Effect of Morphine on Deep Dorsal Horn Projection Neurons Depends on Spinal GABAergic and Glycinergic Tone: Implications for Reduced Opioid Effect in Neuropathic Pain

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ABSTRACT

The μ opioid agonist morphine has distinct effects on spinal dorsal horn neurons in the superficial and deep laminae. However, it is not clear if the inhibitory effect of morphine on dorsal horn projection neurons is secondary to its potentiating effect on inhibitory interneurons. In this study, we tested the hypothesis that removal of GABAergic and glycinergic inhibitory inputs attenuates the effect of morphine on dorsal horn projection neurons and the reduced spinal GABAergic tone contributes to attenuated morphine effect in neuropathic pain. Single-unit activity of deep dorsal horn projection neurons was recorded in anesthetized normal/sham controls and L5 and L6 spinal nerve-ligated rats. Spinal application of 10 μM morphine significantly inhibited the evoked responses of dorsal horn neurons in both normal/sham controls, and this effect was abolished by the specific μ opioid antagonist. However, the effect of morphine on dorsal horn projection neurons was significantly reduced in nerve-injured rats. Furthermore, topical application of the GABAA receptor antagonist bicuculline (20 μM) almost abolished the effect of morphine in normal/sham control rats but did not significantly attenuate the morphine effect in nerve-injured rats. On the other hand, the glycine receptor antagonist strychnine (4 μM) significantly decreased the effect of morphine in both nerve-injured and control animals. These data suggest that the inhibitory effect of opioids on dorsal horn projection neurons depends on GABAergic and glycinergic inputs. Furthermore, reduced GABAergic tone probably contributes to diminished analgesic effect of opioids in neuropathic pain.

The μ opioid agonist morphine is used systemically and spinally to treat moderate and severe pain. The spinal cord dorsal horn is critically involved in pain transmission and modulation and is a major site responsible for the analgesic action of opioids (Yaksh and Nouiehed, 1985; Magnuson and Dickenson, 1991; Chen et al., 2005). However, the mechanisms of opioid analgesia and various factors that influence the opioid efficacy in the spinal cord are not fully known. The spinal dorsal horn is a heterogenous region containing inhibitory and excitatory interneurons as well as different types of ascending projection neurons. Interestingly, morphine has distinct effects on dorsal horn neurons in the superficial and deep laminae. For example, morphine given locally or intravenously increases the primary afferent-evoked excitability of lamina II (substantia gelatinosa) neurons but inhibits deep (presumably ascending) dorsal horn neurons in rats and cats (Woolf and Fitzgerald, 1981; Sastry and Goh, 1983; Magnuson and Dickenson, 1991). Because μ opioid receptors are predominantly located in the superficial dorsal horn (Arvidsson et al., 1995; Chen and Pan, 2003), it is possible that the effect of morphine on deep dorsal horn projection neurons is secondary to its potentiating effect on lamina II neurons. Many lamina II neurons are inhibitory interneurons (Cervero and Iggo, 1980; Lu and Perl, 2003) and project ventrally onto the deeper dorsal horn (Light and Kavookjian, 1988). Nevertheless, it remains unclear if the inhibitory action of morphine on dorsal horn projection neurons depends upon its effect on lamina II neurons. The nature of the synaptic inputs from lamina II neurons to dorsal horn projection neurons involved in the effect of morphine is also uncertain.

Chronic neuropathic pain is a clinical condition in which the analgesic efficacy of morphine is often decreased (Sindrup and Jensen, 1999; Woolf and Mannion, 1999). In animal models of neuropathic pain, systemically or intrathecally administered morphine produces a reduced inhibitory effect on dorsal horn neurons (Suzuki et al., 1999; Chen and Pan, 2003).

ABBR EV IATIONS: CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2.
Spinal nociceptive transmission and dorsal horn neurons in different laminae are under tonic inhibitory control mediated largely by γ-aminobutyric acid (GABA) and glycine (Light and Kavookjian, 1988; Yoshimura and Nishi, 1995; Cronin et al., 2004; Pan and Pan, 2004). Intrathecal bicuculline, a GABA<sub>Α</sub> receptor antagonist, or strychnine, a glycine antagonist, results in hypersensitivity of dorsal horn neurons and allodynia (Yaksh, 1989; Sorkin et al., 1998). Furthermore, spinal disinhibition following nerve injury is considered one of the major mechanisms responsible for central sensitization and neuropathic pain symptoms (Castro-Lopes et al., 1993; Moore et al., 2002). In this regard, several studies have shown a selective loss of GABAergic but not glycinergic inputs to dorsal horn neurons following nerve injury (Castro-Lopes et al., 1993; Ibuki et al., 1997; Moore et al., 2002; Drew et al., 2004). However, it is not known if the reduced GABAergic inhibitory tone in the spinal cord is causally related to the diminished effect of morphine on dorsal horn neurons and nociception in neuropathic pain. In the present study, we tested the hypothesis that removal of GABAergic and glycinergic inhibitory tone attenuates the inhibitory effect of morphine on deep dorsal horn projection neurons. We also determined the role of GABAergic and glycinergic inhibitory tone in the effect of morphine on dorsal horn projection neurons in a rat model of neuropathic pain.

Materials and Methods

Induction of Neuropathic Pain

Male rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 220 to 250 g were used in this study. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine and conformed to the National Institutes of Health guidelines on the ethical use of animals. Anesthesia was induced with 2% halothane in 100% oxygen through a nose cone. Ligation of L5 and L6 spinal nerves in rats was used in this study as an experimental model of neuropathic pain because it produces profound and sustained tactile allodynia, which resembles the condition observed in patients with neuropathic pain because it produces profound and sustained tactile allodynia (Kim and Chung, 1992). The left L5 and L6 spinal nerves were isolated under a surgical microscope, and both nerves were tightly ligated with 4–0 silk suture (Kim and Chung, 1992). The rats were allowed to recover for 2 weeks before evaluating the mechanical sensitivity of the injured hindpaw. Sham control rats were surgically prepared as described above except that the nerve was not ligated.

Behavioral Assessment of Tactile Alldynia

Sham and nerve-injured rats were placed in individual plastic boxes on a mesh floor and allowed to acclimate for 30 min. A series of calibrated von Frey filaments (Stoelting Co., Wood Dale, IL) were applied perpendicularly to the plantar surface of the left hindpaw with sufficient forces to bend the filaments for 6 s. Brisk withdrawal or paw flinching was considered a positive response. In the absence of a response, the filament of next greater force was applied. In the absence of a response, the next lower force was applied (Chaplan et al., 1994; Chen and Pan, 2002). The tactile stimulus producing a 50% likelihood of withdrawal response was calculated by using the up-down method (Chaplan et al., 1994; Chen and Pan, 2002). Each trial was repeated two or three times at approximately 2-min intervals, and the mean value was used as the force to produce withdrawal responses.
but responding more intensely to noxious stimuli (pinch > press). At the end of the experiments, rats were killed by an intravenous injection of an overdose of sodium pentobarbital.

Experimental Protocols

Role of Spinal μ Opioid Receptors in the Effect of Topical Morphine on Dorsal Horn Projection Neurons. In normal rats, after recording the baseline activity of the identified dorsal horn projection neurons for 5 min, the neuronal responses to brush, pressure, and pinch applied to the receptive field were examined before and 10 min after the spinal topical application of 10 μM of morphine. This topical application procedure was chosen to simulate the intra-thecal administration of drugs used in behavioral studies. To determine whether repeated topical morphine application produced a similar inhibitory effect on dorsal horn neurons, topical application of 10 μM morphine was repeated, separated by 30 min, after wash-out of its initial effect and when the firing activity and evoked responses of dorsal horn neurons returned to the control. In the pilot study, we observed that the inhibitory effect of topical morphine on dorsal horn neurons lasted less than 20 to 25 min after washout, and the evoked response of dorsal horn neurons fully recovered 30 min after washout of morphine. Previous studies have shown that 10 μM morphine produces a near maximal inhibition of voltage-gated calcium channels in dorsal root ganglion neurons (Wu et al., 2004) and hyperpolarization of lamina II neurons in spinal cord slices (Yoshimura and North, 1983). The similar dose of morphine also inhibits wide-dynamic-range neurons in the lumbar spinal cord in vivo (Reeve et al., 1998). Furthermore, the inhibitory effect on dorsal horn projection neurons by topical 10 μM morphine (see Results) is similar to the inhibition achieved by intravenous 2.5 mg/kg morphine (Chen and Pan, 2002; Chen et al., 2005), which produces analgesia in conscious rats (Chen and Pan, 2001).

To study the role of spinal μ opioid receptors in the effect of morphine on dorsal horn neurons, the inhibitory effect of topically applied morphine (10 μM) on dorsal horn projection neurons was tested before and 10 min after topical application of 1 μM H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_{2} (CTAP), a highly specific μ receptor antagonist (Kohno et al., 1999). CTAP was applied to the exposed lumbar spinal cord at the concentration of 1 μM, which blocks the effect of intravenous morphine on dorsal horn neurons (Chen et al., 2005).

Role of Spinal GABAergic and Glycinergic Inputs in the Inhibitory Effect of Topical Morphine on Dorsal Horn Projection Neurons in Normal and Sham Control Rats. In this protocol, the inhibitory effect of topical morphine (10 μM) on evoked responses of dorsal horn projection neurons to graded mechanical stimuli was tested before and during spinal application of the GABA_{A} receptor antagonist bicuculline and glycine receptor antagonist strychnine. After testing the initial effect of morphine on evoked responses of dorsal horn neurons and washout of morphine for 30 min, either 20 μM bicuculline or 4 μM strychnine alone or in combination was applied topically to the lumbar spinal cord. Then, the effect of topical morphine on neuronal responses to mechanical stimuli was examined again 10 min after topical application of bicuculline and/or strychnine. Morphine was obtained from Astra Pharmaceuticals (Westborough, MA). CTAP, bicuculline, and strychnine were purchased from Sigma-Aldrich (St. Louis, MO).

Data Analysis

Data are presented as means ± S.E.M. The baseline firing rate of the dorsal horn projection neurons was averaged during a 5-min control period. The evoked responses were quantified as the mean discharge rate over the duration of the stimulus after subtracting the background activity of the neuron (Chen and Pan, 2002, 2004). Significant changes in the drug effect on evoked responses of dorsal horn projection neurons to graded mechanical stimuli were determined using analysis of variance followed by Tukey’s post test. Differences were considered to be statistically significant if P < 0.05.

Results

The mechanical threshold, measured by application of von Frey filaments to the hindpaw on the surgery side, decreased significantly (from 21.3 ± 2.4 to 2.2 ± 0.3 g, P < 0.05) in 10 nerve-ligated rats within 2 weeks. The mechanical withdrawal threshold did not change significantly in six sham control rats during this period (from 22.1 ± 2.7 to 21.6 ± 2.3 g). All the dorsal horn projection neurons chosen for this study were wide-dynamic-range neurons. The ascending dorsal horn neurons recorded in the lumbar spinal cord had a mean depth of 737 ± 42 μm, ranging from 380 to 860 μm.

Role of μ Opioid Receptors in the Effect of Topical Morphine on Dorsal Horn Projection Neurons. Spinal topical application of 10 μM morphine significantly inhibited the evoked activity of dorsal horn projection neurons in response to press and pinch applied to the receptive field in all seven cells tested in normal rats (Fig. 1). Thirty minutes after washout of the initial morphine, the baseline activity and the evoked responses of the dorsal horn projection neurons completely returned to the control. Repeated application of the same concentration of morphine on the same neuron reproducibly inhibited the evoked response of these seven dorsal horn neurons (Fig. 1). There was no significant difference in the inhibitory effect of 10 μM morphine on dorsal horn neurons between the initial and the subsequent two applications of the same concentration of morphine. Spinal topical application of the specific μ opioid receptor antagonist CTAP (1 μM) was used when the baseline and evoked responses of the dorsal horn projection neurons returned completely to the control after morphine. CTAP alone had no significant effect on the evoked response of dorsal horn neurons. In the presence of 1 μM CTAP, subsequent spinal topical application of 10 μM morphine failed to inhibit the evoked response of dorsal horn neurons (n = 7, Fig. 1).

Role of Spinal GABAergic and Glycinergic Tone in the Inhibitory Effect of Topical Morphine on Dorsal Horn Neurons in Normal/Sham Rats. In 10 dorsal horn projection neurons from 10 unoperated normal rats, initial topical application of 10 μM morphine significantly inhibited the evoked neuronal response to pressure (72 ± 6%) and pinch (76 ± 5%) stimuli applied to the receptive field (Figs. 2 and 3A). Then, 20 μM bicuculline was applied topically to the
recording site of the lumbar spinal cord 30 min after washout of the initial effect of morphine. Bicuculline alone significantly increased the evoked response of these dorsal horn projection neurons (Figs. 2 and 3A). In the presence of 20 μM bicuculline, the inhibitory effect of 10 μM morphine on dorsal horn neurons was significantly attenuated, compared with its initial effect (Figs. 2 and 3A). The percentage inhibition by morphine in the presence of bicuculline was 36 ± 3 and 31 ± 4% in response to pressure and pinch, respectively.

Furthermore, 4 μM strychnine was applied topically to the recording site 30 min after the washout of morphine and bicuculline. Strychnine alone also significantly increased the evoked response of these 10 dorsal horn neurons. In the presence of strychnine, the inhibitory effect of topical 10 μM morphine was significantly decreased, compared with the initial effect of morphine (Figs. 2 and 3A). The percentage of inhibition by morphine in the presence of strychnine was 46 ± 4 and 44 ± 4% in response to pressure and pinch, respectively. Subsequently, both 20 μM bicuculline and 4 μM strychnine were coadministered topically to the recording site 30 min after washout of morphine and strychnine. In the presence of both bicuculline and strychnine, topical application of 10 μM morphine showed no significant inhibitory effect on all 10 dorsal horn neurons tested (Figs. 2 and 3A).

Additionally, the above protocol was repeated in another six dorsal horn projection neurons recorded from six sham control rats. Topical application of 20 μM bicuculline and 4 μM strychnine also largely attenuated the inhibitory effect of 10 μM morphine on dorsal horn neurons, similarly to those described in unoperated normal rats (Fig. 3B).

Role of Spinal GABAergic and Glycinergic Tone in the Inhibitory Effect of Topical Morphine on Dorsal Horn Neurons in Nerve-Injured Rats. In 10 dorsal horn projection neurons recorded from nerve-injured rats, both the baseline and evoked neuronal discharges were significantly higher than those in both normal and sham control rats. The baseline activity of dorsal horn projection neurons in six sham control and 10 nerve-ligated rats was 0.17 ± 0.12 and 0.56 ± 0.2 Hz (P < 0.05), respectively. The neuronal responses to press (16.9 ± 1.5 versus 11.5 ± 0.8 Hz, P < 0.05) and pinch (23.1 ± 1.8 versus 16.7 ± 1.6 Hz, P < 0.05) were also significantly greater in nerve-ligated than in sham control rats (Figs. 4 and 5).

In 10 dorsal horn projection neurons recorded from nerve-ligated rats, topical application of 10 μM morphine to the spinal cord produced a significant but attenuated inhibition of the evoked response of dorsal horn neurons to pressure (32 ± 4%) and pinch (26 ± 3%) (Figs. 4 and 5), compared with that in sham controls. Topical application of 20 μM bicuculli-
line alone did not significantly alter the evoked response of these 10 dorsal horn neurons to graded mechanical stimuli. In the presence of 20 μM bicuculline, topical application of 10 μM morphine showed a significant but reduced inhibitory effect on the response of dorsal horn projection neurons to pressure and pinch, compared with that in control animals (Figs. 4 and 5). However, topical application of 4 μM strychnine alone significantly increased the evoked responses of the dorsal horn neurons to press and pinch. In the presence of 4 μM strychnine, 10 μM morphine had no significant inhibitory effect on the evoked response of these 10 dorsal horn neurons to graded mechanical stimuli (Figs. 4 and 5).

**Discussion**

This is the first study demonstrating the importance of GABAergic and glycinergic tone in the inhibitory effect of morphine on deep spinal dorsal horn projection neurons. In this study, we found that removal of spinal GABAergic or glycinergic tone caused a large attenuation of the inhibitory effect of morphine on dorsal horn projection neurons in normal/sham controls and nerve-injured rats. Also, we observed that blockade of GABA_A and glycine receptors in the spinal cord substantially increased evoked responses of dorsal horn projection neurons in normal/sham control rats but not in nerve-injured rats. Importantly, we found that the inhibitory effect of spinal morphine on dorsal horn projection neurons was significantly reduced in nerve-injured rats, and blockade of glycine, but not GABA_A receptors largely reduced the effect of morphine in rats subjected to nerve injury. Collectively, this study provides new evidence that GABAergic and glycinergic inputs in the spinal cord play an important role in the inhibitory effect of morphine on dorsal horn projection neurons. Furthermore, we demonstrate that decreased GABAergic tone is a potential cause for reduced inhibitory effect of morphine on dorsal horn projection neurons in nerve-injured rats. This study sheds new light on our understanding of the mechanisms of spinal opioid analgesia and reduced analgesic efficacy of opioids in neuropathic pain.

Activation of μ opioid receptors in the spinal dorsal horn produces analgesia most likely through attenuation of glutamatergic synaptic inputs and inhibition of dorsal horn neurons (Schneider et al., 1998; Kohno et al., 1999; Light and Willcockson, 1999; Chen et al., 2005). In this study, topical...
the inhibitory effect of morphine is mediated by morphine on dorsal horn projection neurons, suggesting that completely blocked the inhibitory effect of spinally administered receptors. Because the application of the stimulus to the receptive field.

Magnuson and Dickenson, 1991). Opioids administered into rons (Woolf and Fitzgerald, 1981; Sastry and Goh, 1983; Pan, 2003), it is possible that the inhibitory effect of morphine on dorsal horn projection neurons is secondary to its effect on superficial dorsal horn neurons. Hence, increased excitability of lamina II (presumably inhibitory) interneurons by morphine may indirectly modulate the excitability of deep dorsal horn projection neurons. Many lamina II neurons are inhibitory interneurons, and lamina II is an important site for regulation of nociception and the analgesic action of spinally administered opioids. It has been shown that morphine increases the activity of lamina II neurons but suppresses the firing of deeper dorsal horn neurons (Woolf and Fitzgerald, 1981; Sastry and Goh, 1983; Magnuson and Dickenson, 1991). Opioids administered into the substantia gelatinosa (lamina II) reduce the excitability of deep dorsal horn projection neurons in normal and sham control rats. These data suggest that morphine most likely inhibits the response of dorsal horn projection neurons by augmentation of the GABAergic and glycine inputs. Consistent with our finding, it has been shown that the effects of spinally applied morphine on lumbar wide-dynamic-range neurons are largely attenuated by spinal bicuculline (Reeve et al., 1998). Notably, the inhibitory effect of morphine on dorsal horn projection neurons was attenuated to a greater extent by bicuculline than strychnine. Thus, the GABAergic tone appears to be more important than the glycine receptors in the inhibitory effect of morphine on dorsal horn projection neurons.

The mechanisms of potentiation of GABAergic and glycine inputs to dorsal horn projection neurons by opioids are not clear. There is no good evidence showing that µ opioids can directly excite dorsal horn inhibitory neurons. It is possible that the µ opioids increase GABAergic and glycine inputs to dorsal horn projection neurons through at least two indirect mechanisms. As illustrated in Fig. 6, activation of presynaptic and postsynaptic µ opioid receptors can inhibit glutamate release and the glutamatergic interneurons in lamina II (Schneider et al., 1998; Kohno et al., 1999; Light and Willcockson, 1999). The reduced glutamatergic inputs to the next inhibitory interneurons could decrease the excitability of the inhibitory interneuron. Consequently, the reduced release of GABA and glycine (disinhibition) to the interneuron that synapses with the projection neuron could lead to increased inhibitory tone to the projection neuron. Furthermore, stimulation of presynaptic and postsynaptic µ opioid receptors can depress the excitability of the inhibitory interneurons in lamina II by decreasing glutamatergic inputs and direct hyperpolarization of the cell (Fig. 6). As a result, it could reduce GABA and glycine release (disinhibition) to the interneuron that synapses directly with the projection neuron, which would increase the inhibitory tone to the dorsal horn projection neuron.

We found that the baseline activity and evoked responses of dorsal horn projection neurons in nerve-injured rats were increased significantly than those in normal and sham control rats. Increased glutamatergic inputs to spinal dorsal horn neurons constitute an important mechanism for central sensitization and development of neuropathic pain syndromes following nerve injury (Harris et al., 1996; Tolle et al., 1996). Importantly, GABAergic terminals also make synapses with the primary afferent terminals in the superficial dorsal horn to regulate glutamate release through GABAergic receptors (Barber et al., 1978; Li et al., 2002). Nerve injury-induced loss of GABAergic interneurons in the spinal cord is another important mechanism that probably contributes to

![Fig. 4. Original tracings showing the inhibitory effect of topical application of 10 µM morphine on the evoked response of a dorsal horn projection neuron before and 10 min after topical application of 20 µM bicuculline or 4 µM strychnine in a nerve-ligated rat. Arrows indicate the time point of application of the stimulus to the receptive field.](image-url)
hypersensitivity of dorsal horn neurons and neuropathic pain (Castro-Lopes et al., 1993; Ibuki et al., 1997; Moore et al., 2002). In this regard, several studies have shown that selective loss of GABAergic, but not glycinergic, inhibition in the spinal dorsal horn plays a critical role in hyperexcitability of dorsal horn neurons in nerve-injured rats (Castro-Lopes et al., 1993; Ibuki et al., 1997; Moore et al., 2002; Drew et al., 2004). We observed that topical application of bicuculline caused a marked increase in the evoked response of dorsal horn projection neurons in normal and sham controls but not in nerve-ligated rats. On the other hand, topical application of strychnine produced a similar enhancement in the response of dorsal horn projection neurons to graded mechanical stimuli in control and nerve-injured rats. Many inhibitory interneurons in the dorsal horn contain glycine and GABA, although a population of cells contains only GABA without glycine (Todd and Sullivan, 1990). These data provide further evidence that the spinal GABAergic input to dorsal horn projection neurons is selectively reduced following nerve injury, which probably contributes to central sensitization and chronic neuropathic pain.

The most salient finding of this study is that reduced GABAergic/glycinergic tone is directly linked to attenuated morphine effect on dorsal horn projection neurons in neuropathic pain. The mechanisms underlying reduced analgesic effect of opioids in neuropathic pain are not fully understood. It remains controversial whether reduced μ opioid receptors in the spinal cord account for diminished opioid effects in neuropathic pain (Stevens et al., 1991; Porreca et al., 1998; Zhang et al., 1998). We found that topical application of bicuculline had little influence on the inhibitory effect of morphine on dorsal horn projection neurons in nerve-injured rats but almost abolished the effect of morphine in normal and sham control rats. In comparison, spinal application of strychnine significantly attenuated the inhibitory effect of morphine on dorsal horn neurons in both normal and nerve-injured rats. Therefore, it is likely that loss of GABAergic tone following nerve injury not only plays a role in hypersensitivity of dorsal horn neurons but also contributes to the reduced analgesic effect of morphine in the spinal cord. It should be noted that degeneration of central terminals of primary afferents following nerve injury may result in a loss of presynaptic μ opioid receptors in the spinal dorsal horn, which also may reduce the antinociceptive action of morphine at the spinal level in neuropathic pain (Zhang et al., 1998). Nevertheless, data from our study provide an important alternative mechanism that could explain, at least in part, the reduced analgesic effect of morphine in neuropathic pain. It has been shown that μ opioids and GABA<sub>A</sub> receptors can interact synergistically in the spinal cord to produce analgesia (Yanez et al., 1990). The new information from the present study is also important for the design of new strategies to improve opioid efficacy in neuropathic pain.
References