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An NK1 Receptor Antagonist Microinjected into the Periaqueductal Gray Blocks Lateral Hypothalamic-Induced Antinociception in Rats

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Abstract

Substantial data are accumulating that implicate the lateral hypothalamus (LH) as part of the descending pain modulatory system. The LH modifies nociception in the spinal cord dorsal horn partly through connections with the periaqueductal gray (PAG), an area known to play a central role in brainstem modulation of nociception. Early work demonstrated a putative substance P connection between the LH and the PAG, but the connection is not fully defined. To determine whether LH-induced antinociception mediated by the PAG is neurokinin1 (NK1) receptor-dependent, we conducted behavioral experiments in which the cholinergic agonist carbachol (125 nmol) was microinjected into the LH of lightly anesthetized female Sprague-Dawley rats (250–350 g) and antinociception was obtained on the tail flick or foot withdrawal tests. Cobalt chloride (100 nM), which reversibly blocks synaptic activation, blocked LH-induced antinociception. In another set of experiments, the specific NK1 receptor antagonist L-703-606 (5 µg) was microinjected in the PAG following LH stimulation with carbachol abolished LH-induced antinociception as well. Microinjection of cobalt chloride or L-703,606 in the absence of LH stimulation had no effect. These behavioral experiments coupled with earlier work provide converging evidence to support the hypothesis that antinociception produced by activating neurons in the LH is mediated in part by the subsequent activation of neurons in the PAG by NK1 receptors.

Keywords

analgesia; pain; A7 catecholamine cell group; ventromedial medulla

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Introduction

There is now considerable evidence to support the proposal that the lateral hypothalamus (LH) produces antinociception in part through connections with brainstem neurons. The LH produces antinociception [2],[8],[11],[16], [21],[22],[26],[27] that occurs partly through connections with the rostral ventromedial medulla (RVM) [1],[2],[3],[11],[27],[28] and the A7 catecholamine cell group in the dorsolateral pontine tegmentum. [1],[2],[25],[28]

It has long been known that the periaqueductal gray (PAG) plays a central role in modulating nociception, primarily through connections with descending brainstem serotonergic neurons in the RVM [7],[9],[25],[41] and noradrenergic neurons in the dorsolateral pontine tegmentum.[4],[18],[19],[30],[31] It is also known that the LH sends projections to the PAG [8],[10],[12],[40],[53] and produces antinociception through connections with the PAG. [1],[8] The neurotransmitter mechanisms by which this activation occurs have not been fully defined.

One putative neurotransmitter involved in LH-induced antinociception may be substance P, the tachykinin with the highest affinity for NK1 receptors. [54] Substance P-immunoreactive (SP-ir) neurons have been identified in the LH, [28],[37] the PAG contains SP-ir axon terminals[37] and NK1 receptors, [5] and substance P applied to PAG neurons acts at NK1 receptors. [14],[17],[23],[52] It is logical to propose that LH activation mediates antinociception in part through an NK1 receptor-dependent mechanism in the PAG implicating substance P.

To determine whether substance P plays a role in behavioral responses involving the LH and the PAG, we used the tail flick and foot withdrawal tests to measure responses to an acute thermal stimulus. We chose both tests because previous work has shown differences between tail and foot withdrawal latencies. [18],[19],[29] We microinjected 125 nmol carbamoylcholine (carbachol) to stimulate the LH. Carbachol, a cholinergic agonist known to activate neurons throughout the cerebral hemispheres, [34] was previously determined to provide optimum antinociception in a lightly anesthetized preparation at the 125 nmol dose. [26],[27],[28],[29] The LH contains both muscarinic and nicotinic receptors,[15],[45],[51] and we have shown that the actions of carbachol are mediated by cholinergic receptor activity in the LH.[27] Following carbachol-induced antinociception, we microinjected either a non-specific receptor blocker, cobalt chloride, a specific NK1 receptor antagonist, L-703,606, or normal saline for control into the PAG. Preliminary findings have been published as an abstract.[50]

Materials and Methods

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. 80-23, revised 1996. All efforts were made to minimize animal suffering, reduce the numbers of animals used, and use alternatives to in vivo experiments.

Animals

Female Sprague-Dawley rats 250–350 g Charles River, Portage, MI were used in this study because our previous work was done in females of this strain. Rats were allowed to acclimate to the environment for a minimum of two days prior to their use in the study. All rats were housed in cages with free access to food and water with two to three rats per cage, and maintained on a 12-hour day/night schedule. Testing took place between the hours of 8

am and 3 pm. A total of 41 rats were used for the behavioral study as reported, and each rat was used only once. Tail flick and foot withdrawal latencies were measured on each animal. To minimize the effects of estrus cycle on nociceptive responses, rats were randomly selected from multiple cages for each day's experiments.[24],[58],[59]

Analgesiometric testing procedures

The tail flick and foot withdrawal tests were used to measure nociceptive responses. Detailed accounts of the testing procedure have been reported elsewhere [28],[29]. Briefly, a focused beam of high intensity light directed at the dorsal surface of the tail and the hairy surface of the hind feet blackened with ink to facilitate even heating of the skin surface. 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. Three response latencies were measured in succession at three places on the tail and averaged to define the nociceptive response latency. For the foot withdrawal, response latencies were measured at one place on the hairy surface of each hind foot. Baseline response latencies of the paws and tail were approximately 2–3 s.

Carbachol or saline microinjection in the LH

Each rat was lightly anesthetized with sodium pentobarbital (35 mg/kg, IP), and the scalp was infused with the local anesthesia bupivacaine (0.25%; 0.10 ml). Supplemental doses of pentobarbital were given during the procedure if the rats vocalized or moved without stimulation, but were rarely required. Using aseptic technique, a 23-gauge stainless steel guide cannula was lowered into the left LH through a burr hole to a location defined by the following coordinates: AP, –1.5 mm from bregma; lateral, +1.7 mm; vertical, +1.5 mm, with the incisor bar set at –2.5 mm. A burr hole over the PAG was then made using the following coordinates: AP, –1.0 mm from the intra-aural line; lateral, –0.3 mm; vertical, +3.5 mm. A 30-gauge injection cannula connected to a 10 μ l syringe by a length of PE-10 polyethylene tubing filled with 125 nmol carbachol in normal saline in a volume of 0.5 μ l (Sigma, St. Louis, MO) was lowered into the LH and extended approximately 3 mm beyond the end of the guide cannula. A baseline latency measurement was taken and carbachol was injected into the LH over 1 min using an electronic syringe pump. The microinjector was left in place for 60 s to reduce drug flow up the guide cannula. Response latencies were then measured at 5 min intervals for 15 min. A second cannula was filled with a solution of either cobalt chloride (100 nM/0.5 μ l), L-703,606 (5 μ g in 0.5 μ l) or 0.5 μ l normal saline, lowered into the PAG, and microinjection made as described above. Based on findings from previous studies, response latencies were then measured every 5 min for 45 min or until a return to baseline occurred, as previous studies showed that LH stimulation lasts this long in the acute model. [26],[27],[28],[29]

A second group of lightly anesthetized rats was prepared with cannula placement only in the PAG, as described in the preceding experiment. Following a baseline measurement, cobalt chloride 100 nM or L-703,606 5 μ g in 0.5 μ l saline, or saline for control, was injected into the PAG as above. Response latencies were then taken every 5 min for 35 min.

Histology

Following testing, animals were overdosed with sodium pentobarbital and their brains removed and drop fixed in 10% neutral-buffered formalin. The position of the microinjection sites relative to the LH and the PAG were made by microscopic examination of 40- μ m transverse brain sections rinsed in cold phosphate-buffered saline PBS, 10 mM, mounted on gel-coated slides and stained with 0.05% neutral red. Cannula placement was determined by locating the most ventral position of the cannula tip in serial sections by brightfield microscopy and tracings of appropriate sections were made using the

NeuroLucida imaging system (Microbrightfield, Colchester, VT). The tracings were compared with drawings from the atlas of Paxinos and Watson [48] to verify that the cannula was within the LH or the PAG.

Statistical analysis

Based on a priori estimation of power with a significance level of 0.05, a medium effect size of 0.50, and a power of 0.7 (Sigma Stat 3.0), each treatment group consisted of five rats. Tail flick and foot withdrawal latencies are presented as the mean $\pm$ S.E.M. Statistical comparisons among treatment groups across several time points were made using two-way repeated measures ANOVA, and comparisons among means at specific time points were made using the Bonferroni test for multiple post-hoc comparisons. Paired t-tests were used to compare withdrawal latencies for left and right feet. Foot withdrawal latencies were not significantly different between left and right paws for any experiment, so response latencies were averaged for clarity.

Results

Figure 1 shows the approximate locations of microinjections made in the LH (A) and the PAG (B) for which data were used. Of the 41 rats used in the experiments, 11 were excluded because the position of the microinjector was outside the LH, the PAG, or both.

Microinjection of carbachol into the LH at sites similar to those shown in Fig. 1A produced moderate antinociception on the tail flick test compared to baseline response latencies. This antinociception was blocked by microinjection of cobalt chloride or L-703,606 into the PAG as compared to saline controls on the tail flick test (two way repeated measures ANOVA, $F = 13.94$ (2,76), $p < 0.001$; Fig. 2A). Post hoc comparisons showed that the tail flick latencies of rats that received cobalt chloride were almost identical to those that had microinjection of L-703,606 (3.11 ± 0.22 vs. 3.13 ± 0.22 sec respectively). In contrast, physiological saline microinjected into the PAG had no effect on LH-induced antinociception and both treatment groups were significantly different from control rats (4.59 ± 0.22 sec, $p < 0.05$). There was a significant interaction between treatment and time ($p < 0.001$), indicating that the effect of carbachol depended on the time the tail flick latency was measured.

Similar results were seen for the foot withdrawal latency. Carbachol-induced antinociception was significantly reduced following microinjection of either cobalt chloride or L-703,606 into the PAG (two way repeated measures ANOVA, $F = 8.17$ (2,72), $p = 0.006$; Fig. 2B). The mean withdrawal latencies of rats receiving cobalt chloride in the PAG were not significantly different from rats receiving L-703,606 microinjection (2.78 ± 0.22 vs. 2.91 ± 0.22 sec, respectively), but both groups differed from control rats that received saline in the PAG (3.94 ± 0.22 sec, $p < 0.05$). As with the tail flick latency, there was a significant interaction effect for the foot withdrawal latency ($p < 0.001$).

Microinjection of either cobalt chloride or L-703,606 into the PAG in the absence of LH stimulation resulted in tail flick and foot withdrawal latencies that did not differ significantly from control rats ($p > 0.05$). This experiment demonstrated that the connection between the LH and PAG was not tonically active; that the results were not due to injecting a volume of liquid in the PAG; and that L-703,606 has no agonist properties.

Discussion

In this study, LH-induced antinociception was blocked by administration of cobalt chloride in a dose shown to block synaptic activity in the area of microinjection, [28],[29],[35],[44] and by L-703,606. These findings confirm and extend those of Aimone and Gebhart, [1] and

Behbehani, Park, and Clement. [8] Aimone and Gebhart increased electrical stimulation thresholds in the LH by injecting lidocaine or ibotenic acid in the PAG. [1] Electrical stimulation activates both cells and fibers of passage. Our use of chemical stimulation reduced the risk of stimulating axons traveling through the LH *en passant* from areas outside the LH. Behbehani and colleagues [8] applied glutamate to the LH and recorded increased activity in neurons in the PAG. Substance P was not implicated in either of the former studies.

The finding that LH-induced antinociception is blocked by the specific NK1 receptor antagonist L-703,606 [60] is novel, but not definitive. LH stimulation could activate substance P neurons elsewhere that then project to the PAG, or that exist within the PAG itself. [36],[60]. Additionally, the preprotachykinin A gene encodes the precursors not only for substance P, but also for neurokinin A which binds the NK1 receptor. Although substance P exhibits greater affinity for the NK1 receptor than does neurokinin A [54], and evidence for neurokinin A in an LH to PAG pathway does not yet exist, we cannot rule out that neurokinin A plays a role in neurokinin receptor-dependent actions as seen in the present study. However, these lines of converging evidence support the hypothesis that the LH produces antinociception in part through a substance P connection with the PAG. NK1 receptors likely activate neurons in the PAG that innervate spinally projecting serotonergic or noradrenergic neurons which then inhibit nociceptive responses in the dorsal horn.

The mechanism by which substance P in the PAG produces antinociception is not clear. Substance P acts at NK1 non-opioid sensitive neurons [17] and glutamate-containing neurons. [14] It is possible that substance P from LH stimulation acts on PAG glutamate neurons, which then project to spinally-descending noradrenergic or serotonergic neurons, evoking antinociception. Further investigation is needed to determine the mechanisms involved in this circuitry.

Although earlier studies showed that stimulation of all parts of the PAG produce antinociception, [6] recent studies have shown that dorsolateral, and to a lesser extent, ventrolateral PAG neurons express Fos immunoreactivity in response to radiant heat application. [32],[33] Areas in the PAG have also been identified as being distinct in their responses to visceral and somatic pain, [32],[55],[56] with the dorsolateral columns responding to somatic stimulation, [38],[42],[43],[47] and the ventrolateral columns responding to visceral pain input. [13],[33] In the present study, we administered only a somatic stimulus, but found no difference in responses to NK1 antagonist actions whether the microinjector was placed in the ventrolateral, dorsolateral, or dorsal PAG. Although the precise reason for this lack of difference is unknown, it is likely that our microinjections were not precisely located in distinct columnar areas in the PAG, so we are unable to parse out differences in responses based on columnar organization.

Recent work has identified distinct somatotopic organization of the LH and its projections to the dorsal PAG [49], with the more anterior LH projecting to the rostral dorsal PAG, and more caudal LH projecting to more caudally. Most of our microinjections encompassed the tuberal LH and pontine PAG, with only two microinjections in the dorsal PAG, so we cannot make any statements about somatotopic organization based on our behavioral findings. Similarly, while others have identified distinct areas in the lateral aspect of the anterior hypothalamus as projecting to specific columns in the PAG in response to several different pain types, [38],[47],[55] such work remains to be done in the LH.

Stimulating the LH produces hypotension in awake rats [46] and as we did not monitor blood pressure during our studies, we cannot be sure whether carbachol microinjection lowered the blood pressure in our lightly anesthetized rats, although it is possible.

Hypotension can be a side effect of antinociceptive agents, as both hypotension and antinociception occur following systemic injection of a neurotensin analog in rhesus monkeys [20] and morphine in rats [39]. The issue for the present study is whether hypotension, if it did occur, could produce the results we attribute to cobalt chloride or L-703,606 placed in the PAG. Our data show that rats that received carbachol microinjection in the LH and saline in the PAG did not have a decrease in withdrawal latencies on either the tail flick or foot withdrawal tests, compared to rats receiving cobalt chloride or L-703,606, so it is unlikely that our findings can be attributed to changes in blood pressure.

The results of the present study combined with the results from other studies provide converging anatomical and functional evidence in support of the hypothesis that the LH mediates antinociception in part through either a direct or an indirect substance P connection with neurons in the PAG, which then activate spinally projecting neurons to produce antinociception. The full role of the LH in descending nociceptive modulation remains to be determined, including male-female differences in general and the effect of estrus cycle in particular. It is possible that pharmacological or non-pharmacological therapies aimed at activating the LH could ultimately produce improved clinical pain management via existing nociceptive modulatory pathways. These non-pharmacological therapies could include invasive procedures such as deep brain stimulation, or non-invasive behavioral procedures such as exercise, massage, or music therapy, that have the potential to affect hypothalamic function.

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Fig. 1.

A schematic depiction of microinjection sites in rats receiving 125 nmol of carbachol (A). Microinjection sites for carbachol in the LH are differentiated by subsequent microinjection in the PAG. Closed circles = normal saline; open squares = cobalt chloride; open circles = L-703,606. (B) A schematic depiction of microinjection sites for rats receiving normal saline (closed circles), cobalt chloride (open squares) or L-703,606 (open circles). Abbreviations: AMG, amygdala; C, caudate nucleus; D, dorsal PAG; DL, dorsolateral PAG; DM, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; f, fornix; ic, internal capsule; LV, lateral ventricle; LH, lateral hypothalamus; ml, medial lemniscus; PH, posterior hypothalamus; rs, rubrospinal tract; scp, superior cerebellar peduncle; VL, ventrolateral PAG; VM, ventromedial hypothalamic nucleus; VPL, ventral posterolateral thalamic nucleus; ZI, zona incerta. Numbers represent approximate distance from bregma.

Fig. 2.

Antinociception produced by microinjection of carbachol in the LH was blocked by microinjection of cobalt chloride or L-703,606 in the PAG. (A) Following a baseline measurement at -20 min, carbachol was microinjected into the LH, and tail flick latencies measured at -15, -10, and -5 min. Either cobalt chloride, L-703,606, or saline ($n = 5$ per group) was injected into the PAG at time 0. (B) Cobalt chloride or L-703,606 microinjected into the PAG also blocked LH-induced antinociception on the foot withdrawal test as compared to saline controls. Values for foot withdrawal latencies represent pooled data. Mean latency values $\pm$ S.E.M. are plotted on the ordinate as a function of time min.