there was an increase in sensitivity of NMDA receptors to synaptic regulation after inflammation and a large functional reserve or plasticity of NMDA receptor channels coupled with group I mGlu receptors. The DHPG-produced facilitation was prevented by AIDA, confirming the

activation of group I mGlu receptors. Zhong et al. (2000) showed that DHPG-produced long-lasting potentiation of EPSP of dorsal horn neurons was reversed by the NMDA receptor antagonist, consistent with the involvement of NMDA receptors as a down-stream effector of group I mGlu receptor activation.

The group I mGlu receptor-mediated NMDA receptor facilitation likely occurred on the postsynaptic site, although a contribution from the presynaptic site cannot be completely ruled out. A few group I mGlu receptors are present in presynaptic terminals (Jia et al., 1999). However, the activation of presynaptic mGlu receptors functions to reduce presynaptic glutamate release (Pin and Duvoisin, 1995). Instead of facilitation, DHPG reduces monosynaptic and mEPSCs in substantia gelatinosa neurons through a presynaptic mechanism involving the mGlu5 receptor (Giles et al., 2007). In contrast, Park et al. (2004) reported DHPG-produced facilitation of EPSCs of substantia gelatinosa neurons through presynaptic glutamate release. Group I mGlu receptor agonists have been shown to facilitate polysynaptic EPSPs of dorsal horn neurons to C-fiber input, likely involving a postsynaptic mechanism (Zhong et al., 2000). The postsynaptic action of DHPG is further supported by the observation that intracellular dialysis of GDP-beta-S abolished DHPG facilitation of NMDA current, which indicates the involvement of postsynaptic intracellular G-protein in DHPG action.

Ca^{2+} is known to be released from intracellular stores in a Gq/11-PLC-IP₃ pathway (Anwyl, 1999). The elevated Ca^{2+} is also believed to play a pivotal role in inflammatory pain (Vikman et al., 2008; Voitenko et al., 2004) and group I mGlu receptor function at the spinal level (Anwyl, 1999; Heinke and Sandkühler, 2007; Neugebauer, 2002). We show that the DHPG facilitation of NMDA current was blocked by a PLC inhibitor, intracellular Ca^{2+} chelating, or inhibition of Ca^{2+} release from IP₃-sensitive stores. These results are consistent with activation of the group I mGlu receptor-G protein signal transduction pathway and lead to the conclusion that mobilization of intracellular Ca^{2+} underlies the mechanism of the DHPG-produced facilitation of NMDA current. The same signaling pathway is also activated in group I mGlu receptor-induced increase in NR2B tyrosine phosphorylation during inflammatory hyperalgesia (Guo et al., 2004).

4.3. Significance of the mGlu receptor-NMDA receptor interaction in persistent pain

The interaction between group I mGlu receptors and NMDA receptors has important implications for efficiency of nociceptive processing in the dorsal horn, where both receptors are colocalized (Guo et al., 2004). The present study provides direct cellular evidence that supports active group I mGlu receptor-NMDA receptor coupling in a model of inflammatory hyperalgesia. These observations are in line with our previous report that signaling pathways for inflammation-induced NR2B tyrosine phosphorylation involve the coupling of group I mGlu receptor with NMDA receptors (Guo et al., 2004). The present findings extend our knowledge of the modulation of NMDA current by CFA-induced inflammation and the intracellular mechanisms of mGlu receptor-produced potentiation of NMDA current. Previous studies have documented the contribution of group I mGlu receptors to central sensitization and behavioral hyperalgesia (Adwanikar et al., 2004; Guo et al., 2004; Neugebauer, 2002; Scotland et al., 2007). The present study further demonstrates that the involvement of group I mGlu receptors in persistent pain requires subsequent activation of NMDA receptors.

Multiple endogenous substances may be responsible for enhancing excitatory synaptic transmission in the spinal dorsal horn during experimental chronic pain (Garraway et al., 2003; Kohno et al., 2008; Lu et al., 2009; Ren and Dubner, 2010) and the interaction with mGlu receptors may not be the sole upstream pathway leading to enhanced NMDA receptor activation. Previous studies have also suggested an interaction between spinal NMDA and

NK₁ tachykinin receptor systems in the processing of nociceptive information (Dougherty and Willis, 1991; Guo et al., 2002). Additional G protein-linked receptors including muscarinic acetylcholine, bradykinin and somatostatin receptors have been shown to mediate upregulation of NMDA receptor function, suggesting the presence of abundant signaling pathways (Guo et al., 2006; Kohno et al., 2008). In addition, ionotropic receptors such as the 5-HT₃ receptor and the GABA_A receptor also interact with NMDA receptors in producing pain facilitation at the spinal level (LaGraize et al., 2010). These findings are consistent with the hypothesis that signaling coupling between group I mGlu receptors and NMDA receptors underlies mechanisms of spinal synaptic plasticity after injury and that activation of NMDA receptors may be one common final mechanism leading to behavioral hyperalgesia (Guo et al., 2004).

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Fig. 1. Increased NMDA receptor-mediated synaptic responses of spinal cord dorsal horn neurons after inflammation. **A.** Graded electric stimulation of the dorsal root associated with the spinal cord slice evoked excitatory postsynaptic current (eEPSC), which was abolished when tetrodotoxin (1 μ M) was added to the bath. Arrow indicates stimulation artifact. Two traces are superimposed in each response. Stimulus intensity-response relationship is plotted to the right. Holding potential = -70 mV. **B.** NMDA receptor-mediated responses were enhanced after complete Freund's adjuvant (CFA)-induced hind paw inflammation. All recordings were performed in the absence of Mg^{2+} and presence of CNQX (10 μ M), strychnine (1 μ M), and bicuculline (10 μ M) under holding potential of $+40$ mV. Note that

the evoked current persisted in the presence of CNQX, strychnine, and bicuculline, but was abolished when AP-5 (50 μ M) was added to the bath. Dorsal root stimulation-induced NMDA receptor-mediated eEPSC was intensity-dependent and significantly increased in the CFA group between 300–600 μ A stimuli, compared to that of the naïve control rats, shown on the plot to the right. **, $p < 0.01$ vs. naïve.

Fig. 2.

Increased excitability and NMDA responsiveness of spinal cord dorsal horn neurons after hind paw inflammation. **A.** NMDA-induced inward current was increased in CFA-inflamed rats under both normal (1.2 mM) and 0 Mg^{2+} conditions. **B.** The NMDA-induced current was blocked by AP-5, confirming activation of NMDA receptors. Concentration of NMDA in A and B = 15 μ M. **C.** NMDA-induced currents were dose-dependent and significantly increased in the CFA group between 1–500 μ M concentrations (0 Mg^{2+}), compared to that of the naïve control rats, shown on the plot to the right. Note log-scale for the abscissa. Small horizontal bars in all figures indicate the time when NMDA was applied. *, $P < 0.05$; **, $P < 0.01$, vs. naïve. Holding potential = -70 mV.

Fig. 3.

Effects of inflammation on miniature excitatory postsynaptic currents (mEPSCs) and passive membrane properties. **A.** Examples of mEPSCs of dorsal horn neurons from naive control and CFA-inflamed rats. **B.** Summary of the CFA inflammation-induced increases in both frequency and amplitude of mEPSCs. *, $P < 0.05$, vs. naive. **C.** CFA inflammation did not change resting membrane potential or membrane input resistance (N.S., not significant). All recordings were conducted at holding potential = -70 mV in the presence of 1.2 mM Mg^{2+} .

Fig. 4. Blockade of group I mGlu receptors selectively decreases the amplitude of NMDA receptor-mediated eEPSC in neurons of CFA-inflamed but not naive control rats. **A.** Upper row, an example of recordings from a slice preparation from an inflamed animal. Blockade of group I mGlu receptor by AIDA (1 mM, 8 min pretreatment) decreased NMDA receptor-mediated eEPSC. Washout of AIDA recovered NMDA receptor-mediated eEPSC. Lower row, an example of recordings from a naïve control rat. AIDA did not produce an effect on NMDA receptor current. **B.** Summary of the effect of AIDA in CFA and control groups. Note that NMDA receptor currents in the CFA group are larger than that in control group (Left). Pooled data show relative amplitude of NMDA receptor-mediated eEPSC in the two groups

vs. respective pre-AIDA values (Right). **, $P < 0.01$. Stimulation intensity = 300 μ A. Holding potential = +40 mV; Mg^{2+} was absent in the external solution.

Fig. 5.

Blockade of group I mGlu receptors selectively decreases the amplitude of NMDA-induced current in neurons of CFA-inflamed but not naive control rats. **A.** Upper row, an example of recordings from a slice preparation from an inflamed animal. Blockade of group I mGlu receptor by AIDA (1 mM, 8 min pretreatment) decreased NMDA-induced current. Washout of AIDA partially recovered NMDA receptor current. AIDA-induced decrease in NMDA-induced current appeared again on the same neuron after washout of the first AIDA application. Lower row, an example of recordings from a naïve control rat. AIDA did not produce an effect on NMDA current. **B.** Plot summarizing the effect of AIDA on NMDA current in CFA and control groups. Note that NMDA currents in the CFA group are larger

than that in control group (Left). Pooled data show relative amplitude of NMDA current in the two groups vs. respective pre-AIDA values (Right). **, $P < 0.01$. NMDA = 10 μM . Holding potential = -70 mV ; Mg^{2+} was absent in the external solution.

Fig. 6. Activation of group I mGlu receptors potentiates synaptic NMDA receptor current. **A.** Dorsal root stimulation-evoked synaptic activation and the effect of DHPG. All recordings were performed in the presence of CNQX (10 μ M), strychnine (1 μ M), and bicuculline (10 μ M). DHPG facilitated stimulation-evoked NMDA receptor-mediated response when compared to control (veh). Slice was from a rat with CFA-induced hind paw inflammation. **B.** Summary of the effect of DHPG in CAF-inflamed and naïve rats. *, $P < 0.05$; **, $P < 0.01$. Holding potential = +40 mV. Stimulation intensity = 300 μ A. Mg^{2+} was absent in the external solution.

Fig. 7.

Activation of group I mGlu receptors potentiates NMDA-induced current. **A.** An example of recordings from a slice preparation after inflammation. Activation of group I mGlu receptor by DHPG (10 μ M) facilitated NMDA-induced current (middle) in a reversible manner (DHPG Washout). Histogram (right) summarizes DHPG-produced facilitation in CFA and control (veh) groups. **B.** Effect of intracellular dialysis of GDP-beta-S on DHPG facilitation of NMDA current. With the 1st recording electrode containing normal internal solution, DHPG induced an increase in NMDA current (2nd trace to the left) compared to the control (left). Recording with the 2nd recording pipette containing 1 mM GDP-beta-S, the DHPG facilitation was largely prevented. The bar graphs (right) summarize DHPG-induced % changes in NMDA current in CFA and naïve groups between the drug groups. The dashed line indicates the control level. NMDA = 10 μ M. Holding potential = -70 mV. Mg^{2+} was absent in the external solution. *, $P < 0.05$.

Fig. 8.

Involvement of PLC on the DHPG-induced facilitation of NMDA current. **A.** DHPG facilitated NMDA current in a neuron from a CFA-inflamed rat. **B.** In the same neuron, extracellular application of the membrane permeable PLC inhibitor U73,122 (10 μ M) depressed the facilitation. **C.** U73,343 (10 μ M), an inactive analog of U73,122, did not prevent the DHPG facilitation. **D.** Histogram summarizes the effect of U73,122 and U73,343 on DHPG facilitation in CFA and naive groups. The dashed line indicates the control level. NMDA = 10 μ M. Holding potential = -70 mV. Mg^{2+} was absent in the external solution. *, $P < 0.05$, vs. U73,122 groups.

Fig. 9. DHPG-induced facilitation of NMDA-induced current is intracellular Ca^{2+} dependent. **A.** With the 1st recording electrode containing normal internal solution and the 2nd pipette containing a fast-acting Ca^{2+} buffer, BAPTA (+ BAPTA), DHPG-induced facilitation of NMDA current was attenuated by the Ca^{2+} buffer. **B.** DHPG-induced facilitation of NMDA current was attenuated by pretreatment with an IP₃-receptor inhibitor, 2-APB. **C.** Histogram summarizes the effect of BAPTA and 2-APB on DHPG facilitation in CFA and naive groups. The dashed line indicates the control level. NMDA = 10 μ M. Holding potential = -70 mV. Mg^{2+} was absent in the external solution. *, $P < 0.05$, vs. respective BAPTA and 2-APB groups.