

μ Opioid Receptors in the Ventrolateral Periaqueductal Gray Mediate Stress-Induced Analgesia but Not Immobility in Rat Pups

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Rat pups become immobile and analgesic when exposed to an adult male rat. The aim of this study was to determine whether these reactions are under the control of endogenous opioids and to determine the role of the midbrain periaqueductal gray (PAG), which mediates stress-induced immobility and analgesia in adult animals. In Experiment 1, 14-day-old rats were injected systemically with the general opioid receptor antagonist naltrexone (1 mg/kg), which blocked male-induced analgesia to thermal stimulation but did not affect immobility. In Experiment 2, the selective μ opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP; 50 or 100 ng/200 nl) was microinjected into the ventrolateral and lateral PAG. CTOP suppressed male-induced analgesia when injected into the ventrolateral PAG. Male-induced immobility was not affected by CTOP. Male proximity therefore seems to induce analgesia in rat pups by releasing endogenous opioids that bind to μ opioid receptors in the ventrolateral PAG.

Young animals must perform adaptive behavioral and physiological reactions to survive threatening situations. Reactions are age-specific when they are shown only during a limited period of development and then disappear. Previous studies have demonstrated that when rat pups are isolated from their nest and their mother, they emit ultrasonic vocalizations, explore, decrease heart rate and respiration, increase corticosterone levels, and reduce reactivity to noxious stimuli (i.e., become analgesic; Hofer, 1970; Hofer & Shair, 1978; Kuhn, Pauk, & Schanberg, 1990; Spear, Enters, Aswad, & Louzan, 1985). These reactions to social isolation are shown only during a limited preweaning period and seem to diminish the threat to the pup's life posed by isolation (Smotherman, Bell, Hershberger, & Coover, 1978; Spear et al., 1985). Another threat to which the young of altricial rodents are especially vulnerable is predation. Young meadow voles that were exposed to a natural predator, a garter snake, became analgesic (Saksida, Galea, & Kavaliers, 1993), but analgesia decreased and finally disappeared as the risk of predation by the snake decreased

(Saksida et al., 1993). For rat pups, an unfamiliar, unrelated adult male rat is a significant, age-specific social stressor because under natural conditions, male rats intrude into foreign territory and kill the offspring of resident pairs (Mennella & Moltz, 1988; Paul & Kupferschmidt, 1975). Loss of the litter stops lactation in the female and brings her back into estrus, allowing the intruder male to mate and thus increasing his reproductive success. Infanticide disappears around the time of weaning when the female resumes cycling (Mennella & Moltz, 1988; Paul & Kupferschmidt, 1975). Rat pups have evolved the ability to recognize and react to adult males. In proximity to a male, they show elevated plasma levels of adrenocorticotrophic hormone (ACTH), analgesia, and behavioral inhibition including immobility and suppression of ultrasonic vocalization (Gould & Cameron, 1997; Takahashi, 1992; Wiedenmayer & Barr, 1998). These reactions to adult males are shown only during the developmental period before weaning and parallel the changes in severity of the infanticidal threat (Takahashi, 1992; Wiedenmayer & Barr, 1998).

In the adult rat, the neural processes underlying responses to aversive stimuli have been well described. The periaqueductal gray (PAG), a midbrain region surrounding the aqueduct, controls different reactions such as defensive behaviors, autonomic changes, and analgesia (Bandler & Shipley, 1994). The PAG is a major module in the circuitry mediating stress-induced analgesia (Basbaum & Fields, 1984), as it sends descending inhibitory fibers to the medulla, which in turn modulates incoming noxious signals in the spinal cord (Behbehani, 1995; Beitz, 1992; Fields, 1993; Sandkühler, 1996). Stimulation of opioid receptors within the PAG activates these descending inhibitory pathways and suppresses nociception (Bellgowan & Helmstetter, 1998; Helmstetter & Landeira-Fernandez, 1990; Rossi, Pasternak, & Bodnar, 1994). Also, the PAG mediates behavioral reactions such as freezing and defensive immobil-

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ity, which are partly under opioid control (Bandler & Depaulis, 1991; Fanselow, 1991).

Less is known, however, about the neural processes that underlie age-specific aversive reactions in infant animals. Studies focusing on analgesia have demonstrated that either opioid or nonopioid processes are involved, depending on the quality and intensity of the stimulus. Systemically administered opioid antagonists suppressed analgesia induced by social isolation in rat pups (Kehoe & Blass, 1986a; Spear et al., 1985) or by prolonged exposure to a snake in young voles (Saksida et al., 1993), whereas a serotonin-1A agonist suppressed analgesia in young voles after brief exposure to a snake (Saksida et al.) Cold-water immersion induced analgesia in rat pups, which was not affected by a systemic opioid antagonist (Hamm & Knisley, 1988). Pharmacological stimulation studies have indicated a role for the PAG in the induction of opioid and nonopioid analgesia. Microinjections of opioid and glutamate agonists into ventral or dorsal parts of the PAG of infant rats induced analgesia and revealed different roles of these PAG regions in mediating either opioid or nonopioid analgesia to different kinds of stimuli (Barr & Wang, 1994; Tive & Barr, 1992). Little is known about the role of opioids in defensive immobility in infant animals.

The aim of the present study was to determine the role of endogenous opioids in age-specific analgesia and immobility induced by aversive stimuli in infant rats. To generate an ecologically relevant stimulus, a potentially infanticidal situation was produced under laboratory conditions. A small huddle of rat pups was exposed to the stressor of an unfamiliar, adult male rat to induce analgesia and immobility, reactions that peak on Postnatal Day 14 (Wiedenmayer & Barr, 1998). In Experiment 1, the opioid nature of male-induced reactions was assessed by the systemic use of a general opioid receptor antagonist. In Experiment 2, a selective opioid receptor antagonist was microinjected directly into the PAG.

General Method

Animals

Pups were the offspring of Long-Evans hooded rats mated in our laboratory. They were housed in standard laboratory cages in a colony room maintained at 22–24 °C, with a 12-hr light–dark cycle with light onset at 7 a.m. Cages were checked twice daily, at approximately 9 a.m. and 6 p.m. Pups found at either time were designated as 0 days of age. The pups of litters with 12–13 pups were tested on Postnatal Day 14. A sexually experienced, unfamiliar, unrelated adult male was housed under identical conditions in a different room. Treatments were administered according to the guidelines of the Institutional Animal Care and Use Committee.

Apparatus

Tests were conducted in a standard laboratory cage (46 × 25 × 21 cm), in a testing chamber opposite the colony room. The cage was subdivided by a wire-mesh partition positioned in the middle of the cage, thereby forming two equal-sized compartments.

Test Procedure

On the day of testing, 3 pups were taken randomly from a litter, marked with a nontoxic marker on their fur, weighed, and placed in one compartment of the testing cage provisioned with soiled wood shavings that had been taken from the home cage to simulate the nest area. The other compartment of the testing cage was empty. The pups were allowed to acclimate for 10 min. The behavior of the 3 pups was then recorded for a 5-min baseline period, followed by a pain reactivity test (see below). Afterward, the unfamiliar male was placed in the adjacent compartment, and the behavior of the pups was recorded for 5 min, after which the male was removed. Pain reactivity was immediately assessed after removal of the male (Time 0), and assessments were repeated 5 and 10 min later. The pups were then put back into the home cage in the colony room. As a control, the pups were also tested with the adjacent compartment empty. It has been shown that, for rat pups, an empty compartment is equivalent to the presence of the mother and that neither condition affects the pups' nociception and immobility (Wiedenmayer & Barr, 1998). All tests were conducted in the first half of the light cycle.

Behavioral Measurements

Nociceptive reactivity was determined by a thermal paw-lift test. The forepaw was chosen because it is sensitive to supraspinal influences earlier in development than the hindpaw and tail (Barr, 1992; Barr, Miya, & Paredes, 1992). The forepaw of a pup was put on one of two electric resistors, in a random order. The resistors were heated by current and maintained at nominal temperatures of 39 and 44 °C. A higher temperature resistor of 49 °C was also used but caused rapid forepaw withdrawal unaffected by any manipulation (data not shown). The different temperatures were used to generate different intensities of stimulation. The latency to withdraw the paw was recorded with a timer (Lafayette Instruments, Lafayette, IN) operated by a foot pedal. The timer was activated when the pup's forepaw touched the resistor and was terminated when the pup removed its paw from the resistor. To prevent tissue damage, the test was stopped after 20 s if the paw was not withdrawn. Previous work in our laboratory has shown that repeated testing across temperatures and time does not affect paw withdrawal latencies.

Immobility was determined by scan sampling, during which the observer notes whether or not a specific behavior pattern is occurring (Martin & Bateson, 1993). During the 5-min observation periods before and during male exposure (or during control), the behavior of each of the 3 pups was recorded every 15 s on a check list of behavioral categories including "immobile." *Immobile* was defined as any posture in which the pup did not exhibit any movement except that necessary for respiration, and was expressed as a percentage of the scans.

Experiment 1

To determine whether analgesia and immobility in 14-day-old rat pups induced by an adult male are mediated by opioids, the general opioid receptor antagonist naltrexone was systemically administered, thus blocking the binding of endogenous opioid peptides released by the presence of the stressor. Naltrexone is a potent, general antagonist that binds to all three opioid receptor types— μ , δ , κ —with the highest affinity for μ opioid receptors (Goldstein & Naidu, 1989). It was hypothesized that the application of naltrexone would block male-induced analgesia and immobility.

Method

Drugs. Naltrexone was dissolved in isotonic saline and administered intraperitoneally in a dose of 1 mg/kg in a volume of 0.1 ml/10 g body weight. Isotonic saline was used for control injections.

Procedure. On the day of testing, 3 pups with both sexes represented were put in the testing cage (see General Method). After baseline testing, all 3 pups of a group were injected with either naltrexone or saline. The experimenter was unaware of the drug injected. After 1 min, the male was introduced in the adjacent compartment and the test continued as described above. There were four conditions: (a) saline-injected control pups, (b) naltrexone-injected control pups, (c) saline-injected pups tested with the male, and (d) naltrexone-injected pups tested with the male. The order of testing was counterbalanced across the four conditions.

Statistics. No sex differences were found for either nociceptive reactivity or immobility. The data of the 3 littermates in each condition were thus pooled and treated as one data point. Pups from eight litters were tested. Analyses of variance (ANOVAs) were conducted for paw withdrawal latencies. In the first step, the baseline latencies of the pups in each of the four conditions were compared by a two-way ANOVA. Because the latencies did not differ between conditions, the time courses after the injection (Time Points 0–10) were compared by a three-way ANOVA. The four different conditions, the reactivity at the three time points during a test, and the reactivity to the two temperatures were treated as within-subjects variables. Tukey's honestly significant difference (HSD) tests were used for post hoc comparisons. Immobility, which is noninterval data, was analyzed with nonparametric Friedman ANOVAs, a standard for the analysis of such behavioral data (Siegel, 1956), followed by paired comparisons with Wilcoxon's signed rank tests.

Results

Paw withdrawal latencies differed significantly among conditions, $F(3, 21) = 14.07, p < .001$. Immediately after male exposure, saline-injected pups showed significantly increased paw withdrawal latencies at the lower temperature ($p < .001$) compared with control pups and with naltrexone-injected pups tested with the male (see Figure 1). Five minutes after exposure, saline-injected pups tested with the male showed significantly higher latencies at low temperature compared with control pups and with naltrexone-injected pups tested with the male ($p < .05$). Immediately after male exposure, saline-injected pups displayed higher medium-temperature latencies compared with control pups ($p < .01$) but not with naltrexone-injected pups tested with the male ($p < .08$).

The immobility baselines of the four conditions did not differ. During testing, control pups were more immobile than at baseline ($p < .05$), but there was no difference between saline- and naltrexone-injected pups (see Table 1. The immobility baselines of the four conditions are pooled.). When the male was introduced into the adjacent compartment, the pups were significantly more immobile compared with the control pups ($p < .05$), but there was no difference in immobility between saline- and naltrexone-injected pups.

Discussion

Systemic administration of the opioid receptor antagonist naltrexone blocked analgesia in infant rats induced by the

presence of the social stressor adult male. The data suggest that cues from the male induce the release of endogenous opioids that mediate analgesia to moderate thermal stimuli. A similar activation of opioid processes contributes to analgesia induced by isolation stress, when rat pups are separated from siblings, dam, and nest (Kehoe & Blass, 1986b; Spear et al., 1985).

Control pups tested adjacent to an empty compartment were more immobile than at baseline, which may reflect habituation to the test situation. However, when the male was introduced into the adjacent compartment, both saline- and naltrexone-injected pups became more immobile than control pups. Naltrexone did not affect male-induced immobility, a defensive reaction that seems to reduce the probability of detection by an infanticidal male (Wiedenmayer & Barr, 1998). This pharmacological dissociation of immobility from analgesia indicates the existence of two separate behavioral systems that are activated synchronously by male-presence cues but are under control of different neurotransmitter systems. Also, the activation of several distinct systems underlies the reactions shown by socially isolated pups. Ultrasonic vocalization, thermogenesis, and cardiovascular and behavioral reactions are regulated by different neural systems that together form an integrated response to separation (Hofer, 1996).

However, the systemic application of an opioid antagonist does not reveal the site of activation of the endogenous opioid system. Opioid receptors are found in different circuits of the brain (Mansour, Fox, Akil, & Watson, 1995). In the brains of 14-day-old rat pups, all three opioid receptor types are expressed (Leslie & Loughlin, 1992; McDowell & Kitchen, 1987). Different opioid receptor types have complementary or opposing functions. For example, μ and δ receptor agonists both decrease ultrasonic vocalizations in socially isolated 10-day-old rat pups, whereas κ receptor agonists increase vocalization rate (Carden, Barr, & Hofer, 1991). Because different opioid receptor types may have different functions in mediating male-induced analgesia, an inconsistent net effect may have resulted after unspecific blocking by naltrexone. To determine the opioid receptor type and the brain area involved in male-induced analgesia, a selective opioid receptor antagonist must be administered locally into a distinct brain structure.

Experiment 2

A likely brain area for the regulation of male-induced analgesia in rat pups is the midbrain PAG. Opioid stimulation studies have shown that the PAG mediates analgesia to thermal stimuli in rat pups as early as Postnatal Day 3 (Tive & Barr, 1992). The PAG is not a homogenous structure but consists of longitudinal neural columns extending along the rostrocaudal axis (Bandler & Shipley, 1994). In the adult, the ventrolateral column of the PAG (vPAG) mediates opioid analgesia, and the more dorsal part, the lateral column (latPAG), mediates nonopioid analgesia (Bandler & Keay, 1996; Bandler & Shipley, 1994). In the adult animal, opioid analgesia is mediated by μ opioid receptors (Matthes et al.,

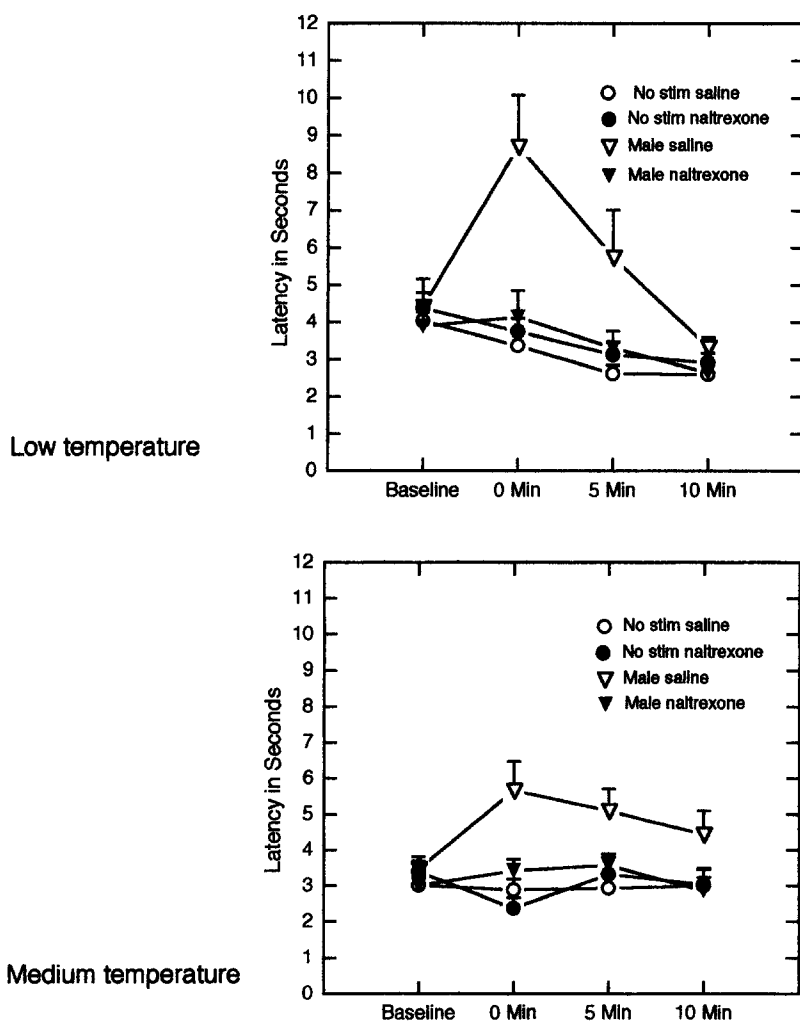


Figure 1. Paw withdrawal latencies ($M \pm SEM$) of 14-day-old rat pups from thermal stimuli (low temperature: 39 °C; medium temperature: 44 °C). The pups were injected after the baseline with either saline vehicle or 1 mg/kg naltrexone. They were then tested adjacent to an empty compartment (control, No stim) or in proximity to an adult male rat. After removal of the male, withdrawal latencies were measured at 0 min, 5 min, and 10 min.

1996; Sora et al., 1997) located in the PAG (Rossi et al., 1994; Smith et al., 1992). The activation of μ opioid receptors activates the descending inhibitory pathways that modulate afferent noxious signals (Bellgowan & Helmstetter, 1998; Helmstetter & Landeira-Fernandez, 1990; Rossi et al., 1994). Because μ opioid receptors are found in the brains of 14-day-old rat pups (Bayon, Shoemaker, Bloom, Mauss, & Guillemin, 1979; Spain, Roth, & Coscia, 1985), they are likely candidates for mediating male-induced analgesia. In this study, the selective μ opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) was microinjected into either the vPAG (Experiment 2A) or the latPAG (Experiment 2B) of rat pups exposed to an adult male. It was hypothesized that the application of CTOP in the vPAG would suppress male-induced analgesia, whereas injections into the latPAG would not, and that immobility would not be affected in either case. CTOP was injected unilaterally into the right PAG. Because the inhibitory pathways descend ipsilaterally (Fitzgerald & Klotzenburg, 1986; Leong, Shieh, & Wong, 1984), the nociceptive reactivity of the left forepaw, contralateral to the injection side, served as a control.

Table 1
Immobility of 14-Day-Old Pups, Expressed as a Percentage of Observation Scans

Baseline	Control saline	Control naltrexone	Male saline	Male naltrexone
31.7	54.7*	56.6*	89.4†	90.0†

Note. After 5 min of baseline, pups were injected with either saline vehicle or 1 mg/kg naltrexone. They were then tested for 5 min adjacent to either an empty compartment (control) or an adult male rat. Data were analyzed with Friedman's analyses of variance followed by Wilcoxon's signed rank tests.

* $p < .05$, significant difference from baseline; † $p < .05$, significant difference from control.

Method

Drugs. The somatostatin analogue CTOP (Peninsula Laboratories, Belmont, CA) was diluted to a stock concentration of 10 mg/ml with isotonic saline, which was also used for control microinjections. Solutions were microinjected in a volume of 200 nl. Doses were 50 ng (low CTOP) and 100 ng (high CTOP), diluted from the stock.

Surgery. In the afternoon of Postnatal Day 13, 3 male pups from the same litter were removed from the home cage and kept in a cage with home cage shavings on a heating pad. Pups were anesthetized by methoxyflurane (Metofane) inhalation and put in a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA). A hole was drilled 0 mm posterior and 0.7 mm lateral to lambda. A 26-gauge guide cannula (Plastics One, Roanoke, VA) was implanted unilaterally into the right PAG. For Experiment 2A, the tip of the guide cannula was aimed 1 mm above the vPAG, 4.5 mm below the skull. For Experiment 2B, the guide cannula was aimed 1 mm above the latPAG, 3.6 mm below the skull. The guide cannula was fixed to the skull with dental cement, and a dummy cannula was kept in the guide cannula to prevent clogging. After recovering pups were put back with their mothers and littermates.

Procedure. The next morning, on Postnatal Day 14, the test was carried out. A cannulated pup was put with 2 untreated littermates into the testing chamber. After baseline testing (see General Method) the pup was injected with either CTOP or saline. During infusion, a 33-gauge, microinjection cannula was inserted, which projected a further 1 mm ventral to the tip of the guide cannula. The microinjection cannula was connected to a 10- μ l glass syringe operated by hand. The solution was microinjected over a period of 30 s, and the microinjection cannula was left in place for 1 min to permit diffusion. Afterward, the male was introduced into the testing chamber, and the test continued as described above. There were three conditions: (a) saline-injected pups exposed to the male, (b) low CTOP-injected pups exposed to the male, and (c) high CTOP-injected pups exposed to the male. The order of testing was counterbalanced across the three conditions. After testing, 0.2 μ l of India ink was injected through the guide cannula to assess cannula placement. Rats were given an overdose of sodium pentobarbital (Nembutal), and their brains were removed by dissection and placed in a 10% buffered formalin–30% (vol/vol) sucrose solution. Fixed and frozen brains were sectioned coronally at 50 μ m through the PAG, and the sections were mounted on glass slides, stained with cresyl violet, and coverslipped. The histological placement of the cannula was determined for each rat by using a brain atlas (Paxinos & Watson, 1998), by an experimenter unaware of the treatment groups and the responses of the pups.

Statistics. Some pups were excluded because their cannulas had been damaged by the dam overnight. Consequently, all three conditions were not tested in some litters. In Experiment 2A, of 12 litters, 9 pups were injected with vehicle, 8 with low CTOP, and 9 with high CTOP; and in Experiment 2B, 8 pups in each condition were tested from 11 litters. ANOVAs were conducted for paw withdrawal latencies. In the first step, the baseline latencies of the pups from the three conditions (saline, low CTOP, and high CTOP) were compared by two-way ANOVAs. Because the latencies did not differ between conditions, the time courses after the injection (Time Points 0–10) were compared by three-way ANOVAs. The three conditions were treated as between-group factors; the reactivity at the three time points during the test and the reactivity to the two temperatures were treated as within-subject factors. Tukey's HSD tests were used for post hoc comparisons. Immobility was analyzed with nonparametric Kruskal–Wallis ANOVAs, followed by paired comparisons using Mann–Whitney *U* tests.

Results

vPAG. Brain slices were examined under a light microscope to determine cannula placements. Ink staining was found ventral to the tip of the cannula and was limited to the vPAG (see Figure 2). Two cases were discarded because condition group could not be verified.

Withdrawal latencies of the *right* paw ipsilateral to the injection side differed significantly between conditions, $F(2, 23) = 7.22$, $p < .01$. Immediately after male exposure, saline-injected pups demonstrated increased paw withdrawal latencies at low temperature that were significantly higher than those of the high CTOP pups ($p \leq .05$; see Figure 3). Latencies did not differ between the low CTOP pups and saline-injected pups, or between the low and high CTOP pups. After 5 min, there was no difference between conditions. Latencies at medium temperature did not differ between saline and CTOP pups. Withdrawal latencies of the *left* paw contralateral to the injection side did not differ between conditions. After male exposure, saline and CTOP pups all increased latencies at low temperature, but not at medium temperature (Figure 3).

Baseline immobility did not differ between the three conditions. During male exposure, the pups were more immobile than at baseline ($p < .01$), with no differences between saline- and CTOP-injected pups (see Table 2).

latPAG. Ink staining demonstrated that cannula placement was limited to the lateral part of the PAG (see Figure 4). One case was excluded because placement was above the PAG.

Neither the *right* nor the *left* paw withdrawal latencies differed among pups injected with either saline or CTOP. Immediately after male exposure, all pups increased paw withdrawal latencies at low temperature, but not at medium temperature (see Figure 5).

Baseline immobility did not differ between the three conditions. During male exposure, the pups were more immobile than at baseline ($p = .01$), with no differences between saline- and CTOP-injected pups (Table 2).

General Discussion

CTOP injected into the vPAG of 14-day-old rat pups suppressed analgesia induced by the presence of an adult male rat. CTOP is a selective μ opioid receptor antagonist that blocks binding of endogenous opioid receptor ligands. The results of the present study indicate that male rat cues induce the release of endogenous opioids that activate μ opioid receptors located in the vPAG in rat pups, and that this activation lowers reactivity to noxious thermal stimulation, a mechanism also found in stress-induced analgesia in the adult rat.

First, some methodological issues have to be addressed. In contrast to Experiment 1, the male-exposed pups did not demonstrate analgesia to the medium temperature and, in general, showed more variability in response latencies. It is possible that handling, anesthesia, and the surgical procedures performed the day before testing affected the pups' ability to modulate nociception. Although other studies found that aversive experiences early in life produce long-lasting alterations in nociceptive function (McDowell &

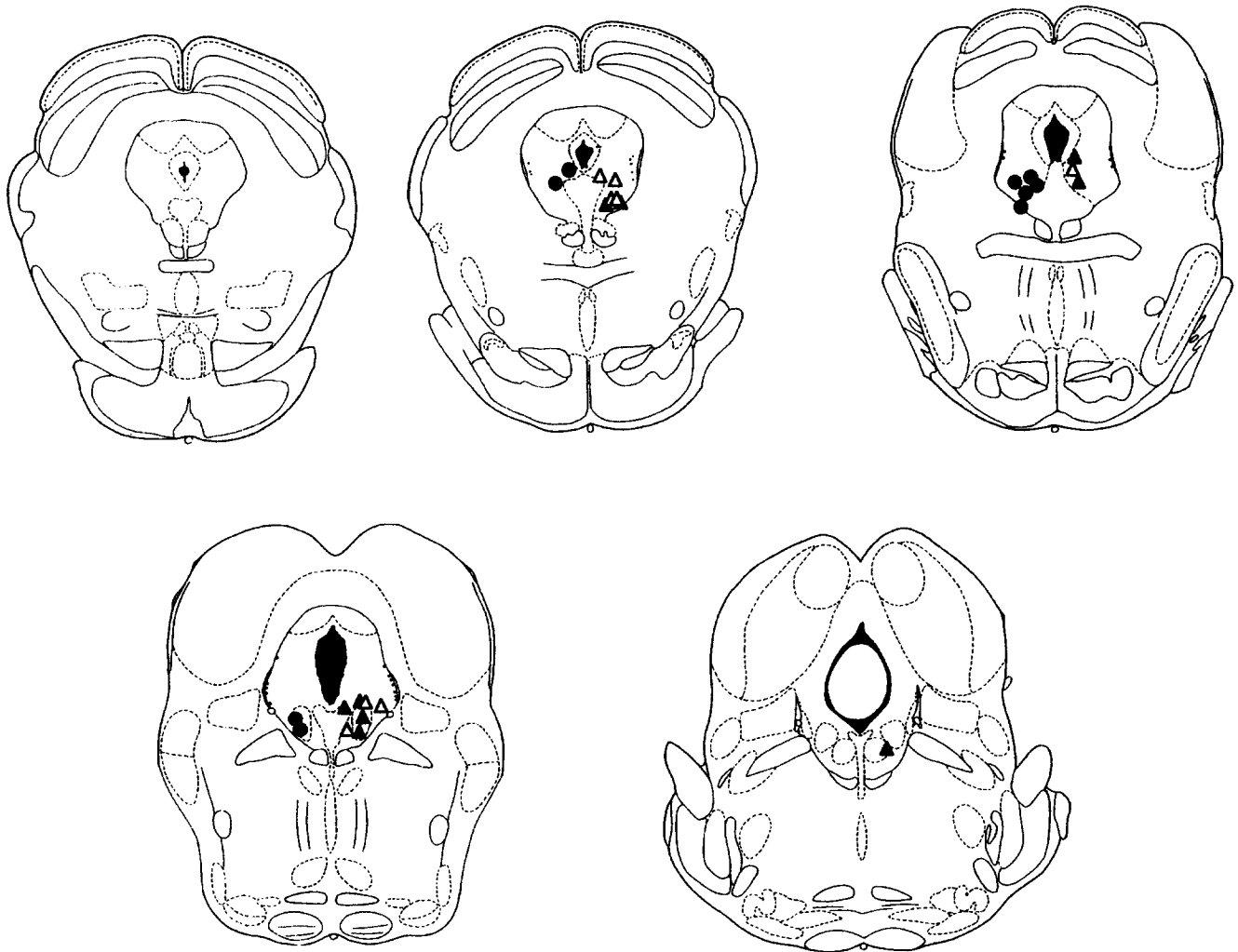


Figure 2. The location of cannula placements within the ventrolateral periaqueductal gray of 14-day-old rat pups into which 200 ng saline vehicle (solid triangles), 50 ng D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, open triangles), or 100 ng CTOP (solid circles) was injected. All placements were made on the rat's right side. Symbols are drawn on both sides for clarity.

Kitchen, 1987; Smythe, McCormick, Rochford, & Meaney, 1994), possible effects of surgery-related stress on subsequent nociceptive modulation have not been investigated.

The data indicate that the effect of CTOP was restricted to the PAG area into which it was injected. The ventrolateral and the lateral injection sites were approximately 0.9 mm apart. The lack of an effect of the high dose of CTOP in the latPAG argues against a diffusion of CTOP from the site of administration into adjacent areas. In addition, the drug did not seem to diffuse into the aqueduct and affect the untreated PAG side, as paw withdrawal latencies were not decreased contralaterally. Neuroanatomical studies suggest that the descending inhibitory pathways are already lateralized in the infant (Fitzgerald & Klotzenburg, 1986; Leong et al., 1984), and a recent amygdala lesion study demonstrated that behavioral analgesia is lateralized in the adult rat as well (Manning, 1998). Our results support and extend these

findings by demonstrating a lateralization in infant analgesia mediated by a brain site more caudal to the amygdala.

CTOP may also act as an agonist at nonopioid receptors (Chieng, Connor, & Christie, 1996). Because no control pups (CTOP-injected pups not exposed to the male) were tested in the present study, a possible effect of CTOP on nociception in the absence of stress could not be determined.

Another limitation of the study concerns the time course of the immobility response. Immobility was measured after baseline at only one time point, during male exposure, but not after removal of the male because the pups were manipulated for nociceptive testing. Therefore, it remains to be determined how the temporal course of immobility relates to the poststress decrease in analgesia.

The neural mechanisms that underlie opioid analgesia have been explored extensively in the adult animal, and the operations of the descending inhibitory pathways are, in

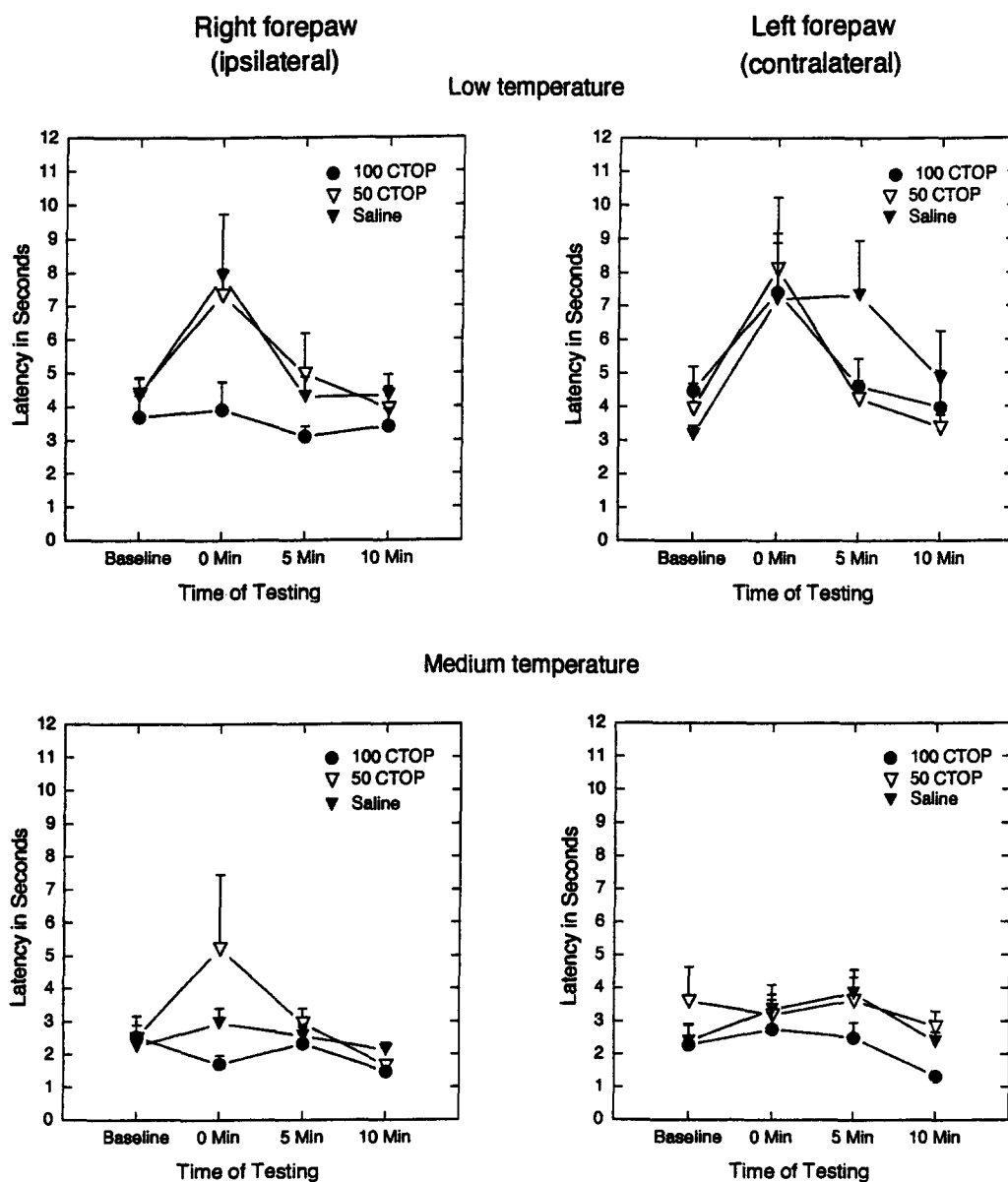


Figure 3. Paw withdrawal latencies ($M \pm SEM$) of 14-day-old rat pups from thermal stimuli (low temperature: 39 °C, medium temperature: 44 °C). After baseline testing, the pups were injected unilaterally into the right ventrolateral periaqueductal gray with 200 ng saline vehicle, 50 ng D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) or 100 ng CTOP. They were then exposed to an adult male rat. After removal of the male, withdrawal latencies were measured at 0 min, 5 min, and 10 min.

principle, well understood. Aversive stimuli release endogenous opioids such as β -endorphin in the PAG (Külling, Siegfried, Frischknecht, Messiha, & Pasi, 1989; Millan, Czlonkowski, Millan, & Herz, 1987). These peptides bind to μ opioid receptors located on GABAergic interneurons in the PAG that are tonically inhibitory (Behbehani, 1995; Chieng & Christie, 1994b; Kalyuzhny & Wessendorf, 1997). Activation of the opioid receptors induces hyperpolarization by increased K⁺ conductance and decreased Ca²⁺ influx into neurons and reduces neurotransmitter release (Chieng & Christie, 1994a; Standifer & Pasternak, 1997). Therefore, opioid binding reduces both the release of GABA and the

tonic inhibition of these interneurons (Osborne, Vaughan, Wilson, & MacDonald, 1996; Vaughan & Christie, 1997; Vaughan, Ingram, Connor, & Christie, 1997). The PAG projects, via the rostral ventromedial medulla, to the dorsal horn of the spinal cord (Basbaum & Fields, 1984; Beitz, 1992). As a result of the decreased inhibition, the PAG output becomes excitatory (Roychowdhury & Fields, 1996) and modulates afferent noxious signals in the spinal cord (Budai & Fields, 1998). In principle, the same neural processes apply to infants, but during early postnatal development the descending inhibitory pathways are subjected to maturational processes until they become fully functional

Table 2
Immobility of 14-Day-Old Pups, Expressed as a Percentage of Observation Scans

Brain area	Baseline	Saline	50 ng CTOP	100 ng CTOP
vPAG	18.9	81.7*	73.1*	70.5*
latPAG	22.1	81.2*	83.7*	83.7*

Note. After 5 min of baseline, pups were injected unilaterally into the right ventrolateral periaqueductal gray (vPAG) or the lateral PAG (latPAG) with 200 ng saline vehicle, 50 ng D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) or 100 ng CTOP. Pups were then exposed for 5 min to an adult male. Data were analyzed with Kruskal-Wallis's analyses of variance followed by Mann-Whitney *U* tests.

**p* ≤ .01, significant difference from baseline.

(Fitzgerald, 1994; McDowell & Kitchen, 1987). For example, in 3-day-old pups, μ opioid receptor stimulation within the PAG induces analgesia only in the forepaws, but it is not until Postnatal Day 14 that analgesia occurs in the

hindpaws and tail as well (Barr & Wang, 1994). This rostral-to-caudal development of analgesia indicates a somatotopic maturation of the descending inhibitory pathways (Barr, 1992). The present data suggest that the descending inhibitory pathways are functional on Postnatal Day 14 when activated by male cues.

The effect of CTOP on male-induced analgesia seemed to be column-specific, which extends the findings from adult literature. In adults, although μ opioid receptors are expressed in both the vPAG and the latPAG (Mansour, Fox, Akil, & Watson, 1995; Mansour, Fox, Burke, Akil, & Watson, 1995), only μ opioid receptor blocking in the vPAG suppressed stress-induced analgesia (Bellgowan & Helmstetter, 1998). Whether μ opioid receptors are also expressed in the latPAG of infant rats and what role they play remains to be investigated.

Only a few studies have investigated how descending inhibitory pathways are activated by biologically relevant

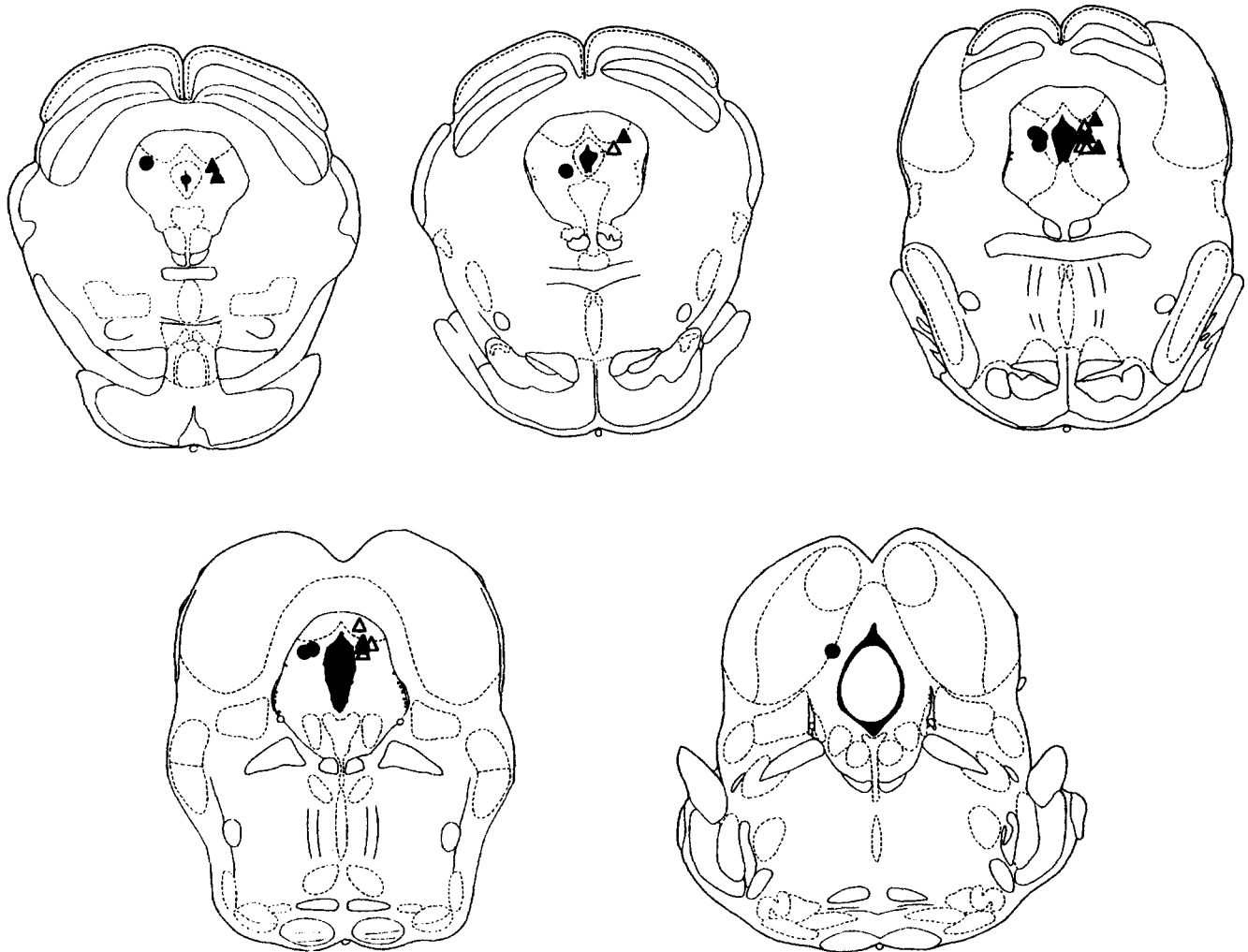


Figure 4. The location of cannula placements within the lateral periaqueductal gray of 14-day-old rat pups into which 200 ng saline vehicle (solid triangles), 50 ng D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, open triangles) or 100 ng CTOP (solid circles) was injected. All placements were made on the rat's right side. Symbols are drawn on both sides for clarity.

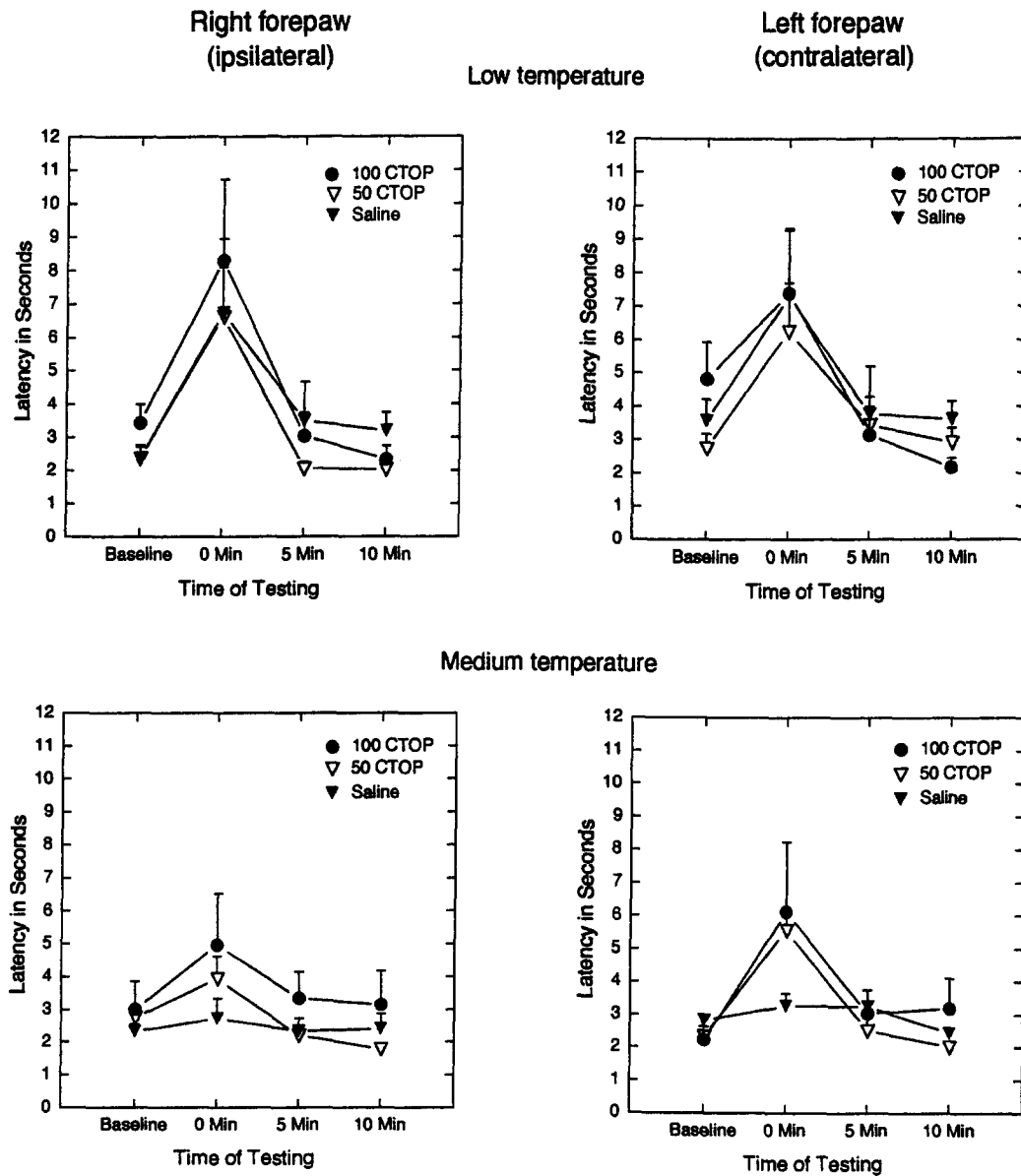


Figure 5. Paw withdrawal latencies ($M \pm SEM$) of 14-day-old rat pups from thermal stimuli (low temperature: 39 °C, medium temperature: 44 °C). After baseline testing, the pups were injected unilaterally into the right lateral periaqueductal gray with 200 ng saline vehicle, 50 ng D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) or 100 ng CTOP. They were then exposed to an adult male rat. After removal of the male, withdrawal latencies were measured at 0 min, 5 min, and 10 min.

stimuli and how they modulate nociception in naturally occurring situations. Social defeat stress induced the release of β -endorphin in the PAG of adult male mice (Külling, Frischknecht, Pasi, Waser, & Siegfried, 1988) and rats (Nikulina, Marchand, Miczek, & Kream, 1997), and the injection of an opioid antagonist into the PAG blocked defeat-induced analgesia in adult mice (Miczek, Thompson, & Shuster, 1985). The present study is, to our knowledge, the first to demonstrate the role of the PAG in mediating opioid analgesia induced by a natural, ecologically relevant stressor in the infant rat. The results demonstrate that similar opioid processes in the PAG in both infant and adult rats

mediate environmentally induced analgesia, although the effective stimuli depend on the age of the subject.

Stimulation and lesion studies in adult animals have demonstrated that the PAG is an important brain area not only for the mediation of analgesia, but also for defensive behaviors (for a review, see Behbehani, 1995). In a number of animal species, becoming immobile is a prominent defensive reaction to a variety of aversive stimuli (Blanchard & Blanchard, 1988; Boissy, 1995; Lima & Dill, 1990). In the adult rat, immobility or freezing can be induced by injecting excitatory amino acids (Bandler & Depaulis, 1991; Depaulis, Keay, & Bandler, 1994; Morgan, Whitney, &

Gold, 1998) or morphine (Morgan et al., 1998) into the PAG. Other neurotransmitter systems in the dorsal PAG such as serotonin (Amat, Matus-Amat, Watkins, & Maier, 1998), nitric oxide (Chiavegatto, Scavone, & Canteras, 1998), and tachykinin (Mongeau, De Oca, Fanselow & Marsden, 1998) seem to contribute to stress-induced freezing. Further investigation is necessary to determine the roles of the different opioid receptor types within the PAG in the control of immobility. The present findings argue against a role of PAG μ opioid receptors in male-induced immobility in the infant rat. Other brain areas and transmitter systems have been implicated in male-induced immobility in rat pups. Immobility depends on maturational effects of glucocorticoids on dentate granule cells in the hippocampus (Takahashi, 1995) and is facilitated by septohippocampal cholinergic release (Takahashi & Goh, 1996). An intact hippocampus seems to be important for male-induced immobility because blockade of the NMDA receptors on Postnatal Day 5, which interferes with the normal development of the dentate gyrus, decreases immobility (Gould & Cameron, 1997). The only other studies investigating the PAG as the substrate of defensive behaviors in infants used a different aversive situation, in which a rat pup is separated from dam, nest, and siblings, resulting in reactions such as ultrasonic vocalization and escape behaviors (Hofer, 1996). When 14-day-old, socially isolated pups are infused with κ opioid receptor or glutamate receptor agonists into the PAG, escape behaviors and immobility are increased (Goodwin & Barr, 1998a, 1998b). Isolation-induced immobility may be elicited by stimuli other than male-induced immobility, and it has yet to be investigated whether immobility shown in different situations involves the same processes in the PAG.

The lack of an effect of CTOP on male-induced immobility confirms the results of Experiment 1, which dissociated the two male-induced reactions of analgesia and immobility. In the adult rat, several studies have indicated that distinct neural substrates within the PAG underlie analgesia and immobility, both of which were evoked separately by morphine microinjections into either the vPAG or the latPAG (Morgan, Whitney, & Gold, 1998). Naltrexone injections into the vPAG blocked stress-induced analgesia but did not disrupt defensive freezing (Helmstetter & Landeira-Fernandez, 1990). The dissociation of analgesia and immobility on the level of the PAG supports the model of two separate and competitive motivational systems: defense and pain (Bolles & Fanselow, 1980; Fanselow, 1986). This model stipulates that the defense system inhibits the pain system through endogenous opioids, but the brain areas mediating this inhibition are unknown. Our findings do not allow us to draw conclusions on whether and where these two neurobehavioral systems interact. In addition to the role of the PAG, the amygdala is also involved in the control of both analgesia and defensive behavior through projections to the PAG (Bellgowan & Helmstetter, 1996; Da Costa Gomez & Behbehani, 1995; Helmstetter, Tershner, Poore, & Bellgowan, 1998; Oliveira & Prado, 1994; Pavlovic & Bodnar, 1998; Pavlovic, Cooper, & Bodnar, 1996). The amygdala is a likely candidate as a site of an interaction because defensive behavior and analgesia seem to be

mediated by the same processes, whereas these two reactions rely on different neural substrates in the PAG (Harris & Westbrook, 1995). It remains to be investigated what pathway activated by aversive male cues mediates defensive immobility, and where in the brain an interaction between this pathway and the pathway inhibiting nociception may take place.

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