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Conditional hypoalgesia is attenuated by naltrexone applied to the periaqueductal gray

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The ventrolateral periaqueductal gray (vPAG) is an important component in a brainstem system involved in the endogenous modulation of nociception and defensive behavior. The present study was conducted to determine if opioid receptors within the vPAG contribute to the hypoalgesia seen in rats during presentation of a Pavlovian CS for footshock. Independent groups of animals received microinjections of either 5.0 μ g naltrexone HCl or vehicle into the vPAG prior to being placed in an observation chamber in which shock had been delivered 24 h earlier. Nociceptive reactivity was measured with the formalin test. Naltrexone treatment attenuated conditional hypoalgesia but did not affect formalin-induced behavior in non-shocked rats. Naltrexone had no effect on the amount of defensive freezing behavior observed during the test session. These results indicate that conditional hypoalgesia as measured by the formalin test involves the activation of documented brainstem antinociceptive systems.

INTRODUCTION

Considerable evidence indicates that the midbrain central gray (PAG) is a critical component within a brainstem system capable of modulating nociceptive reactions^{3,4}. Projections from cells within the ventrolateral PAG terminate in the nucleus raphe magnus and other nuclei within the rostral ventral medulla which in turn project via the dorsolateral funiculus to the dorsal horn. Through these connections, the activity of PAG cells may modulate nociceptive afferent information at the level of the spinal cord. Electrical and pharmacological stimulation of the ventrolateral PAG in several preparations results in a profound inhibition of spinally and supraspinally mediated reactions to presumably painful or noxious stimuli^{5,17,22,27}. Importantly, the rich concentration of receptors for endogenous opioid peptides within the PAG may play a critical role in the antinociception produced by both electrical brain stimulation⁷ and opioid-mediated forms of environmentally induced hypoalgesia²¹.

Conditional hypoalgesia (CHA) refers to the process by which 'neutral' environmental stimuli that have been paired with aversive events such as footshock are able to modulate nociceptive reactions. CHA may be considered a form of 'stress-induced analgesia' in which the stressor

is a previously ineffective stimulus that has gained the ability to activate endogenous antinociceptive systems through Pavlovian conditioning^{10,15,25,26}. The performance of CHA in the rat, as indicated by the suppression of stereotyped reactions to a subcutaneous injection of formalin, critically depends on a population of opioid receptors within the CNS⁶. Furthermore, a series of recent studies suggest that CHA involves the selective activation of both μ and δ opioid receptor types^{12,13}.

While the neural substrates of hypoalgesia as a conditional response are largely unknown, several lines of evidence suggest that the ventrolateral PAG may play an important role. Lesions of the caudal PAG have been reported to disrupt CHA as measured with the tailflick test¹⁸. Hammer and Kapp¹⁴ reported that when the opioid antagonist naloxone was applied to the PAG prior to aversive conditioning with footshock, drug treated animals displayed larger amounts of defensive freezing, a conditional response (CR) to stimuli present at the time of shock, when tested 24 h later. While CHA was not measured directly in this study, the enhanced performance of freezing is consistent with antagonism of CHA by PAG naloxone during the training session^{14,15}.

The purpose of the present study was to determine if opioid receptors within the PAG are involved in the performance of CHA.

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MATERIALS AND METHODS

Subjects, surgery and histology

A total of 40 adult female Long-Evans rats bred locally from stock obtained from Blue Spruce Farms (Altamont, NY) served as subjects. Animals were housed individually in hanging stainless-steel cages with free access to lab chow (Agway) and water. Lights in the colony room were on a 14 h on, 10 h off schedule. All testing occurred during the light portion of the cycle.

Prior to surgery, animals were anesthetized with ketamine HCl (100 mg/kg, i.p.) followed by a supplemental injection of sodium pentobarbital (2.5 mg/rat, i.p.). Animals were mounted in a standard stereotaxic apparatus and stainless-steel guide cannula (26 gauge, Plastic Products C315G) were implanted unilaterally in the left ventrolateral PAG. The following coordinates were used based on the atlas of Paxinos and Watson²³: AP -7.7 mm, L 0.65 mm, V -5.0 mm relative to bregma with the skull level between lambda and bregma. Guide cannulae were secured to the skull with the aid of stainless-steel screws, epoxy, and cranioplastic cement. All subjects were allowed to recover from surgery for at least one week prior to testing. Beginning 3 days prior to testing, animals were exposed daily to the handling and injection procedure to be used during the experiment. During this time their cannula obturators were removed, cleaned with 50% Betadine solution, and replaced.

Following behavioral testing, all subjects were overdosed with sodium pentobarbital and perfused transcardially with saline followed by 10% phosphate-buffered formalin. Brains were removed and frozen 40 μ m sections were collected through the cannula track. Tissue was stained with Cresyl violet and the location of injection sites was determined with the aid of a rat brain atlas²³. The data from animals with cannula placements outside the ventrolateral PAG were not included in subsequent analysis.

Drugs and injection

Naltrexone HCl (Research Biochemicals Inc.) was dissolved in pyrogen filtered sterile isotonic saline at a concentration of 10 μ g/ μ l. Sterile saline served as a control. Injections were performed via a

33 gauge cannula connected to a 1 μ l Hamilton syringe mounted in an infusion pump. A total volume of 0.5 μ l was infused evenly over a 40 s period while the animal was gently restrained by hand. Cannulae were left in place for at least 30 s following the injection.

Apparatus and procedure

All conditioning and testing took place in standard rodent observation chambers (23.5 \times 25 \times 19.5 cm) which were housed in sound-attenuating enclosures. The floor of each chamber was composed of 18 stainless-steel rods through which scrambled electric shock could be delivered. A Plexiglas window in each enclosure allowed the experimenter an unobstructed view of the subject.

On Day 1, one half of the rats were randomly assigned to receive footshock or serve as non-shocked controls. Subjects in the shock condition were placed in the observation chamber and after 4 min were given a series of three 1 mA/0.5 s footshocks spaced 20 s apart and returned to the home cage 20 s after the final shock. Non-shocked animals received equivalent treatment without shock.

On the following day all subjects were given a 0.05 ml subcutaneous injection of 15% formalin in saline (5.5% formaldehyde) into the dorsal surface of the right hind paw and returned to the home cage. Ten min after the formalin injection one half of the animals in both the shocked and non-shocked groups were randomly assigned to receive either 5 μ g naltrexone HCl or a vehicle injection. Thus a total of 4 groups were formed prior to testing representing a factorial combination of shock and drug conditions.

Twenty min after intracranial injections rats were removed from the home cage and placed in the observation chambers in which they had received shock on the previous day. With the aid of a metronome, an observer blind to group assignments recorded the animals behavior for 10 min using a time sampling procedure^{11,15}. Every 4 s, each rat's behavior was scored as belonging to one of 3 categories which were defined as follows: (1) Freezing — the absence of all detectable body movement except that required for respiration. (2) Formalin-induced behavior — any licking or contact of the injected paw with the animal's mouth, or lifting and maintaining the injected paw off the grid floor. (3) General activity

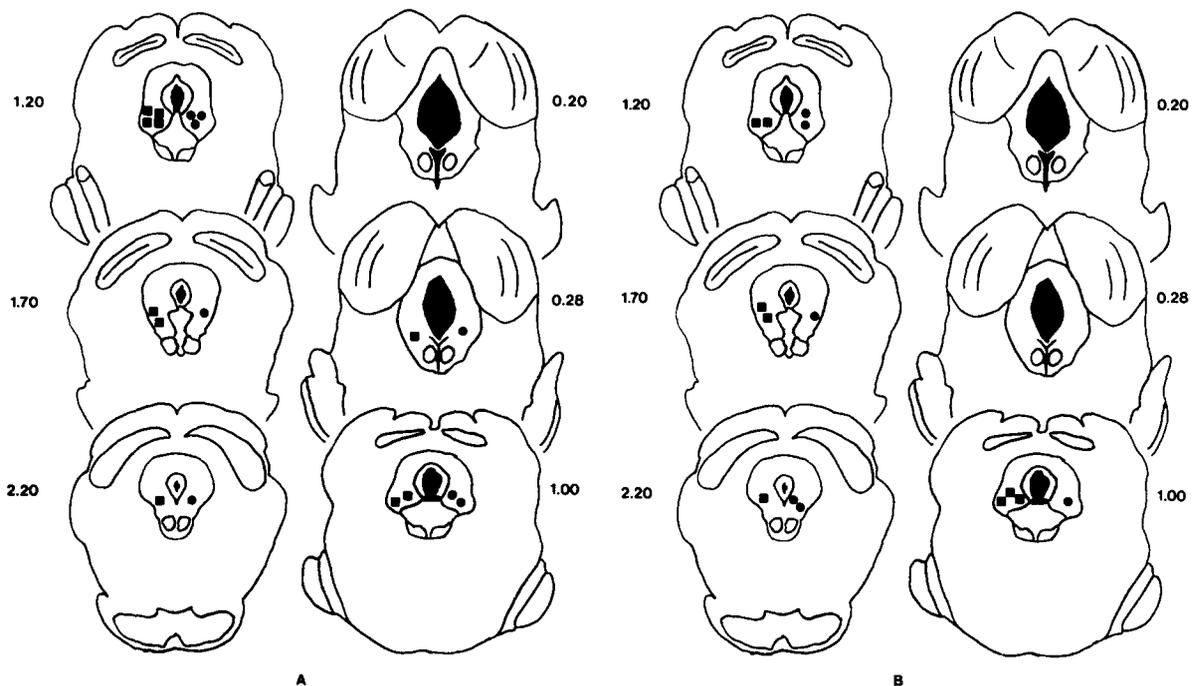


Fig. 1. Injection sites for shocked (A) and non-shocked (B) animals included in the analysis. All placements were made on the rats' left side, contralateral to the formalin-injected paw. Symbols are drawn on both sides of the sections for clarity. Numbers indicate distance in mm from the interaural line. ●, naltrexone; ■, saline.

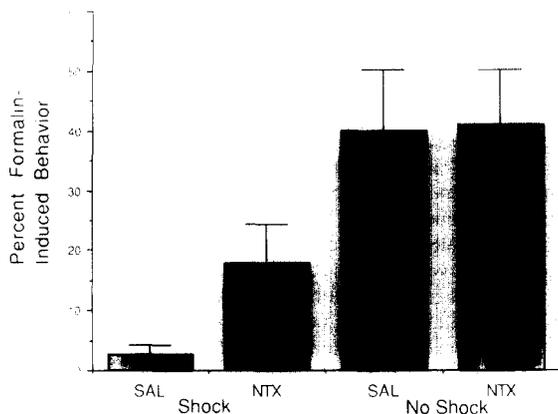


Fig. 2. Mean (\pm S.E.M.) proportion of observation time spent engaged in formalin-induced behavior. Presentation of footshock 24 h earlier suppressed formalin behavior. Naltrexone (5.0 μ g) attenuated this suppression. *n*'s for each group are Shock/NTX = 8, Shock/SAL = 10, No Shock/NTX = 6, No Shock/SAL = 8.

— all other behavior not defined as freezing or formalin-induced behavior. The percentage of time each animal spent engaged in these behaviors was calculated.

RESULTS

Injection sites for animals with cannulae within the ventrolateral PAG are depicted in Fig. 1. The data from 8 rats whose placements were located outside the ventrolateral PAG were not included in the analysis. The resulting *n*s for each group were: Shock/NTX = 8, Shock/SAL = 10, No Shock/NTX = 6, No Shock/SAL = 8.

Fig. 2 presents the results of the formalin test conducted 24 h after conditioning. Formalin scores were subjected to a square root transformation to improve normality of the distribution prior to analysis of variance (ANOVA)¹⁵. As can be seen in the figure, rats that did not receive footshock during conditioning displayed larger amounts of formalin-induced behavior than did shocked rats. Formalin behavior in the shocked animals receiving saline was suppressed, indicating that apparatus cues paired with shock were effective in producing CHA. Importantly, NTX injected into the vPAG resulted in a greater amount of formalin behavior, indicating that vPAG opioids play a role in the performance of CHA. These observations were confirmed by a 2 (Drug) \times 2 (Shock) ANOVA which indicated significant main effects for both drug treatment, $F_{1,28} = 5.09$, $P < 0.032$, and shock, $F_{1,28} = 25.09$, $P < 0.001$. Subsequent planned comparisons indicated that shocked animals given NTX displayed significantly more formalin-induced behavior relative to shocked animals receiving saline, $F_{1,28} = 7.06$, $P < 0.012$, while NTX had no effect on this behavior in non-shocked animals, $F_{1,28} < 1.0$. The mean percent

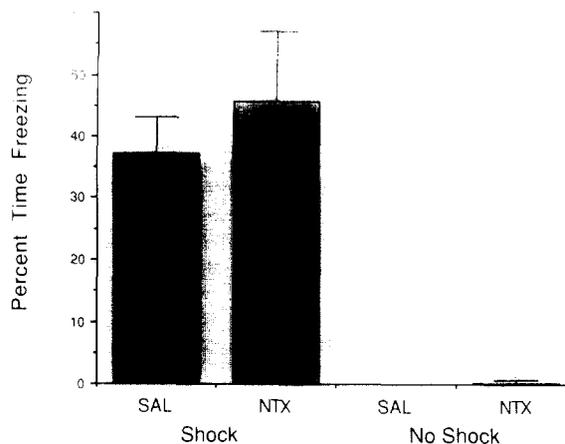


Fig. 3. Mean percentage of time spent engaged in freezing behavior during the test session. Shock-naive rats tend not to display this behavior. Naltrexone treatment had no effect on the performance of freezing 24 h after shock.

formalin-induced behavior for SHK/NTX animals not included in the analysis because of injection sites located slightly lateral to the vPAG was quite low (mean = 2.5% \pm 1.18 S.E.M.), supporting the idea that naltrexone is acting on receptors within the PAG.

The percentage of the observation period subjects spent engaged in freezing behavior is shown in Fig. 3. Presentation of footshock during training resulted in significantly greater amounts of freezing relative to non-shocked controls, $F_{1,28} = 36.68$, $P < 0.001$. Naltrexone treatment did not affect the performance of freezing, $F_{1,28} < 1.0$.

DISCUSSION

The present results indicate that opioid receptors within the ventrolateral PAG participate in the conditional activation of endogenous antinociceptive systems. These observations are consistent with prior work using lesions of the PAG¹⁸ or measuring PAG opioid effects on CHA indirectly¹⁴. NTX at the dose used significantly attenuated but did not completely reverse CHA relative to non-shocked controls. This result may indicate that antagonism of only a subpopulation of receptors within the PAG was achieved, or that additional parallel inhibitory systems are also involved in the hypoalgesia observed in this preparation. In non-shocked rats NTX did not alter baseline levels of formalin behavior. This lack of effect is consistent with other data on systemic and intracerebroventricular administration of opioid antagonists^{11,12} and may indicate that the central mechanisms involved in the performance of CHA do not contribute significantly to the tonic modulation of nociceptive input.

In addition to its demonstrated role in endogenous

antinociception, the ventrolateral PAG is also involved in the expression of defensive behavior in rats and cats^{1,2,9}. Lesions of the PAG disrupt freezing as well as other defensive behaviors in the rat^{19,20}. In the present study freezing, measured concurrently with formalin behavior, was not affected by a dose of NTX that attenuated CHA. This evidence combined with the fact that systemic administration of opioid antagonists tend also not to affect the performance of freezing¹⁵ may indicate that while freezing and CHA share common gross anatomical substrates, they may be dissociated pharmacologically. Thus, within the ventrolateral PAG a subpopulation of opioid-sensitive cells may contribute directly to nociceptive modulation while other non-opioid cells are involved in the performance of freezing that are affected by lesions or more general pharmacological manipulations. Additional research is needed to determine if this is indeed the case.

Evidence for the participation of the PAG in hypoalgesia elicited by conditionally aversive environmental cues supports the position that CHA represents the activation of known antinociceptive systems within the brainstem^{14,18,25,26}. Based on this, one can begin to describe in detail the 'neural circuits' that mediate the acquisition and expression of this response. The ventrolateral PAG receives direct projections from the central nucleus of the amygdala [8] and electrical stimulation of

the amygdala has been shown to influence unit responses in the PAG²⁴. Lesions of the amygdala exert similar disruptive effects on the performance of freezing in response to conditional aversive stimuli as do lesions of the PAG^{16,19}. Importantly, recent studies have shown that lesions of the central or basolateral amygdala and the local application of benzodiazepines in the basolateral amygdala also disrupt CHA as measured by the formalin test¹⁶. Taken together, this evidence supports the position that the amygdala may be one example of the 'rostral structures' assumed to be important for the activation of brainstem antinociceptive systems in response to signals for aversive events^{4,25}.

In conclusion, the present data indicate that CHA in this preparation represents the activation of a brainstem antinociceptive system, one component of which is a population of opioid-sensitive cells within the vPAG. Further research is needed to characterize the opioid pharmacology of these cells and the neuroanatomical organization of cells responsible for CHA and other defensive behaviors within the PAG.

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