Lateral Hypothalamic-Induced Alpha-Adrenoceptor Modulation Occurs in a Model of Inflammatory Pain in Rats

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Previous work from our lab showed that stimulation of the lateral hypothalamus (LH) produces analgesia (antinociception) in a model of thermal nociceptive pain. This antinociceptive effect is mediated by \( \alpha_2 \)-adrenoceptors in the spinal cord dorsal horn. However, a concomitant, opposing hyperalgesic (pronociceptive) response also occurs, which is mediated by \( \alpha_1 \)-adrenoceptors in the dorsal horn. Antinociception predominates but is attenuated by the pronociceptive response. To determine whether such an effect occurs in a model of inflammatory pain, we applied mustard oil (allyl isothiocyanate; 20 \( \mu \)l) to the left ankle of female Sprague-Dawley rats. We then stimulated the LH using carbamylcholine chloride (carbachol; 125 nmol). The foot withdrawal latencies were measured. Some rats received intrathecal \( \alpha \)-adrenoceptor antagonists to determine whether the opposing \( \alpha \)-adrenoceptor response was present. Mustard oil application produced hyperalgesia in the affected paw, while the LH stimulation increased the foot withdrawal latencies for the mustard oil paw as compared to the control group. Following carbachol microinjection in the LH, WB4101, an \( \alpha_1 \)-adrenoceptor antagonist, produced significantly longer foot withdrawal latencies compared to saline controls, while yohimbine, an \( \alpha_2 \)-antagonist, decreased the foot withdrawal latencies from 10 min postinjection \((p < .05)\). These findings support the hypothesis that the LH-induced nociceptive modulation is mediated through an \( \alpha \)-adrenoceptor opposing response in a model of inflammatory pain.

Keywords: antagonist; A7 cell group; norepinephrine

Substantial evidence is accumulating to show that the lateral hypothalamus (LH) plays an important role in the endogenous modulation of pain (nociception). The LH receives nociceptive impulses from the spinal cord dorsal horn via the spinohypothalamic tract (Burstein, Cliffer, & Giesler, 1987; Burstein, Dado, Cliffer, & Giesler, 1991). In models of nociceptive pain, stimulation of the LH produces significant analgesia (antinociception) in rats (Aimone & Gebhart, 1987; Behbehani, Park, & Clements, 1988; Dafny et al., 1996; Franco & Prado, 1996; Fuchs & Melzack, 1995). Although these studies were done in male rats, we stimulated the LH with the cholinergic agonist carbamylcholine chloride (carbachol) to produce antinociception in female rats on the tail flick and foot withdrawal tests of thermal nociceptive pain that lasts for more than 60 min and is blocked by pretreatment with the cholinergic antagonist atropine sulfate (Holden & Naleway, 2001; Holden, Naleway, & Jeong, 2005). We also found that the LH stimulation produces an opposing \( \alpha \)-adrenoceptor mediated response. In this response, \( \alpha_2 \)-adrenoceptors in the spinal cord dorsal horn mediate antinociception, while a concurrent pronociceptive, or hyperalgesic, response to the painful stimulus is mediated by \( \alpha_1 \)-adrenoceptors in the dorsal horn.
The antinociceptive effect predominates but is attenuated by the concurrent pronociception. This opposing response is also seen when the A7 catecholamine cell group, a group of spinally descending noradrenergic neurons, is activated directly with microinjection of morphine (Holden, Schwartz, & Proudfit, 1999) or bicuculline (Nuseir & Proudfit, 2000) or indirectly by stimulating the ventromedial medulla (Brodie & Proudfit, 1986), which innervates the A7 cell group (Buhler, Proudfit, & Gebhart, 2004). As spinally descending noradrenergic neurons have not been identified in the LH, it is likely that the LH innervates the A7 cell group and that this connection mediates the LH-induced, \( \alpha \)-adrenergic-opposing response in the nociceptive model of pain.

Pain that has a strong inflammatory component differs from the brief pain stimulus used in the thermal nociceptive model described earlier. Persistent inflammatory pain can alter anatomical, physiological, and genetic functions of dorsal horn neurons and can promote hyperactive responses (Kidd & Urban, 2001; Sweitzer, White, Dutta, & DeLeo, 2002; Traub, 1996). These increased responses contribute to the development of symptoms such as primary hyperalgesia (an increased response to noxious stimuli at the injury site) and secondary hyperalgesia (hyperalgesia in tissue adjacent to the injury site). Whether LH stimulation produces antinociception in inflammatory pain or whether the \( \alpha \)-adrenergic system is involved is not known.

Topical application of mustard oil produces inflammation and exaggerated responses to a variety of noxious stimuli via the c-fiber primary afferent activation (Jiang & Gebhart, 1998; Urban, Jiang, & Gebhart, 1996). To determine the role of the LH stimulation in modulating inflammatory pain elicited by mustard oil application, we applied mustard oil to the left ankle of female Sprague-Dawley rats and stimulated the LH using carbachol, a nonselective cholinergic agonist that activates neurons in the LH and elsewhere (Brodie & Proudfit 1986; Holden & Naleway 2001; Holden et al., 2005; Klamt & Prado 1991; Leite-Panissi, Brentegani, & Menescal-de-Oliveira, 2004). In previous studies, we demonstrated that 125 nmol of carbachol microinjected into the LH produces optimum antinociception in the acute thermal model of nociceptive pain (Holden & Naleway, 2001; Holden et al., 2005), and this dose was used in the present experiments. A thermal heat source was used as the stimulus to measure the foot withdrawal latencies. Some groups of rats received \( \alpha \)-adrenoceptor antagonists to determine whether the opposing bidirectional response was present. Because our previous studies were conducted in female rats, we used female Sprague-Dawley rats for the present study. Preliminary findings have been published as an abstract (Jeong, Pizzi, & Holden, 2004).

**Method**

The Institutional Animal Care Committee at the University of Illinois at Chicago approved the experimental protocols used in this study. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 90-23, revised 1978). All efforts were made to minimize animal suffering, reduce the numbers of animals used, and use alternatives to in vivo experiments.

**Design and Sample**

The pre–posttest experimental design with control groups was used for this series of behavioral experiments. Adult female Sprague-Dawley rats (250-350 g; Charles River, Portage, MI) were randomly assigned either to the treatment group or to the control group. A total of 29 rats were used and each rat was only used once. To reduce the possibility of estrous cycle influence, the rats were randomly assigned to groups and no 2 rats were taken from the same cage on the same day. Nociceptive modulation was manipulated through intracranial and intrathecal injections and outcomes measured through nociceptive testing using the foot withdrawal test.

**Procedures**

Adult female Sprague-Dawley rats were lightly anesthetized with intraperitoneal pentobarbital injection (35 mg/kg) and immobilized in a stereotaxic frame. Light anesthesia was maintained throughout the testing procedure as evidenced by the absence of vocalization, writhing, and other signs of conscious withdrawal from painful stimuli. Supplemental doses of pentobarbital were given during the 80-min procedure if the rats vocalized or moved without stimulation, but supplementation was rarely required. Rats were placed on a warming pad during the procedure and the body temperature was maintained within normal limits as shown by the rectal temperature. The midline scalp was infused with bupivacaine.
which provides local anesthetic relief for approximately 24 hr (J. E. Artwohl, personal communication). An incision was made along the midline of the scalp, and a burr hole was drilled into the skull to allow insertion of a microinjection guide cannula into the LH. A 23-gauge stainless steel guide cannula was lowered into the region of the left LH through the burr hole to the predetermined position. A 32-gauge stainless steel microinjection cannula was connected to a 10-\(\mu\)l syringe by a length of PE-10 polyethylene tubing. This tubing was filled with a solution of carbachol (Sigma, St. Louis, MO) dissolved in physiological saline and filtered through a syringe filter. Normal saline was used for control (see Figure 1).

For intrathecal injection, an incision was made over the occiput of the skull, from a line between the ears, and a small incision made in the atlanto-occipital membrane. A 32-gauge intrathecal catheter was carefully advanced to a position 8 cm caudal of the cisterna at the level of lumbar enlargement (Yaksh & Rudy, 1976).

**Behavioral Tests**

To determine the effect of carbachol microinjection in the LH on mustard oil–induced pain, the foot withdrawal test of nociception was used. Briefly, the hairy surface of the hind paws was blackened with India ink to facilitate more uniform heating of the skin surface by the noxious thermal stimulus. A focused beam of high-intensity light was directed at the lateral aspect of the hairy surface of the hind paws and a single-response latency was measured on each hind paw. The time interval between the onset of skin heating and the withdrawal response was measured electronically. In the absence of a response, the skin heating was terminated after 8 s to prevent burning of the skin. The low cutoff, or the shortest measurable time that it takes the rat to withdraw its paw, was 1 s. The average response latencies of the paws and tail were approximately 2–3 s. The stimulation of the LH was accomplished as follows: An initial response latency was measured 1 min prior to microinjection, then 125 nmol carbachol was microinjected in a volume of 0.5 \(\mu\)l over a 1-min period using an electronic syringe pump. The microinjector was left in place for an additional 60 s prior to removal to reduce the flow of drug up the guide cannula. The foot withdrawal latencies were then measured at 5-min intervals for 50 min after microinjection or until they returned to baseline.

**Experiment 1. The Effect of Mustard oil Application on LH-induced Antinociception.**

Rats were prepared for intracranial injection as described earlier. Mustard oil (allyl isothiocyanate, 100%; Sigma) in a dose of 20 \(\mu\)l has been shown to produce hyperalgesic responses (Urban et al., 1996), and this dose was carefully placed directly on the shaved, lateral left ankle bone using a microliter syringe. Saline was applied to the lateral surface of the shaved right ankle as a control. After the application of either mustard oil or saline, a baseline response latency was taken, then carbachol was microinjected into the LH and the foot withdrawal latencies were measured.

**Experiment 2. The Effect of Intrathecal \(\alpha\)-Adrenoceptor Antagonists on the Foot Withdrawal Latencies.**

To determine whether the \(\alpha\)-adrenoceptor–mediated opposing response occurred in inflammatory pain, the rats were prepared for intracranial and intrathecal cannulation as previously described, and mustard oil was applied to the left ankle. Following a baseline

![Figure 1. Distribution of sites in the lateral hypothalamus (LH) at which carbachol was microinjected. AHC = anterior hypothalamic area, central; AMG = amygdala; C = caudate nucleus; CPU = caudate putamen; DH = dorsal hippocampus; DM = dorsomedial hypothalamic nucleus; f = fornix; ic = internal capsule; LV = lateral ventricle; PH = posterior hypothalamus; SI = substantia innominata; VH = ventral hippocampus; VM = ventromedial thalamic nucleus; VPL = ventroposterolateral thalamic nucleus; VPM = ventral posteromedial thalamic nucleus; ZI = zona incerta. Distance from bregma is approximately 3.80 mm.](image-url)
withdrawal measurement, carbachol (125 nmol/0.5 µl) was microinjected into the LH. After 1 min, 3 foot withdrawal measurements were made at 5-min intervals. Either the α1-adrenoceptor antagonist WB4101 or the α2-adrenoceptor antagonist yohimbine (97 nmol in 15 µl) or normal saline for control was then administered intrathecally. The foot withdrawal latencies were measured at 5-min intervals until a return to baseline was achieved.

Histology
At the end of each experiment, each rat was deeply anesthetized with sodium pentobarbital (80 mg/kg). The brains were removed and placed in a formalin fixative overnight followed by 20% sucrose solution. The brains were cut through the LH in 40-µm transverse sections on a cryostat microtome. The sections were rinsed 3 times in cold phosphate-buffered saline (10 mM), mounted on gelatin-coated slides, stained with 0.05% neutral red, and coverslipped. The placement of the microinjection cannula was determined by plotting the most ventral portion of the cannula tip in serial sections using the brightfield microscopy. Tracings of the appropriate sections were then made using the Neurolucida imaging system (Microbrightfield, Colchester, VT) and compared to the coordinates described by Paxinos and Watson (1998). Because the previous work has shown that areas near the LH can also produce antinociception (Holden & Naleway, 2001; Holden et al., 2005), data were excluded when the microinjection cannula was outside the LH. The position of the intrathecal cannula was determined by laminectomy. Data were excluded when the intrathecal cannula was in the spinal cord tissue to eliminate the possibility that prolonged foot withdrawal responses were due to motor deficits from ventral horn activation rather than from antinociceptive responses.

Statistical Analysis
Statistical comparisons of the foot withdrawal latencies among treatment groups across time points were made using means and standard errors of the means and multiple post hoc comparisons were made using repeated measure two-way analysis of variance (ANOVA) with Student Neuman-Kuels tests. Data from 25 rats were used in the analyses. Data from 4 rats were excluded because of microinjector misplacement.

Results
The Effect of The LH Stimulation on The Foot Withdrawal Latencies Following Mustard Oil Application
To determine whether mustard oil produces hyperalgesia in the affected paw, we applied mustard oil to the lateral surface of the shaved left hind ankle and saline to the shaved right ankle, then microinjected only saline into the LH. Mustard oil application significantly decreased the withdrawal latencies on the left paw compared to the right control paw \((F(1,54) = 487.25, p < .05; \text{Figure 2})\). Given that the low cutoff is 1 s, the left paw that had mustard oil applied to the ankle responded to the thermal stimulus 50% faster than the control paw, a hyperalgesic response.

To determine whether the LH stimulation produces antinociception in mustard oil–induced hyperalgesia, a second group of rats received carbachol (125 nmol) microinjection in the LH. Mustard oil was applied to the left ankle, while normal saline was applied to the right ankle for control. As seen in Figure 3, LH stimulation with carbachol increased the withdrawal latencies for the left mustard oil paw as compared to the control group that had mustard oil application but was given saline in the LH from the previous experiment \((F(1,4) = 18.18, p < .05)\). When compared to the group that was given carbachol in the LH but no mustard oil, there was no significant difference in the foot withdrawal responses, indicating that carbachol stimulation of the LH reduced mustard oil–induced hyperalgesia to the level of controls \((p > .05; \text{Figure 3})\).

The Effect of Intrathecal α-Adrenoceptor Antagonists on the Foot Withdrawal Latencies
To determine whether the LH-induced, bidirectional opposing effect mediated by α-adrenoceptor antagonists occurs with inflammatory pain, we applied mustard oil to the left ankle of rats, microinjected 125 nmol carbachol in the LH, then gave either WB4101, yohimbine, or normal saline intrathecally. Foot withdrawal latencies were then measured. The intrathecal injection of α-adrenoceptor antagonists produced significant differences \((F(2,12) = 10.99; p < .05; \text{Figure 4})\), and there was a significant interaction effect between antagonist and time.
The one-way ANOVA at a readjusted significance level \( (p = .005) \) revealed that antagonism of \( \alpha_1 \)-adrenoceptors by intrathecal WB4101 significantly increased foot withdrawal latencies compared to controls at 5 min \( (F(2,12) = 13.55; p < .005) \) and 10 min \( (F(2,12) = 16.75; p < .005) \) after intrathecal injection, indicating that \( \alpha_1 \)-adrenoceptors mediate early pronociceptive responses when the LH is stimulated. The \( \alpha_2 \)-adrenoceptor antagonist yohimbine significantly decreased withdrawal latencies \( (n = 5) \) but the difference was not statistically significant. Values are expressed as mean foot withdrawal values (seconds) \( \pm SEM \) and plotted on the ordinate as a function of time (minutes).

**Figure 2.** In the absence of carbachol stimulation, mustard oil applied to the left ankle of rats prior to the foot withdrawal measurements produced a significant hyperalgesic response to a thermal stimulus \( (n = 5; p < .05) \) compared to the right ankle, on which normal saline was applied. LH = lateral hypothalamus.

**Figure 3.** Lateral hypothalamus (LH) stimulation with carbachol increased foot withdrawal latencies on the side with mustard oil \( (n = 5) \) compared to those of the right paw (saline controls). Mean latency values \( \pm SEM \) are plotted on the ordinate as a function of time (minutes). Closed triangles represent rats that had mustard oil applied to the left ankle and saline microinjection in the LH, as in Figure 2, and are used for comparison purposes.

**Figure 4.** The effects of intrathecal antagonists on antinociception produced by microinjection of carbachol in the lateral hypothalamus (LH) as measured by foot withdrawal latencies following mustard oil application to the left ankle of rats. Following a baseline measurement at \(-15\) min, carbachol \( (125\ nmol) \) was microinjected into the LH and 3 foot withdrawal measurements were taken at \(-10, -5, \) and \(-1\) min. At time 0, intrathecal injection of \( 97\ nmol \) of WB4101 \( (n = 5) \) or yohimbine \( (n = 5) \) was given; 1 min later, the foot withdrawal latencies were measured. WB4101 produced a significant antinociceptive effect \( (n = 5) \) compared to the controls \( (n = 5) \). Yohimbine decreased withdrawal latencies \( (n = 5) \) but the difference was not statistically significant. Values are expressed as mean foot withdrawal values (seconds) \( \pm SEM \) and plotted on the ordinate as a function of time (minutes).

**Discussion**

In this study, we showed that mustard oil applied to the left ankle of rats produced significantly faster foot withdrawal latencies in the left paw (Figure 2). Application of mustard oil produces central sensitization (Park et al., 2006; Zhang et al., 2006) and acts specifically at the wasabi or TRPA1 receptor that plays an important role in nociceptor excitability and neurogenic inflammation due to tissue damage (Akopian, Ruparel, Jeske, & Hargreaves, 2007; Bautista et al., 2006; Trevisani et al., 2007). Such central sensitization
leads to primary hyperalgesia at the site of application and to secondary hyperalgesia adjacent to the treated skin area (Mansikka & Pertovaara, 1997; Urban et al., 1996; Walker & Fitzgerald, 2007). As we elicited a hyperalgesic response from the paw distal to the location of the mustard oil, our findings are supportive of the idea that mustard oil–induced pain produces secondary hyperalgesia.

An important finding of this study is that stimulating the LH with carbachol reduced foot withdrawal latencies to the level of controls in rats with mustard oil application. This finding supports the hypothesis that LH stimulation produces antinociception in inflammatory pain and extends earlier findings that demonstrate LH-induced antinociception in the thermal nociceptive model (Aimone & Gebhart, 1987; Behbehani et al., 1988; Dafny et al., 1996; Holden & Naleway, 2001; Holden et al., 2005).

In our final experiment, we gave receptor antagonists to block the effect of α1 and α2 receptors in the dorsal horn. An antagonist has no pharmacological effect itself and does not activate the receptor it binds to. Rather, it prevents or blocks the binding of a neurotransmitter or drug to the receptor. When a neurotransmitter is released at a synapse, it binds to a receptor and produces an effect. Giving an antagonist will block that effect and the outcome will be opposite to the effect produced by the neurotransmitter-receptor binding. In this study, we observed that LH stimulation recruited the α-adrenoceptor opposing bidirectional effect (Figure 4) and that the effect depended on the time that the foot withdrawal measurement was taken following antagonist administration. Part of the bidirectional response was that blocking α1 receptors increased the withdrawal latencies in a relatively intense fashion, which declined at 15-min postinjection. Because blocking the receptor produced antinociception, it shows that when norepinephrine binds to α1 receptors the effect is a pronociceptive, or hyperalgesic, response. The second part of the bidirectional response occurred following antagonism, or blockade, of α2-receptors. Intrathecal injection of yohimbine produced faster withdrawal latencies, or hyperalgesia. Therefore, the effect of norepinephrine binding to α2 receptors is analgesia or antinociception. The effect of yohimbine took over 10 min to develop and was of small magnitude. Although the effect of yohimbine was not strong, the overall behavioral effect was to reduce the withdrawal latencies by at least a full second at 5 time points. As hyperalgesic responses tend to be smaller in magnitude than analgesic responses, this finding, though small, is suggestive of behavioral involvement of the α2-adrenoceptors and represents antinociception (Holden et al., 1999; Nuseir & Proudfit, 2000; Yeomans & Proudfit, 1992; Yeomans, Clark, Paice, & Proudfit, 1992). Within the spinal cord dorsal horn, there is indisputable evidence that α-adrenoceptors modulate nociception. α2-Receptors exist on primary afferents as well as on intrinsic neurons in the superficial and deep dorsal horn and exert antinociception in diverse types of pain, including mustard oil–induced pain in rat pups (Walker & Fitzgerald, 2007) and neuropathic pain (see Millan, 2002 for review). α1-Adrenoceptors are located mainly on intrinsic neurons in the dorsal horn and, through inhibition of K+ channels and activation of Ca2+ currents, exert strong excitatory actions on nociceptive inputs. Their upregulation in models of primary afferent fiber injury and neuropathic pain has been well documented (Fuchs, Meyer, & Raja, 2001; Hord, Denson, Stowe, & Haygood, 2001; Millan, 1997; Nicholas, Hokfelt, & Pieribone, 1996; Pieribone, Nicholas, Dagerlind, & Hökfelt, 1994), and there is evidence that persistent activation of supraspinal sites by inflammatory pain actually increases the spinal sensitivity to pain (Guo et al., 2006; Pinto, Lima, & Tavares, 2007; Porreca, Ossipov, & Genhart, 2002; Urban & Gebhart, 1999; Smith, Beyer, & Brandt, 2006). The intense α1 pronociceptive response and the relatively small α2 antinociceptive response may contribute to this increased sensitivity. Further study is needed to elucidate the role of the LH-induced α-adrenergic response in inflammatory pain.

An alternative hypothesis for the effect of yohimbine is that this antagonist has been shown to act as a partial 5-HT1A agonist in the formalin test (Shannon & Lutz, 2000). We do not think yohimbine acts as such in the dorsal horn for several reasons. First, we found that 5-HT1A receptors mediate the LH-induced antinociception in a thermal nociceptive model (Holden et al., 2005). If yohimbine acted as a partial agonist in the dorsal horn, it would produce antinociception rather than the pronociceptive response we observed (Figure 4). There is substantial evidence that yohimbine blocks antinociception following direct activation of the A7 cells (Nuseir & Proudfit, 2000; Yeomans & Proudfit, 1992; Yeomans et al., 1992) and of the LH (Holden & Naleway, 2001). Furthermore, intrathecal yohimbine does not appear to act at 5-HT1A receptors in the hot plate (Takano & Yaksh, 1992) or the spinal nerve ligation model (Yaksh, Pogrel, Lee, & Chaplan, 1995),
findings that Shannon and Lutz (2000) point out. Given that all drugs in the Shannon and Lutz study were injected subcutaneously in a model where formalin is injected peripherally, it is likely that yohimbine was acting at peripheral rather than at central receptors. In further support of this argument, 2 recent studies identified a role for α2-adrenoceptor activity in a model of peripheral neuritis (Romero-Sandoval & Eisenach, 2006; Romero-Sandoval, McCall, & Eisenach, 2005), providing further evidence that yohimbine was acting at peripheral sites when given peripherally. As yohimbine was given intrathecal in the present study, it probably was not acting at central 5HT1A receptors.

In the present study, we used rats lightly anesthetized with sodium pentobarbital, and our findings support those of Han and colleagues, who also used mustard oil to induce hyperalgesia and inflammatory pain in a light anesthesia model (Han et al., 2008). Although pentobarbital has little effect on withdrawal responses in the absence of mustard oil, it has been shown to partially reduce the early component of the hyperalgesia (2–6 min following application) and prevents late hyperalgesia (42–120 min post application) following mustard oil application (Cleland, Lim, & Gebhart, 1994). Under conditions of pentobarbital anesthesia, we observed a hyperalgesic response from mustard oil that occurred from the first measurement at 1 min post application (Figure 2) and lasted for the 50 min of our experiment. The reason for this difference is not clear but may be related to the differences between the female rats we used in the present study and the male rats used in the study of Cleland and colleagues (1994). However, our last measurement occurred at the beginning of the late phase, so we may not have captured late hyperalgesic response as reported by Cleland and colleagues (Cleland et al., 1994).

In summary, we demonstrated that LH-induced, nociceptive modulation occurred in a model of inflammatory pain that was mediated in part through an α-adrenoceptor bidirectional opposing response in the dorsal horn. Our findings support the hypothesis that the LH modulates nociception in both acute and inflammatory pain in part through connections with spinally descending neurons in the A7 catecholamine cell group in the pons. A better understanding of normally functioning hypothalamic pain control systems has the potential to define some of the underlying mechanisms for pharmacological and nonpharmacological pain relief interventions devised by nurses, leading to improved pain management.

References


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