Increased Nociceptive Input Rapidly Modulates Spinal GABAergic Transmission Through Endogenously Released Glutamate

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Abstract

Stimulation of nociceptive primary afferents elicits pain by promoting glutamatergic transmission in the spinal cord. Little is known about how increased nociceptive input controls GABAergic tone in the spinal dorsal horn. In this study, we determined how increased nociceptive inflow affects GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs) of lamina II neurons by using whole-cell recordings in rat spinal cord slices. Bath application of capsaicin for 3 min induced a long-lasting inhibition of sIPSCs in 50% of the neurons tested. In the other half of the neurons, capsaicin either increased the frequency of sIPSCs (34.6%) or had no effect on sIPSCs (15.4%). The GABA_A current elicited by puff application of GABA was not altered by capsaicin. Capsaicin did not inhibit sIPSCs in rats treated with intrathecal pertussis toxin. Also, capsaicin failed to inhibit sIPSCs in the presence of ionotropic glutamate receptor antagonists or in the presence of both LY341495 and CPPG (group II and III metabotropic glutamate receptor antagonists, respectively). However, when LY341495 or CPPG was used alone, capsaicin still decreased the frequency of sIPSCs in some neurons. Additionally, bradykinin significantly inhibited sIPSCs in a population of lamina II neurons, and this inhibitory effect also abolished by LY341495 and CPPG. Our study provides novel information that stimulation of nociceptive primary afferents rapidly suppresses GABAergic input to many dorsal horn neurons through endogenous glutamate and activation of presynaptic group II and III metabotropic glutamate receptors. These findings extend our understanding of the microcircuitry of the spinal dorsal horn involved in nociception.
Introduction

Nociceptive information generated from noxious stimuli is relayed primarily by unmyelinated primary afferents to second-order neurons in the superficial dorsal horn of the spinal cord. The resulting pain intensity and duration often increase following the initial tissue or nerve damage. Tissue and peripheral nerve injury can sensitize primary afferents and spinal cord dorsal horn neurons, which results in secondary pain hypersensitivity (LaMotte et al. 1982; Torebjork et al. 1992; Woolf et al. 1988). The release of inflammatory mediators from injured tissues and inflammatory cells, including prostaglandins (Martin et al. 1987; Trebino et al. 2003) and bradykinin (Liang et al. 2001; Rueff and Dray 1993), can stimulate and sensitize nociceptors (peripheral sensitization). Furthermore, increased release of glutamate from primary afferents and activation of postsynaptic ionotropic glutamate receptors (iGluRs) in the spinal cord can enhance the excitability of dorsal horn neurons (central sensitization) (Dougherty et al. 1992; Leem et al. 1996). However, the cellular mechanisms for the hypersensitivity of dorsal horn neurons after tissue and nerve injury are not fully known.

The lamina II of the spinal cord is a critical site for the relay and processing of dynamic modulation of sensory information. Yet the detailed circuitry and synaptic control of lamina II neurons involved in the integration and regulation of nociceptive input rare largely unclear. Both excitatory and inhibitory interneurons in the lamina II can modulate the input of primary afferents through presynaptic and postsynaptic mechanisms (Lu and Perl 2003; Pan and Pan 2004; Yoshimura and Nishi 1995). Glutamate is an important excitatory neurotransmitter, and iGluRs are essential for nociceptive transmission and sensitization of dorsal horn neurons in the spinal
cord (Dougherty et al. 1992; Leem et al. 1996; Lu and Perl 2003; Pan and Pan 2004; Yoshimura and Nishi 1995). By contrast, GABA is the dominant inhibitory neurotransmitter in the spinal cord, and spinal nociceptive transmission and dorsal horn neurons in different laminae are under tonic inhibitory control mediated largely by GABA (Light and Kavookjian 1988; Pan and Pan 2004; Yoshimura and Nishi 1995). In this regard, blockade of GABA$_A$ receptors results in hypersensitivity of dorsal horn neurons and in allodynia (Sivilotti and Woolf 1994; Sorkin et al. 1998). Reduced GABAergic input to lamina II neurons is considered an important contributor to chronic neuropathic pain (Moore et al. 2002). The primary afferent terminals and the glutamatergic and GABAergic interneurons are closely intermingled in the superficial dorsal horn. Although stimulation of primary afferents potentiates glutamatergic transmission in second-order and higher-order neurons in the spinal dorsal horn, little is known if, and how, increased nociceptive input affects local inhibitory GABAergic input to dorsal horn neurons.

The aim of this study was to determine if, and how, stimulation of nociceptive primary afferents rapidly modulates GABAergic synaptic transmission in the spinal cord. We found that glutamate spillover following primary afferent stimulation produces a heterosynaptic action to affect GABAergic transmission. Reduction of GABAergic tone can occur rapidly (within minutes) after stimulation of primary afferents, which could facilitate transmission of nociceptive information in the spinal cord. Through groups II and III metabotropic glutamate receptors (mGluRs) on GABAergic interneurons, modulation of spinal GABAergic tone is directly linked to increased glutamatergic input from nociceptive primary afferents. This new information is important for our understanding of the integrative mechanisms underlying the regulation of nociceptive inflow by spinal dorsal horn neurons.
Materials and Methods

Animals

Male Sprague-Dawley rats (3-4 weeks old; Harlan, Indianapolis, IN) were used in this study. All the surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center. Some rats were treated with intrathecal pertussis toxin (PTX) to inactivate inhibitory Gi/o proteins (Chen and Pan 2004; Zhang et al. 2005). Intrathecal catheters were inserted in rats anesthetized using 2-3% isoflurane. The catheters (polyethylene-10 tubing) were inserted through an incision in the cisternal membrane and advanced 4.5 cm caudal so that the tip of each catheter was positioned at the lumbar spinal level. Rats were injected with intrathecal 1 µg of PTX 5-7 days before the final electrophysiology experiments.

Spinal Cord Slice Preparation

Under isoflurane anesthesia, the lumbar segment of the spinal cord was removed through laminectomy at the L2-L5 level. The segment of the spinal cord was immediately placed in an ice-cold sucrose artificial cerebrospinal fluid (aCSF) presaturated with 95% O₂ and 5% CO₂. The sucrose aCSF contained 234 mM sucrose, 3.6 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 12.0 mM glucose, and 25.0 mM NaHCO₃. The tissue was then placed in a shallow groove formed in a gelatin block and glued onto the stage of a vibratome (Technical Product International, St. Louis, MO). Transverse spinal cord slices (400 µm) were cut in the ice-cold sucrose aCSF and incubated in Krebs' solution oxygenated with 95% O₂ and 5% CO₂ at
34 C for at least 1 h before they were transferred to the recording chamber. The Krebs' solution contained 117.0 mM NaCl, 3.6 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 11.0 mM glucose, and 25.0 mM NaHCO₃.

**Electrophysiological Recordings**

Recordings of postsynaptic currents were performed using the whole-cell voltage-clamp method, as we described previously (Li et al. 2002; Pan and Pan 2004; Zhang et al. 2005). The slice was placed in a glass-bottomed chamber (Warner Instrument, Hamden, CT) and fixed with parallel nylon threads supported by a U-shaped stainless steel weight. The slice was continuously perfused with Krebs' solution at 5.0 ml/min at 34 C maintained by an inline solution heater and a temperature controller (TC-324; Warner Instrument). The lamina II was identified as a distinct translucent band across the superficial dorsal horn under a microscope with transmitted illumination. The neurons in the lamina II in the spinal cord slice were visualized under a fixed-stage microscope (BX50WI; Olympus, Tokyo, Japan) with differential interference contrast/infrared illumination. Lamina II neurons with a smooth surface were selected for recordings from the translucent region of the spinal dorsal horn (typically 50-150 µm from the dorsal surface the spinal dorsal horn). Neurons in both the outer and inner zones of lamina II were selected for recording, as described in detail previously (Pan and Pan 2004). The electrode for the whole-cell recordings was pulled from borosilicate glass capillaries with a puller (P-97; Sutter Instruments, Novato, CA). The impedance of the pipette was 3-5 MΩ when filled with internal solution containing 110 mM Cs₂SO₄, 5 mM TEA, 2.0 mM MgCl₂, 0.5 mM CaCl₂, 5.0 mM HEPES, 5.0 mM EGTA, 5.0 mM ATP-Mg, 0.5 mM Na-GTP, and 10 mM lidocaine N-
ethyl bromide, adjusted to pH 7.2-7.4 with 1 M CsOH (290–320 mOsm). Lidocaine N-ethyl bromide was added to the internal solution to suppress the action potential generation from the recorded cell.

Recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) at the holding potential of 0 mV began about 6 min after whole-cell access was established and the current reached a steady state. The input resistance was monitored, and the recording was abandoned if it changed more than 15%. Signals were recorded using an amplifier (MultiClamp 700B; Axon Instruments Inc., Union City, CA), filtered at 1-2 kHz, digitized at 10 kHz, and stored in a computer with pCLAMP 9.2 (Axon Instruments Inc.). All GABAergic sIPSCs and the current elicited by puff application of GABA were recorded in the presence of 0.5 µM strychnine, a glycine receptor antagonist. The internal solution for recording of the GABAergic current elicited by puff application of GABA contained: 130 mM KCl, 1.0 mM MgCl₂, 1.0 mM CaCl₂, 5.0 mM HEPES, 5.0 mM ATP-Mg, 0.5 mM Na-GTP, and 1 mM guanosine 5’-O-(2-thiodiphosphate) (GDP-β-S), and 10 mM lidocaine N-ethyl bromide, adjusted to pH 7.2 to 7.4 with 1 M KOH (290-300 mOsm).

Capsaicin, GDP-β-S, strychnine, CNQX, MK801, and GABA were obtained from Sigma-Aldrich (St. Louis, MO). (R,S)-cyclopropyl-4-phosphonomethylglycine (CPPG) and LY341495 were obtained from Tocris Cookson (Ellisville, MO). PTX was purchased from List Biological Laboratories (Campbell, CA). QX314 was obtained from Alomone Labs (Jerusalem, Israel). Drugs were dissolved in Krebs' solution and perfused into the slice chamber by using syringe pumps.
Data Analysis

Data are presented as means ± S.E.M. The sIPSCs were analyzed off-line with a peak detection program (MiniAnalysis; Synaptosoft, Decatur, GA). Measurements of the amplitude and frequency of sIPSCs were performed for at least 1 min during control, drug application, and recovery. The peak effect of capsaicin on sIPSCs was analyzed over a period of 1.5-2 min after capsaicin application and used for the comparison of the capsaicin effect with the baseline value. The sIPSCs were detected by the fast rise time of the signal over an amplitude threshold above the background noise signal (typically 6-10 pA). We manually excluded the event when the noise was erroneously identified as the sIPSCs by the software program. The background noise level was typically constant throughout the recording of a single neuron. The cumulative probability of the amplitude and inter-event interval of sIPSCs before and after drug application was compared using the Kolmogorov-Smirnov test, which estimates the probability if the two cumulative distributions are statistically different. The effects of capsaicin and bradykinin on the amplitude and frequency of sIPSCs were determined by paired two-tailed Student's t-test or one-way analysis of variance. The percentage of increase- and decrease-type neurons affected by capsaicin in different protocols was compared using Fisher's exact test. P < 0.05 was considered to be statistically significant.
Results

**Diverse effects of capsaicin on GABAergic sIPSCs in the spinal dorsal horn**

To test if GABAergic synaptic transmission in the spinal cord is altered by increased nociceptive input from primary afferents, we tested the effect of capsaicin, a known agonist for TRPV1 channels, on GABAergic sIPSCs of lamina II neurons. TRPV1 is located predominantly on primary afferent neurons and terminals in the superficial laminae of the spinal cord (Chen and Pan 2006; Guo et al. 2001; Pan et al. 2003). We used capsaicin to stimulate primary afferents (Pan and Pan 2004; Yang et al. 1998). Bath application of 2 µM capsaicin for 3 min decreased significantly both the frequency and amplitude of GABAergic sIPSCs in 13 of 26 (50%) neurons recorded (Fig. 1). These 13 neurons consisted of 9 inner and 4 outer lamina II neurons. The cumulative probability analysis of sIPSCs revealed that the distribution pattern of the inter-event interval but not the amplitude of sIPSCs was shifted toward the right in response to capsaicin (Fig. 1A). The mean sIPSC frequency and amplitude were reduced 53.0 ± 5.2% (n = 13) and 16.4 ± 3.7% (n = 13), respectively, by capsaicin. In contrast, in 9 of the 26 neurons (34.6%), capsaicin significantly increased the frequency but not the amplitude of sIPSCs (Fig. 1B and C). The latency of both the inhibitory and stimulatory effects of capsaicin was about 1.5 min, and these effects lasted for at least 6 min after capsaicin application was discontinued (Fig. 1). In the remaining 4 (15.4%) neurons, capsaicin had no significant effect on the frequency and amplitude of sIPSCs (Fig. 1). We did not observe the biphasic effect of capsaicin on sIPSCs. Bath application of 10 µM bicuculline, a GABA_A receptor blocker, abolished sIPSCs of all lamina II neurons tested (Fig. 1). These results suggest that activation of nociceptive primary afferents
inhibits GABAergic input to a population of dorsal horn neurons.

**Effect of capsaicin on postsynaptic GABA\(_A\) currents of lamina II neurons**

To determine if changes in the postsynaptic GABA\(_A\) receptors account for the inhibitory effect of capsaicin on sIPSCs, we recorded the GABA\(_A\) current elicited by puff application of GABA in lamina II neurons before and after bath application of 2 \(\mu\)M capsaicin for 3-10 min. In all 10 neurons tested, the current elicited by puff application of 1 mM GABA was not altered significantly by capsaicin. The mean amplitude of the current elicited by GABA was 1169.0 \(\pm\) 289.7 and 1142.2 \(\pm\) 323.5 pA (P > 0.05) before and after capsaicin, respectively (Fig. 2). These data suggest that stimulation of nociceptive primary afferents acutely modulates GABAergic transmission through a presynaptic mechanism.

**Role of G\(_{i/o}\) proteins in the inhibitory effect of capsaicin on synaptic GABA release**

To determine if the G\(_{i/o}\) protein-coupled receptors are involved in the inhibitory effect of capsaicin on synaptic GABA release, a group of rats was pretreated with intrathecal PTX to inactivate inhibitory G\(_{i/o}\) proteins (Chen and Pan 2004; Zhang et al. 2005). In none of the lamina II neurons from PTX-treated rats did capsaicin cause an inhibition of sIPSCs. In most (23 of 25, 92%) neurons tested, 2 \(\mu\)M capsaicin significantly increased the frequency but not the amplitude of GABAergic sIPSCs and shifted the distribution pattern of the inter-event interval of sIPSCs to the left (Fig. 3). In the remaining 2 (8%) neurons, capsaicin had no evident effect on the frequency and amplitude of GABAergic sIPSCs. These data suggest that the G\(_{i/o}\)-coupled receptors on GABAergic interneurons are involved in the inhibitory effect of capsaicin on...
GABAergic transmission in the spinal cord.

**Role of ionotropic glutamate receptors in the effect of capsaicin on synaptic GABA release**

Stimulation of primary afferents evokes glutamate release, which activates ionotropic glutamate receptors (iGluRs) on the postsynaptic dorsal horn neurons in the spinal cord (Pan and Pan 2004; Wang et al. 2005; Yoshimura and Nishi 1995). We next examined if endogenous glutamate release plays a role in the effect of capsaicin on GABAergic transmission in the spinal cord. In PTX-treated rats, in the presence of both 20 μM CNQX (a non-NMDA receptor antagonist) and 20 μM MK801 (an NMDA receptor antagonist), bath application of 2 μM capsaicin failed to inhibit GABAergic sIPSCs in all cells recorded (n = 21, Fig. 4A). Capsaicin did not significantly alter the frequency of sIPSCs in 17 of 21 (81%) cells in the presence of CNQX and MK801. In the remaining 4 cells (19%, Fig. 4A), capsaicin still significantly increased the frequency of sIPSCs. These data suggest that the potentiating effect of capsaicin on synaptic GABA release in the spinal cord of PTX-treated rats is primarily caused by increased glutamate release and iGluRs.

Similarly, in untreated rats, in the presence of both 20 μM CNQX and 20 μM MK801, 2 μM capsaicin failed to inhibit the GABAergic sIPSCs in all 22 lamina II neurons recorded. In the majority (17 of 22, 77.3%) of lamina II neurons tested, capsaicin had no significant effect on the frequency and amplitude of sIPSCs (Fig. 4B). In the remaining 5 (22.7%) neurons, capsaicin significantly increased the frequency of GABAergic sIPSCs (Fig. 4B). These results strongly suggest that the inhibitory effect of capsaicin on synaptic GABA release is di-synaptic or multi-synaptic, which is mediated by endogenous glutamate release and activation of iGluRs in the
spinal cord.

**Effect of capsaicin on GABAergic mIPSCs of lamina II neurons**

To determine if the dorsal horn interneurons are involved in the stimulating effect of capsaicin on synaptic GABA release, we tested the effect of capsaicin on GABAergic mIPSCs in 21 separate lamina II neurons. In most neurons (17/21, 81%) tested, bath application of 2 µM capsaicin did not significantly alter the frequency and the amplitude of mIPSCs in the presence of 1 µM of TTX (Fig. 4C). In the remaining 4 (19%) neurons, 2 µM capsaicin increased significantly the frequency but not the amplitude of mIPSCs (Fig. 4C). Thus, increased nociceptive inflow can stimulate the GABAergic and/or glutamatergic interneurons to evoke synaptic GABA release to most dorsal horn neurons.

**Role of groups II and III metabotropic glutamate receptors in the inhibitory effect of capsaicin on GABA release**

Groups II and III metabotropic glutamate receptors (mGluRs) are Gi/o protein-coupled receptors (Prezeau et al. 1992; Tanabe et al. 1992). It has been shown that activation of presynaptic groups II and III mGluRs depresses GABA release in spinal dorsal horn neurons (Gerber et al. 2000). The results described in the previous section suggest that inhibition of synaptic GABA release by capsaicin is mediated by endogenous glutamate release. Also, because the inhibitory effect of capsaicin on synaptic GABA release was abolished by PTX treatment, we hypothesized that the groups II and III mGluRs play a role in the attenuation of synaptic GABA release during stimulation of primary afferents with capsaicin. In the presence
of 200 µM CPPG and 100 nM LY341495, which block groups II and III mGluRs, respectively (Conn and Pin 1997; Gerber et al. 2000; Schoepf et al. 1999), 2 µM capsaicin had no inhibitory effect on sIPSCs. In 9 of 17 (52.9%) neurons studied, capsaicin significantly increased the frequency of sIPSCs (Fig. 5). These 9 neurons consisted of 6 inner and 3 outer lamina II neurons. In the remaining 8 neurons, capsaicin had no significant effect on the frequency and amplitude of sIPSCs (Fig. 5B).

We then examined the relative contribution of group II mGluRs to the inhibitory effect of capsaicin on synaptic GABA release in the spinal cord. In the presence of 100 nM LY341495, 2 µM capsaicin significantly increased the frequency of sIPSCs in 13 of 21 (62%) neurons recorded (Fig. 6A). However, in 4 of 21 (19%) neurons, capsaicin still significantly decreased the frequency of sIPSCs (Fig. 6A). These four neurons consisted of three inner and one outer lamina II neurons. In the remaining 4 (19%) neurons, capsaicin had no significant effect on the frequency and amplitude of GABAergic sIPSCs.

We also determined the relative role of group III mGluRs in the inhibitory effect of capsaicin on synaptic GABA release in the spinal cord. In the presence of 200 µM CPPG, 2 µM capsaicin significantly increased the frequency of sIPSCs in 12 of 21 (57%) neurons (Fig. 6B,C). However, in 4 of 21 (19%) neurons, capsaicin significantly decreased the frequency of sIPSCs (Fig. 6B,C). These four neurons consisted of one inner and three outer lamina II neurons. In the remaining 5 (24%) neurons, capsaicin had no significant effect on the frequency or amplitude of sIPSCs. Collectively, these results suggest that acute stimulation of primary afferents inhibits synaptic GABA release by activating both groups II and III mGluRs, located presynaptically on GABAergic interneurons, in the spinal cord.
Role of groups II and III mGluRs in the inhibitory effect of bradykinin on GABA release

Because capsaicin can cause rapid desensitization of TRPV1 (Szallasi and Blumberg 1999), we were unable to examine the capsaicin effect before and after application of groups II and III mGluRs antagonists in the same dorsal horn neuron. Bradykinin is a nonapeptide, which can be used repeatedly to stimulate glutamate release from nociceptive primary afferents in the spinal cord (Jeftinija 1994; Wang et al. 2005). We chose bradykinin to stimulate nociceptive primary afferent terminals not restricted to TRPV1-expressing nerves in the spinal cord. Bath application of 10 µM bradykinin for 3 min significantly decreased the frequency of GABAergic sIPSCs in 18 of 49 (36.7%) neurons studied (Fig. 7). These 18 neurons consisted of 15 inner and 3 outer lamina II neurons. In separate (14 of 49, 28.6%) neurons, bradykinin significantly increased the frequency of sIPSCs (Fig. 7). The bradykinin effect persisted for at least 6 min during washout of bradykinin. In the remaining 17 (34.7%) neurons, bradykinin had no significant effect on the frequency or amplitude of sIPSCs (Fig. 7). Repeated application of 10 µM bradykinin had a reproducible effect on sIPSCs (data not shown).

In 12 lamina II neurons in which bradykinin reduced the frequency of sIPSCs, we determined the role of groups II and III mGluRs in this action. In 9 of 12 cells, 200 µM CPPG alone abolished the inhibitory effect of bradykinin on GABAergic sIPSCs (Fig. 8). These 9 neurons consisted of 8 inner and 1 outer lamina II neurons. In the remaining 3 cells, CPPG alone did not significantly reduce the effect of bradykinin on sIPSCs. However, subsequent application of 100 nM LY341495 completely blocked the reduction of the frequency of sIPSCs by bradykinin (Fig. 8). These 3 neurons consisted of 2 inner and 1 outer lamina II neurons. In no
case was a decrease-type response converted to an increase-type response in the presence of CPPG and LY341495. These results provide further evidence that stimulation of nociceptive primary afferents inhibits GABAergic input to dorsal horn neurons through combined groups II and III mGluRs in the spinal cord.
Discussion

The increased pain sensitivity following tissue and nerve injury is likely due to a net result of peripheral and central sensitization and an imbalance between excitatory and inhibitory synaptic inputs to spinal dorsal horn projection neurons. This is the first study that has demonstrated that increased nociceptive inflow can rapidly alter GABAergic synaptic transmission in the spinal cord. We found that stimulation of nociceptive primary afferents with either capsaicin or bradykinin increased the frequency of GABAergic sIPSCs in many lamina II neurons. Interestingly, capsaicin or bradykinin also caused a rapid and long-lasting inhibition of sIPSCs in a population of lamina II neurons. Subsequent experiments with iGluR antagonists provided evidence that the reduction in GABAergic input to dorsal horn neurons is through endogenously released glutamate. Using PTX and specific groups II and III mGluR antagonists, we provided further evidence that groups II and III mGluRs function as heteroreceptors to inhibit GABAergic tone following stimulation of primary afferents. Therefore, our study provides novel information that groups II and III mGluRs play a critical role in the rapid modulation of GABAergic transmission in the spinal dorsal horn in response to increased nociceptive input. Acute disinhibition of lamina II neurons by endogenous glutamate may contribute to central sensitization and pain hypersensitivity.

The lamina II is critical for the relay of nociceptive information in the spinal cord (Cervero and Iggo 1980; Lu and Perl 2003). In this study, we used capsaicin and bradykinin to stimulate nociceptive primary afferents in the spinal cord slice preparation. The capsaicin receptors (TRPV1 channels) have been localized to the central terminals of primary afferent
neurons in the superficial dorsal horn (Chen and Pan 2006; Guo et al. 2001; Pan et al. 2003). Immunocytochemical and electrophysiological studies have documented that TRPV-expressing primary afferent terminals are glutamatergic (Chen and Pan 2006; Hwang et al. 2004; Pan et al. 2003). Furthermore, bradykinin receptors (kinin B2 receptors) are also expressed on primary sensory neurons (Prado et al. 2002; Wang et al. 2005), and bradykinin can elicit glutamate release from primary afferent terminals (Jef tinija 1994; Wang et al. 2005). We found that while capsaicin and bradykinin increased synaptic GABA release in a population of lamina II neurons, they also induced a long-lasting inhibition of GABAergic input to other lamina II neurons. Because we measured GABAergic sIPSCs (not mIPSCs), the decrease in the sIPSC amplitude by capsaicin or bradykinin can be explained by the reduced presynaptic GABA release. This presynaptic action is further supported by our finding that the postsynaptic GABA_A current was not altered by capsaicin. Notably, capsaicin was reported to have no effect on the GABAergic and glycinergic inhibitory synaptic transmission (Yang et al. 1998). The lack of capsaicin effect on synaptic GABA release in the previous studies might have been due to the short duration (30 s) of capsaicin application. We found in the present study that the effect of capsaicin on GABAergic sIPSCs took about two minutes, reflecting a di-synaptic or multi-synaptic effect. Also, a longer onset latency of capsaicin effect on sIPSCs compared to that on excitatory postsynaptic currents (< 30 s) (Pan and Pan 2004; Yang et al. 1998) suggests that the effect of capsaicin on GABAergic transmission is probably an indirect effect secondary to increased glutamatergic input. The use of iGluR antagonists to isolate IPSCs in the previous study also may account for the failure to observe the inhibitory effect of capsaicin (Pan and Pan 2004; Yang et al. 1998). As shown in this study, the iGluR antagonists abolished the inhibitory effect of
capsaicin on GABAergic input, suggesting that this capsaicin effect is critically dependant on endogenous glutamate and activation of iGluRs. This finding suggests that stimulation of primary afferents can rapidly (within minutes) reduce GABAergic tone in the spinal cord, which possibly can increase glutamatergic synaptic efficacy in the dorsal horn. Thus, “disinhibition” of lamina II neurons could contribute to the sensitization of dorsal horn neurons, leading to long-lasting pain hypersensitivity after tissue and nerve injury.

We found that in rats treated with PTX to inactivate G_{i/o} proteins, capsaicin failed to inhibit synaptic GABA release in all lamina II neurons tested. Therefore, the receptors coupled to G_{i/o} proteins are probably involved in the inhibitory effect of capsaicin. Glutamate released from primary afferents and interneurons acts through two broad classes of glutamate receptors, ionotropic (AMPA, NMDA, and kainate) receptors and G protein-coupled metabotropic receptors (mGluRs). Eight mGluRs have been cloned and are classified into three groups. Group I receptors (mGluRs 1 and 5) couple to phospholipase C via G_{q/11} proteins (Houamed et al. 1991; Masu et al. 1991) and increase neuronal firing and synaptic transmission. Stimulation of group II (mGluRs 2 and 3) and group III (mGluRs 4, 6, 7, and 8) receptors coupled to G_{i/o} proteins (Prezeau et al. 1992; Tanabe et al. 1992; Tanabe et al. 1993), on the other hand, generally reduces neuronal excitability and synaptic transmission (Conn and Pin 1997; Macek et al. 1996; Schoepp et al. 1999). Both groups II and III agonists can reduce chronic neuropathic pain (Chen and Pan 2005; Fisher et al. 2002). Bath application of exogenous groups II and III mGluR agonists reduces both glutamatergic and GABAergic transmission in the rat spinal cord (Gerber et al. 2000). We hypothesized that when glutamate release is enhanced from primary afferents and glutamatergic interneurons following high-intensity stimulation, glutamate can overspill at the
synapse to activate presynaptic inhibitory mGluRs. This use-dependent activation of mGluRs may influence the strength of synaptic transmission in the spinal dorsal horn (Scanziani et al. 1997). We found that the inhibitory effect of both capsaicin and bradykinin on the frequency of GABAergic sIPSCs in lamina II neurons was completely blocked by specific groups II and III mGluRs antagonists LY341495 and CPPG. Because either antagonist alone only partially reduced the inhibitory effect of capsaicin and bradykinin on GABAergic sIPSCs, both groups II and III mGluRs must be involved. Since bradykinin also decreased the frequency of GABAergic sIPSCs in a population of lamina II neurons, those non-TRPV1-expressing afferent terminals can also inhibit spinal GABAergic tone. Collectively, our results suggest that increased nociceptive input leads to endogenous glutamate release from glutamatergic interneurons in the dorsal horn, which activates both groups II and III mGluRs expressed on GABAergic interneurons (to function as heteroreceptors) to reduce GABAergic transmission. Capsaicin-induced reduction in GABAergic tone and increase in glutamatergic input can act in concert to facilitate nociceptive transmission in the spinal cord.

The mGluR2/3 is present at the afferent terminals in the spinal superficial dorsal horn (Jia et al. 1999; Tang and Sim 1999). Furthermore, two subtypes of group III mGluRs, mGluR4 and mGluR7, are located in the rat spinal dorsal horn (Azkue et al. 2001; Ohishi et al. 1995). Immunocytochemical labeling studies suggest that group II mGluRs appear to be in the inner zone of lamina II, but group III mGluRs are mainly in the lamina I and the outer zone of lamina II (Jia et al. 1999; Ohishi et al. 1995; Tang and Sim 1999). In our study, group II mGluRs that mediated the inhibitory effect of capsaicin and bradykinin on sIPSCs appeared to occur mostly in the outer lamina II neurons. Also, the group III mGluRs that mediated the decrease in sIPSCs by
capsaicin and bradykinin were predominately in the inner lamina II neurons. However, the histological evidence does not necessarily conflict with the results of our electrophysiological study. It is important to note that the GABAergic interneurons expressing groups II and III mGluRs may be located at a distance from the recorded lamina II neurons. While groups II and III mGluRs may be present on glutamatergic, GABAergic, or glycinergic neurons in the neuroanatomical studies, we focused on only mGluRs on GABAergic neurons in the present study. Since iGluR antagonists abolished the inhibitory effect of capsaicin, the source of glutamate that stimulates groups II and III mGluRs on GABAergic interneurons is probably released from glutamatergic interneurons rather than from primary afferents. If glutamate released directly from primary afferents were involved in activation of groups II and III mGluRs on GABAergic interneurons, the inhibitory effect of capsaicin on sIPSCs would have been observed in the presence of iGluR antagonists. Furthermore, the finding that capsaicin had no significant effect on GABAergic mIPSCs in most lamina II neurons is also consistent with the involvement of glutamatergic interneurons in the spinal dorsal horn.

Although we focused primarily on the mechanism responsible for the depression of GABAergic transmission by increased nociceptive inflow, we observed that primary afferent stimulation produced a stimulatory effect on synaptic GABA release to a subpopulation of lamina II neurons. This effect was especially prominent in PTX-treated rats. However, in the presence of CNQX and MK801, capsaicin only increased GABA release in a few lamina II neurons recorded from PTX-treated rats. These data suggest that iGluRs expressed on GABAergic interneurons are also involved in the stimulatory effect of capsaicin on synaptic GABA release. Consistent with these data, it has been shown that electrical stimulation of
primary afferents elicits GABAergic IPSCs primarily through non-NMDA receptors (Yoshimura and Nishi 1995). Nevertheless, we observed that the stimulatory effect of capsaicin was not blocked by iGluR antagonists in a small group of lamina II neurons. Hence, neurotransmitters other than glutamate, such as substance P (Vergnano et al. 2004) or ATP (Hugel and Schlichter 2000), may be responsible for this action. Alternatively, group I mGluRs on GABAergic interneurons (Dang et al. 2002; Jia et al. 1999; Tao et al. 2000) may mediate part of this capsaicin effect. Thus, glutamate released from the primary afferents may increase GABAergic input to some lamina II neurons, which could modulate nociception during acute stimulation of primary afferents. It is uncertain how increased synaptic GABA release influences nociceptive transmission in the spinal dorsal horn. Precisely which phenotype of recorded postsynaptic neurons receiving GABAergic input will ultimately dictate their contribution to modulation of nociceptive information. This heterosynaptic interaction may be important for dynamic integration of sensory signals at the spinal levels, through the lamina II along subsequent projection pathways. An important observation is that the increase- and decrease-type lamina II neurons in response to primary afferent stimulation do not overlap. Had this been the case, groups II and III mGluR antagonists would have reversed the bradykinin effect. But we never observed such a response. The dual effect of capsaicin and bradykinin on GABAergic input to two separate populations of lamina II neurons suggests a rather complex mechanism of integrating nociceptive inflow at the spinal level. The major limitation of this study is that the phenotype of the recorded postsynaptic neurons was not characterized. The spinal lamina II has excitatory and inhibitory (both GABAergic and glycinergic) interneurons. Characterizing the phenotype of the postsynaptic neuron would be important in explaining the differential effect of
capsaicin on synaptic GABA release.

In summary, the findings from our study provide new evidence for the interaction between glutamatergic and GABAergic synapses in the spinal dorsal horn following stimulation of the nociceptive primary afferents. Distinct populations of dorsal horn interneurons are involved in the integration of nociceptive information from primary afferents. As illustrated in Fig. 9, the groups II and III mGluRs located on the somatodendritic and/or terminal sites of GABAergic interneurons are critically involved in the inhibition of GABAergic input to dorsal horn neurons after stimulation of the primary afferents. Glutamate released from primary afferents can heterosynaptically reduce GABAergic input to dorsal horn neurons via groups II and III mGluRs. Therefore, increased glutamatergic input is directly linked to the reduction of GABAergic tone in the spinal dorsal horn, and the reduction of GABAergic transmission appears to be an important “downstream” effect of increased glutamatergic input from primary afferents. This information is important for our understanding of the synaptic interaction in the integration of nociceptive information at the spinal level and extends our understanding of the microcircuitry of the spinal cord dorsal horn involved in pain signaling.
Acknowledgments

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Figure Legends

Fig. 1. Effect of capsaicin on GABAergic sIPSCs of spinal lamina II neurons. A, raw tracings showing sIPSCs during control and application of 2 µM capsaicin in a decrease-type neuron. Cumulative plot analysis of sIPSCs of the same neuron showing the distribution of the inter-event interval and amplitude during control and application of 2 µM capsaicin. B, raw tracings showing sIPSCs during control and application of 2 µM capsaicin in an increase-type neuron. Cumulative plot analysis of sIPSCs of the same neuron showing the distribution of the inter-event interval and amplitude during control and application of 2 µM capsaicin. The frequency histograms in panels A and B show the time course and duration of the capsaicin-induced changes in the sIPSC frequency. Note that 10 µM bicuculline abolished the sIPSCs in both decrease- and increase-type neurons. C, summary data showing the differential effect of capsaicin on the frequency and amplitude of sIPSCs in 26 lamina II neurons. *, P < 0.05 compared with control.

Fig. 2. Lack of effect of capsaicin on the postsynaptic GABA\textsubscript{A} current of lamina II neurons. A, original tracings of the current elicited by puff application of 1 mM GABA in a lamina II neuron before and after bath application of 2 µM capsaicin. B, summary data showing that capsaicin had no significant effect on the current elicited by GABA in 10 lamina II neurons.

Fig. 3. Effect of capsaicin on GABAergic sIPSCs of spinal lamina II neurons in PTX-treated rats. A, raw tracings showing sIPSCs of a lamina II neuron during control and application of 2
µM capsaicin. Cumulative plot analysis of sIPSCs of the same neuron showing the distribution of the inter-event interval and amplitude during control and application of 2 µM capsaicin. B, summary data showing the effect of capsaicin on the frequency and amplitude of GABAergic sIPSCs in 23 lamina II neurons. *, P < 0.05 compared with control.

Fig. 4. Effect of capsaicin on GABAergic sIPSCs and mIPSCs of spinal lamina II. A, summary data showing the effect of 2 µM capsaicin on the frequency and amplitude of sIPSCs in 21 lamina II neurons of PTX-treated rats in the presence of 20 µM CNQX and 20 µM MK-801. B, effect of 2 µM capsaicin on the frequency and amplitude of sIPSCs in 22 lamina II neurons of untreated rats in the presence of 20 µM CNQX and 20 µM MK-801. C, effect of 2 µM capsaicin on the frequency and amplitude of mIPSCs in 21 lamina II neurons of untreated rats. *, P < 0.05 compared with control.

Fig. 5. Effect of capsaicin on GABAergic sIPSCs of spinal lamina II neurons in the presence of CPPG and LY341495. A, raw tracings showing sIPSCs in an increase-type neuron during control, application of 200 µM CPPG and 100 nM LY341495, and 2 µM capsaicin plus 200 µM CPPG and 100 nM LY341495. Cumulative plot analysis of sIPSCs of the same neuron showing the distribution of the inter-event interval and amplitude of sIPSCs during control, application of 200 µM CPPG and 100 nM LY341495, and 2 µM capsaicin plus 200 µM CPPG and 100 nM LY341495. B, summary data showing the effect of 2 µM capsaicin on the frequency and amplitude of sIPSCs in 17 lamina II neurons. *, P < 0.05 compared with control.
Fig. 6. Effect of capsaicin on GABAergic sIPSCs of spinal lamina II neurons in the presence of LY341495 or CPPG. A, summary data show the effect of 2 µM capsaicin on the frequency and amplitude of sIPSCs in the presence of 100 nM LY341495 in 21 lamina II neurons. B, summary data show the effect of 2 µM capsaicin on the frequency and amplitude of sIPSCs in the presence of 200 µM CPPG in another 21 lamina II neurons. *, P < 0.05 compared with control. C, summary data show the percentage of increase- and decrease-type neurons in which capsaicin significantly altered the frequency of sIPSCs in different protocols. *, P < 0.05 compared with the percentage of decrease-type neurons in the control group. #, P < 0.05 compared with the percentage of increase-type neurons in the control group (Fisher's exact test).

Fig. 7. Effect of bradykinin on GABAergic sIPSCs of lamina II neurons. A, original tracings showing sIPSCs during control and application of 10 µM bradykinin in a decrease-type neuron. Cumulative plot analysis of sIPSCs of the same neuron shows a significant shift in the distribution of the inter-event interval and amplitude by bradykinin compared with that during control (Kolmogorov-Smirnov test). B, raw tracings showing sIPSCs during control and application of 10 µM bradykinin in an increase-type neuron. Cumulative plot analysis of sIPSCs of the same neuron showing the distribution of the inter-event interval and amplitude during control and application of 10 µM bradykinin. C, summary data showing the effect of bradykinin on the frequency and amplitude of GABAergic sIPSCs in 49 lamina II neurons. *, P < 0.05 compared with control.

Fig. 8. Effect of CPPG and LY341495 on the inhibitory effect of bradykinin on GABAergic
sIPSCs of spinal lamina II neurons. A, raw tracings and the cumulative plot showing sIPSCs of a lamina II neuron during control, application of 10 µM bradykinin, 200 µM CPPG plus 10 µM bradykinin, and 100 nM LY341495 plus 200 µM CPPG and 10 µM bradykinin. B, raw tracings and the cumulative plot showing sIPSCs of another lamina II neuron during control, application of 10 µM bradykinin, 200 µM CPPG plus 10 µM bradykinin, and 100 nM LY341495 plus 200 µM CPPG and 10 µM bradykinin. C, summary data showing the inhibitory effect of bradykinin on the frequency of GABAergic sIPSCs in 11 lamina II neurons before and after application of CPPG and LY341495. *, P < 0.05 compared with control. BK, bradykinin.

Fig. 9. Schematic drawing illustrating the interaction between glutamatergic and GABAergic synapses in the spinal dorsal horn in response to increased nociceptive input. Stimulation of primary afferents with capsaicin and bradykinin (via TRPV1 and kinin B2 receptors, respectively) evokes glutamate release from primary afferent terminals, which excites glutamatergic interneurons through ionotropic glutamate receptors. Subsequently, the excited glutamatergic interneurons release glutamate to activate groups II and III mGluRs (located on the somatodendritic sites or terminals) of GABAergic interneurons. As a result, the GABAergic input to a postsynaptic lamina II neuron is reduced (see text for details). Note that this drawing only summarizes the inhibitory effect of capsaicin on GABAergic sIPSCs. For the potentiating effect of capsaicin on GABAergic sIPSCs, capsaicin can enhance the input from primary afferents directly to GABAergic neurons.
Fig 1

A
Control

Capsaicin

Bicuculline

Cumulative Probability

0.0 0.5 1.0
Inter-event interval (ms)
0 5000 10000

0.0 0.5 1.0
Amplitude (pA)
0 10 20 30 40

0 14
Number of events
0 180 360 540 720 900

B
Control

Capsaicin

Bicuculline

Cumulative Probability

0.0 0.5 1.0
Inter-event interval (ms)
0 4000 8000

0.0 0.5 1.0
Amplitude (pA)
0 10 20 30 40 50

0 60
Number of events
0 180 360 540 720 900

C

sPSC Frequency (Hz)

0.0 0.5 1.0 1.5 2.0

Control

Capsaicin

n=13
n=4
n=9

sPSC Amplitudes (pA)

0.0 5.0 10.0 15.0 20.0 25.0

n=13
n=4
n=9

*
Fig 2

A

Control  Capsaicin

200 pA

1 s

B

Amplitude (pA)

1600

1400

1200

1000

800

600

400

200

0

Control  Capsaicin
**Figure 3**

**A**

Comparison of sPSC traces between control and capsaicin treatments. The traces show a significant increase in both frequency and amplitude in the capsaicin group compared to the control group.

**B**

Cumulative probability plots for inter-event interval and amplitude distributions. The plots indicate a higher cumulative probability in the capsaicin group for both metrics, suggesting a greater likelihood of sPSCs occurring at lower intervals and amplitudes.

Statistical analysis shows a significant difference in sPSC frequency and amplitude between the two groups, with capsaicin-treated neurons exhibiting higher values.

n = 23
fig 4
A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

**fig 6**
Figure 8
Fig. 9