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Centralization of Noxious Stimulus-induced Analgesia (NSIA) is Related to Activity at Inhibitory Synapses in the Spinal Cord

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Abstract

The duration of noxious stimulus-induced antinociception (NSIA) has been shown to outlast the pain stimulus that elicited it, however, the mechanism that determines the duration of analgesia is unknown. We evaluated the role of spinal excitatory and inhibitory receptors (NMDA, mGluR-5, mu-opioid, GABA-A, and GABA-B), previously implicated in NSIA initiation, in its maintenance. As in our previous studies, the supraspinal trigeminal jaw-opening reflex (JOR) in the rat was used for nociceptive testing because of its remoteness from the region of drug application, the lumbar spinal cord. NSIA was reversed by antagonists for two inhibitory receptors (GABA-B and mu-opioid) but not by antagonists for either of the two excitatory receptors (NMDA and mGluR-5), indicating that NSIA is maintained by ongoing activity at inhibitory synapses in the spinal cord. Furthermore, spinal administration of the GABA-B agonist baclofen mimicked NSIA in that it could be blocked by prior injection of the mu-opioid receptor antagonist H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP) in nucleus accumbens. CTAP also blocked baclofen antinociception when administered in the spinal cord. We conclude that analgesia induced by noxious stimulation is maintained by activity in spinal inhibitory receptors.

Introduction

Analgesia equivalent in magnitude to that of high dose morphine can be produced by noxious (i.e., painful) stimulation; subdermal capsaicin injection attenuates the trigeminal jaw-opening reflex (JOR) equivalent in magnitude to inhibition by 10 mg/kg morphine for more than an hour [13]. Antinociception induced by noxious stimulation (NSIA) is mediated by an ascending pain modulation system that originates in the spinal cord and includes nucleus accumbens circuitry in the ventral striatum. At the level of nucleus accumbens, NSIA can be blocked by prior microinjection of μ - or δ -opioid receptor antagonists [13,25,27].

Under basal physiological conditions ongoing activity ascending from the spinal cord inhibits nucleus accumbens-mediated antinociception. Peripheral noxious stimulation activates inhibitory spinal mechanisms that suppresses this ascending activity, disinhibiting an

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opioidergic/dopaminergic link in nucleus accumbens to produce antinociception. The spinal mechanisms activated by noxious stimulation include both excitatory (NMDA and mGluR₅) and inhibitory (μ -opioid, GABA_B, and GABA_A) receptors [31,32].

An important feature of NSIA that needs to be explained is its duration. Although, peripheral nerve block before noxious stimulation blocks NSIA, the same block given afterwards has no effect on the time course of NSIA [13]. Thus, the long duration of NSIA (i.e., greater than one hour) cannot be explained by ongoing activity in primary afferent nociceptors. The aim of this study was to investigate whether the time course of NSIA is determined by a spinal mechanism.

Materials and methods

Animals

Experiments were performed on 280 – 350 g male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA). Experimental protocols were approved by the University of California San Francisco Committee on Animal Research and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, revised 1996. All efforts were made to minimize the number of animals used and their suffering.

Nociceptive assay

Changes in nociception were measured as attenuation (i.e., antinociception) or enhancement (i.e., hyperalgesia) of the trigeminal jaw-opening reflex (JOR) electromyographic (EMG) signal [13,14,22]. This assay was employed because it is segmentally remote from the hind paw where the noxious stimulus is applied, thus allowing separation of heterosegmental effects from any intrasegmental effects that might influence assays such as the paw-withdrawal reflex or the tail flick reflex. Previous studies show examples of the effect of intraplantar capsaicin on the JOR EMG traces [27].

Anesthesia

Experiments were performed in rats anesthetized with an intraperitoneal injection of 0.9 g/kg urethane and 45 mg/kg α -chloralose (both from Sigma-Aldrich, St. Louis, MO). This method provides a state of anesthesia with stable physiological parameters [8] and a stable JOR EMG signal [14] over the time period required to complete the experiments.

Electrode implantation

To evoke the JOR, a bipolar stimulating electrode, consisting of two insulated copper wires (36 AWG), each with 0.2 mm of insulation removed from the tips, one tip extending 2 mm beyond the other, was inserted into the pulp of a mandibular incisor to a depth of 22 mm from the incisal edge of the tooth to the tip of the longest wire and cemented into place with dental acrylic resin. A bipolar recording electrode, consisting of two wires of the same material as the stimulating electrode with 4 mm of insulation removed, was inserted into the anterior belly of the digastric muscle ipsilateral to the implanted tooth to a depth sufficient to completely submerge the uninsulated end of the wire.

JOR electromyogram

Because the JOR is a nociceptive reflex only when the stimulus intensity is sufficient to activate nociceptors, the stimulation current was set at 3 times threshold to assure that this would be the case [22]. Each data point consisted of the average peak-to-peak amplitude of 12 consecutive jaw-opening reflex EMG signals evoked by stimulating the tooth pulp with 0.2 ms square wave pulses at a frequency of 0.33 Hz. Pre-intervention baseline amplitude was defined as the average of the last 3 data points, recorded at 5-minute intervals, before an

experimental intervention. As is customary for JOR studies [1-3,9,10,13,14,26,29,36,37], data were normalized for differences in baseline by calculating the percentage change from baseline for each post-intervention data point. These values were used in the statistical analyses and were also plotted (mean $\pm$ s.e.m.) in the figures such that JOR attenuation is represented on the y-axis as greater positive numbers (*i.e.* antinociception); JOR enhancement (*i.e.* “hyperalgesia”) is represented as greater negative numbers. In all figures the x-axis represents the time in minutes following the first (or only) experimental intervention, which, in most cases, was an intraplantar capsaicin injection; in cases where the effect of the i.t. treatment alone was tested, the x-axis represents the time from the i.t. injection.

Nucleus accumbens drug administration

Bilateral 23 gauge stainless steel guide cannulae were stereotaxically positioned and fixed into place with orthodontic resin (L.D. Caulk Co., Milford, DE, USA). Intra-accumbens drug administration was accomplished via insertion of a 30 gauge stainless steel injection cannula, which extended 2 mm. beyond the guide cannula, connected to a 2 μ l syringe (Hamilton, Reno, NV, USA). The stereotaxic coordinates for the tip of the injection bilaterally positioned cannulae were 1.3 mm rostral, 7.2 mm ventral, and 1.8 mm lateral from bregma. Injection volumes in all experiments were 0.5 μ l and were carried out over a period of 2 minutes after which the cannula was left in place an additional 30 seconds.

Administration sites were verified by histological examination (100 μ m sections stained with cresyl violet acetate) and were plotted on coronal maps adapted from the atlas of Paxinos and Watson [24].

I.t. drug administration

I.t. administration of drugs to the lumbar region of the spinal cord was performed through a polyethylene catheter 10 μ l in volume (PE-10, Intramedic, Clay Adams, Becton-Dickinson, Franklin Lakes, NJ) inserted 8.5 cm caudally into the subarachnoid space through a slit in the atlanto-occipital membrane [35]. Rats were placed on an inclined surface (approximately 30° with the head higher than the tail) to retard rostral spread of the injected drug [14]. Drugs were injected in 15 μ l volumes followed by 10 μ l of vehicle to flush the tubing. Injections were performed at a rate of 6 μ l/minute.

Drugs and doses

Capsaicin (E-capsaicin, Sigma) was initially dissolved in Tween 80 (50%) and ethanol (50%) to a concentration of 50 μ g/ μ l and then diluted with 0.9% saline to a concentration of 5 μ g/ μ l; subdermal capsaicin injection volume was 50 μ l (250 μ g) in all experiments.

The following receptor antagonists were administered intrathecally: LY235959 ((-)-6-phosphonomethyl-decahydroisoquinoline-3-carboxylic acid) the active isomer of LY274614, both of which are highly selective competitive NMDA receptor antagonists with no appreciable affinity for AMPA/kainate receptors [28]; MPEP (methylphenylethynylpyridine), an mGluR₅-receptor antagonist originally reported to lack cross reactivity with other glutamate receptors [12]; H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP), a highly selective μ -opioid receptor antagonist [18]. Bicuculline and CGP35348 are generally regarded as selective antagonists for GABA_A and GABA_B receptors, respectively [15,17,23] see [16] for review}. All drugs were dissolved in phosphate buffered saline (PBS). LY235959, MPEP and CGP35348 were obtained from Tocris, Ellisville, MO and all other drugs were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Data analysis

A two-way repeated measures ANOVA with one between-subjects factor (i.e., treatment) and one within subjects factor (i.e., time) was used to determine if there were significant ($p \leq 0.05$) differences in antinociceptive responses among the groups. For experiments with only two groups, only the group $\times$ time interaction (which, if significant, indicates that the groups differed from each other over time) is reported. For experiments with three or more treatment groups, the main effect of treatment group is also reported in order to run Tukey post hoc analyses to determine which groups were significantly different. The main effect of time was significant in all experiments, but is not reported because it doesn't help to distinguish the groups from each other.

Results

To identify spinal receptors required for NSIA maintenance, receptor selective antagonists were administered intrathecally 30 minutes *after* intraplantar capsaicin administration, the time at which NSIA is maximal [13]. Phosphate buffered saline (PBS), the vehicle for the antagonists, was administered as a control. Antagonists were chosen on the basis of previous findings implicating these receptors in NSIA initiation [31,32].

Excitatory receptors

Intraplantar capsaicin (250 μ g) was administered following baseline recordings. Thirty minutes later, the NMDA receptor antagonist LY235959 (8.1 ng); the mGluR₅ antagonist MPEP (12 μ g), or PBS were administered intrathecally in separate groups of rats (Fig. 1). The two groups receiving the antagonists did not differ significantly from the group receiving PBS or from each other (group $\times$ time interaction: $F_{(8,60)} = 1.501$, $p=0.216$; main effect of treatment group: $F_{(2,15)} = 0.316$, $p=0.734$), indicating lack of involvement of these receptors in NSIA maintenance. (See Discussion section for comments on AMPA/kainate receptors.)

Inhibitory receptors

Intraplantar capsaicin (250 μ g) was administered following baseline recordings. Thirty minutes later, the μ -opioid receptor antagonist CTAP (2 μ g, Fig. 2A); the GABA_B receptor antagonist CGP35348 (270 μ g, Fig. 2B); the GABA_A receptor antagonist bicuculline (3 μ g, Fig. 2C), or PBS were administered intrathecally in separate groups of rats. The groups receiving CTAP or CGP35348 both differed significantly from the group receiving PBS (CTAP: $F_{(4,40)} = 5.643$, $p=0.0005$; CGP35348: $F_{(4,56)} = 4.901$, $p=0.010$) in ability to reverse NSIA, indicating that, after initiation, the maintenance of NSIA is dependent on both μ -opioid and GABA_B receptors. The group receiving bicuculline showed a trend toward NSIA reversal, but the effect was not significant ($F_{(4,48)} = 2.495$, $p=0.101$).

GABA_B receptor agonism

Because GABA_B receptors are required for NSIA maintenance (Fig. 2B), we investigated whether activation of these receptors is *sufficient* to induce accumbens-mediated antinociception. I.t. administration of the selective GABA_B-receptor agonist baclofen (10 μ g) induced significant antinociception (Fig. 3), an effect that was not observed when baclofen was combined with CTAP (2 μ g) in the same spinal injection. Baclofen-induced antinociception was also blocked by CTAP microinjected into nucleus accumbens 10 minutes prior to the spinal injection, indicating that it is mediated by the same nucleus accumbens circuitry that mediates NSIA [13] (group $\times$ time interaction $F_{(8,60)} = 3.350$, $p=0.012$; main effect of treatment group $F_{(2,15)} = 8.910$, $p=0.003$, post-hoc analyses showed that the group receiving i.t. baclofen alone differed from the group receiving i.t. CTAP ($p = 0.012$) as well as the group

receiving intra-accumbens CTAP ($p=0.004$). The two groups receiving CTAP, however, did not differ significantly from each other ($p=0.840$).

Discussion

The current study extends earlier work in which we have described a novel neural circuit that mediates pain-induced analgesia/noxious stimulus-induced antinociception (NSIA). These studies have shown that NSIA is mediated by circuits in nucleus accumbens [13,25,27] and the spinal cord [30-32]. A previously unexplained finding was the observation that nerve block prevents but does not reverse NSIA [13], suggesting that primary afferent nociceptive input is necessary for NSIA initiation but not for maintenance. Since NSIA lasts more than one hour, the present study was undertaken to investigate whether a spinal mechanism explains NSIA persistence in the absence of peripheral input.

To initiate NSIA, glutamate, released by primary afferent nociceptors, acts at spinal NMDA and mGluR₅ receptors [32]. Although doses of the receptor antagonists administered were able to *block* NSIA in the previous study, these same doses were unable to *reverse* NSIA in the current study, an observation that is compatible with the lack of ongoing peripheral input. To confirm this result, however, a full dose response analysis would be needed. AMPA/kainate receptors, which also play a role in NSIA initiation [30], could not be tested in the current study because these receptors have opposing effects on nociception, depending on where they are located in the spinal circuit [30,32].

NSIA initiation also depends on activation of inhibitory receptors, namely GABA_A, GABA_B and μ -opioid receptors [31]. In the present study we demonstrated that spinal intrathecal administration of GABA_B and μ -opioid receptor antagonists, but not a GABA_A receptor antagonist, can reverse NSIA, suggesting that the proposed inhibitory interneuron (see schematic diagram, Fig. 4) remains tonically active for a period of time following cessation of peripheral input, releasing GABA/endogenous opioids. Consistent with this, GABA and enkephalins are known to co-localize in spinal neurons [33,34]. Although the mechanism of this tonic inhibitory activity remains to be investigated, it is unlikely to result from non-specific effects of the inhibitory antagonists, since these drugs have no significant effect on nociceptive responses when administered alone [31]. It is possible that prolonged metabolic changes induced by Ca²⁺ influx into these neurons contribute. Consistent with this idea, activation of mGluR₅ and NMDA receptors (presumably, during NSIA initiation) increases cytosolic Ca²⁺ [11,20] and the function of GABAergic neurons following peripheral capsaicin injection is mediated by NMDA receptors [38].

Effect of receptor agonists

In the current study we found that spinal administration of the GABA_B-receptor agonist baclofen induces heterosegmental antinociception that can be blocked by intra-accumbens administration of a selective μ -opioid receptor antagonist, suggesting that activation of spinal GABA_B receptors is sufficient to mimic the antinociceptive effect of noxious stimulation. However, because in an earlier study we found that spinal administration of the selective μ -opioid receptor agonist DAMGO also induces supraspinally mediated antinociception [14], we investigated whether GABA_B receptors alone are sufficient or whether μ -opioid receptors must also play a role. Coadministration of the μ -opioid receptor antagonist CTAP with baclofen blocked the antinociceptive effect of baclofen, indicating that both μ -opioid and GABA_B receptors are required in this circuit.

NSIA and counter-irritation

Counter-irritation (CI) analgesia produced by noxious or painful stimulation has been used for millennia to treat pain of diverse etiology. Capsaicin, the pungent ingredient of chili hot peppers, and arguably the most commonly used counter-irritation therapy world wide, and heat have both been used extensively as CI therapy to relieve pain. Capsaicin therapy has been judged to be “clinically successful” [21]. Sauna, moxa, paraffin baths for arthritic extremities, and physical therapy hot packs, acupuncture, and transcutaneous electrical nerve stimulation (TENS) have been widely advocated as counter-irritation remedies.

NSIA may account mechanistically for some of these forms of counter-irritation therapies for the treatment of pain, but probably not for all of them. Diffuse noxious inhibitory controls (DNIC) is produced by noxious stimulation and has been proposed as a mechanism for counter-irritation. However, in contrast to NSIA, DNIC lasts less than five minutes in most studies and is dependent on the duration of the noxious stimulus [19]. Consistent with this, some counter-irritation modalities, for example, TENS, are most effective during active application, suggesting that ongoing peripheral stimulation is required. As shown in the current study, however, once initiated, NSIA undergoes centralization, without need for ongoing peripheral input. DNIC and NSIA also differ in that DNIC is principally a spinal system that is unaffected by total medullary transection rostral to subnucleus reticularis dorsalis in the caudal medulla [4-7], whereas, in addition to the spinal cord, NSIA is mediated in nucleus accumbens in the ventral striatum [13].

In summary, this study reports that maintenance of NSIA is mediated by inhibition of the same ascending pathway involved in its induction. It is proposed that activation of μ -opioid and GABA_B receptors by tonically released endogenous opioids and GABA in the spinal cord in response to intense peripheral noxious stimulation suppresses tonic ascending activity to induce heterosegmental antinociception mediated by nucleus accumbens.

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Figure 1. Effect of excitatory receptor antagonists

Capsaicin (Cap) was administered immediately after the last baseline recording. The antagonist or vehicle (PBS) was administered intrathecally immediately after the recording at 30 minutes. Compared to PBS (n=8), neither the competitive NMDA receptor antagonist LY367385 (n=5) nor the mGluR₅ receptor antagonist MPEP (n=5) significantly affected NSIA.

In this and subsequent figures data are presented as percentage attenuation of the JOR EMG (mean $\pm$ s.e.m.); see Methods for additional details regarding data presentation and analysis.

Figure 2. Effect of inhibitory receptor antagonists

Capsaicin (Cap) was administered immediately after the last baseline recording. Follow capsaicin administration, the antagonist or vehicle (PBS, n=8) was administered intrathecally immediately after the recording at 30 minutes.

A. The selective μ -opioid receptor antagonist CTAP (n=4) significantly *reversed* capsaicin-induced attenuation of the JOR.

B. The selective GABA_B-receptor antagonist CGP35348 (n=8) significantly *reversed* capsaicin-induced attenuation of the JOR.

C. The GABA_A-receptor antagonist bicuculline (n=6) did not significantly reverse capsaicin-induced attenuation of the JOR.

Figure 3. Effect of GABA_B receptor agonist baclofen

Baclofen injected i.t. as a single agent significantly attenuated the JOR (n=6), an effect that was blocked by prior administration of CTAP into nucleus accumbens (n=6) or by intrathecal co-administration of CTAP with baclofen (n=6).

Figure 4. Schematic diagram of proposed spinal NSIA circuit

Top panel. Under basal conditions, in the absence of noxious stimulation, tonically active neurons (red) project supraspinally to inhibit accumbens (“NAc”)-mediated antinociception. Note lack of activity in the incoming primary afferent nociceptor (“PAN”) and the inhibitory interneuron (both black). The activity in the ascending neuron results from ongoing glutamatergic synaptic input arising from an unknown intrinsic source [30]. *Middle panel.* When noxious stimulation, depicted as a red chili pepper, is applied, activating the PAN (now red), the inhibitory interneuron (also now red) is activated by various glutamate receptors. Activity in this neuron in turn inhibits activity in the ascending neuron (now black), thereby disinhibiting accumbens-mediated antinociception (i.e., NSIA). *Bottom panel.* After cessation

of primary afferent nociceptor (“PAN”) activity (now black), the inhibitory interneuron remains active (red), maintaining tonic inhibition of the ascending neuron (black). This decoupling of the inhibitory interneuron from peripheral input is what we refer to as “centralization.”