

Research report

The effect of periaqueductal gray lesions on responses to age-specific threats in infant rats

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Accepted 28 December 1999

Abstract

During early ontogeny infant rats show specific responses to a variety of age-dependent threatening situations. When isolated from nest and dam, they emit ultrasonic vocalizations and show decreased reactivity to noxious stimulation, or analgesia. When exposed to an unfamiliar adult male, they become immobile and analgesic. The midbrain periaqueductal gray (PAG) is an important area within the circuitry that controls responses to threatening stimuli in the adult. Little is known about the functions of the PAG in early life. It was hypothesized that the PAG mediates the responses to the age-specific threats social isolation and male exposure in the infant rat. Rat pups were lesioned electrolytically either in the lateral or the ventrolateral PAG on postnatal day 7, tested in social isolation on day 10, and exposed to a male on day 14. On day 10 during isolation, ultrasonic vocalizations and isolation-induced analgesia were decreased in both lesion groups. On day 14, male-induced immobility and analgesia were decreased in ventrally lesioned animals. In conclusion, the PAG seems to play a developmentally continuous role in age-specific responses to threat such as ultrasonic vocalization, analgesia, and immobility. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Periaqueductal gray; Lesion; Ultrasonic vocalization; Analgesia; Immobility; Rat

1. Introduction

Characteristic of early development are rapid changes in an individual's environment. The changing requirements that the environment imposes on the growing animal are met by changes in the individual's behavioral repertoire. New behavior patterns appear and disappear throughout development. In the young of altricial rodents a situation may be dangerous during a certain period of maturation, elicit specific responses, then lose its threat and no longer trigger these responses. For example, preweaning rat pups strongly depend on the mother for protection, warmth and nutrition [19]. Therefore, isolation from nest, littermates, and dam, which may be caused by falling out of the nest or

by being dropped by the mother, represents a severe threat to the pup's life [36,39]. Social isolation elicits an array of behavioral and physiological responses such as increased ultrasonic vocalization [29] and decreased reactivity to noxious stimuli (analgesia) [22,34], which may increase the chances to be retrieved by the mother and thus survive the separation [33,34]. These isolation-induced responses are shown shortly after birth, but decrease gradually and disappear as the pups get older [29]. The disappearance of the isolation-induced responses parallels an increase in mobility as the rat pups no longer spend their entire time in the nest close to littermates and dam, but begin to extend their area of activity outside the nest [9,37]. Another age-specific threat is imposed on rat pups by unfamiliar male rats that intrude into foreign territories and kill the offspring of resident pairs, increasing the intruder's reproductive success by bringing the female into estrous [28,30]. Infanticide ends with weaning because after lactation females resume cycling and mating [28,30]. At an age

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of 7 days, rat pups do not show responses to the presence of an unfamiliar male. On day 14 the male elicits immobility and analgesia, which may counteract the male threat [41]. These responses disappear at the end of the third week around weaning [35,41] paralleling the decrease of the infanticidal threat.

In the adult animal, the midbrain periaqueductal gray (PAG) coordinates adaptive reactions to a variety of aversive stimuli by integrating different sensory inputs and organizing appropriate behavioral and physiological output [6,12]. Lesion and stimulation studies in the adult rat have shown that the PAG is made of longitudinal neuronal columns extending along the rostrocaudal axis of the PAG [1]. Its columnar organization relates to different behavioral, physiological and antinociceptive reactions. Different classes of input neurons target specific columnar circuits and adjust somatic, autonomic and antinociceptive functions by triggering defensive behavior patterns, cardiovascular changes and analgesia [2,10,32]. For example, the lateral column in the dorsal PAG (IPAG) controls active defensive behaviors such as threat display or flight, vocalization, increases cardiovascular activity, and mediates non-opioid analgesia, whereas the ventrolateral column (vIPAG) controls passive defensive behaviors such as immobility, decreases cardiovascular activity and mediates opioid analgesia [2,6,12]. Note that earlier papers did not always make this distinction.

Few studies have addressed the role of the different columns of the PAG to generating responses to aversive stimuli in the developing animal. Injection of the amino acid agonist kainic acid into the IPAG increased immobility in socially isolated rat pups on day 14 [14]. When opioid agonists were microinjected into the vIPAG, analgesia was produced in forepaw as early as on postnatal day 3 [5,38]. Yet stimulation studies do not answer the question of whether the responses triggered by central stimulation correspond to the responses triggered by external aversive stimuli. In contrast, presenting external aversive stimuli to an animal with a localized lesion may show whether this brain area is a necessary part of the neural circuitry underlying a specific response.

The aim of the present study was to determine whether responses of rat pups shown in different aversive situations at different ages are mediated by the PAG. Accordingly, we hypothesized that PAG lesions would diminish defensive behavior and analgesia in rat pups in aversive situations at different ages. We tested pups that were lesioned bilaterally in either the IPAG or the vIPAG in two age-specific aversive situations, in social isolation and exposed to an unfamiliar adult male rat. The pups were tested in these two situations at an age when their responses peak on postnatal day 10 in isolation [16], and on day 14 in the

presence of a male [40]. We expected that IPAG lesions would decrease isolation-induced ultrasonic vocalization, and that vIPAG lesions would decrease isolation-induced and male-induced analgesia and male-induced immobility.

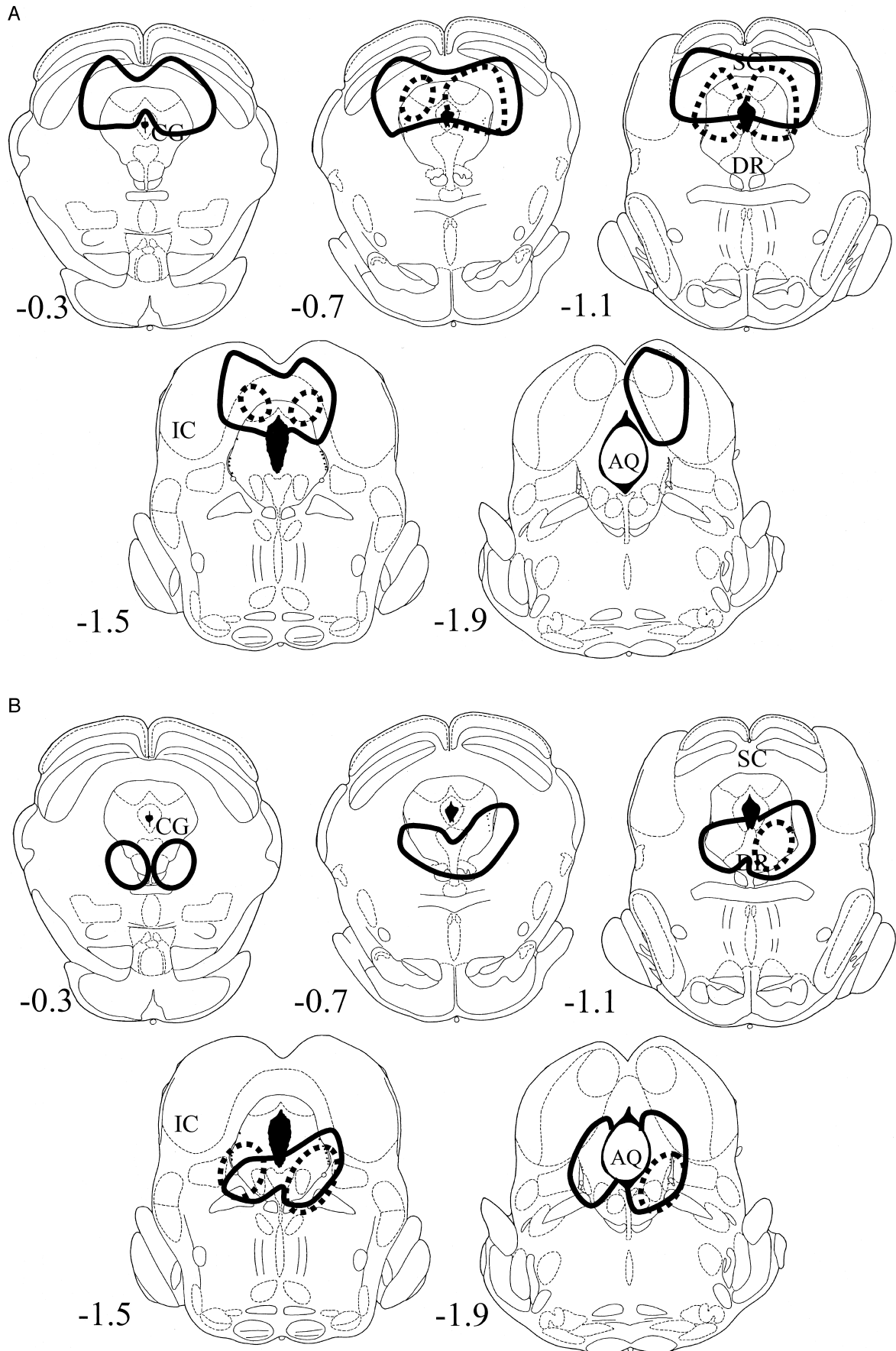
2. Materials and methods

2.1. Animals

Subjects were 79 rat pups derived from 17 litters. They were the offspring of Long–Evans hooded rats mated in our laboratory. The parental animals were housed in standard laboratory cages in a colony room maintained at 22–24°C with a 12-h light/12-h dark photocycle with light onset at 0800 h. Cages were checked twice daily, at approximately 0900 and 1800 h. Pups found at either time were termed 0 days of age. A sexually experienced unfamiliar, unrelated adult male was housed under identical conditions in a different room.

2.2. Surgery

On the day of lesioning, on postnatal day 7, litters were removed from the dam and placed in a cage on a heating pad maintained at $35 \pm 2^\circ\text{C}$. Subjects were weighed and sexed. They were anesthetized with methoxyflurane (Metofane, Pitman-Moore) and then placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) modified for pups. Two holes were drilled through the skull, a stainless steel electrode insulated except for the tip (1 mm) was stereotaxically lowered into position, using the following coordinates: 1.8 mm posterior to lambda, 0.8 mm lateral to midline on both sides (bilateral lesion); dorsoventral coordinates varied according to PAG sites: 4.0 mm for IPAG, 5.0 mm for vIPAG. A current of 10 mA was applied on both sides during 10 s through a lesion maker (Grass LM4, Quincy, MA). The electrode was removed, the holes covered with cyanoacrylate glue and the wound was closed with the same adhesive. Sham lesioned animals underwent the same procedure with the electrode lowered to the vIPAG site; however, no current was delivered. After surgery the pups were returned to the holding cage with the littermates and allowed to recover before being returned to the dam. In each litter, three pups received the same lesion treatment and two of the three treatments (IPAG, vIPAG, sham) were carried out, yielding a total of six treated pups per litter. The three treatments were counterbalanced across litters. Recovery from surgery was assessed by taking body weights on postnatal day 10. Nine animals were excluded because they had low body weights (≤ 15 g).



2.3. Testing procedure and behavioral measures on postnatal day 10

An observer who was blind to the lesion treatment took a pup out of the home cage and carried it to the testing room adjacent to the colony room. Nociceptive reactivity was immediately assessed by a paw-withdrawal test. The forepaw was chosen because it is sensitive to supraspinal influences earlier in development than the hind paw and tail [3,4]. The left forepaw of a pup was put on one of three electric resistors in a random order. The resistors were heated by current and maintained nominal temperatures of 39, 44 and 49°C. Different temperatures were used to generate multiple intensities of stimulation. The latency to withdraw the paw was recorded using a timer (Lafayette Instruments, Lafayette, IN) operated by a foot pedal. The timer was activated when the forepaw touched the resistor and was terminated when the animal removed the paw from the resistor. If the pup did not lift its paw after 20 s, it was removed from the resistor to prevent tissue damage. Axillary body temperature was assessed and the pup was put in the testing cage (46 × 25 × 21 cm). The number of ultrasonic vocalizations (USV) was counted for 10 min. USVs were transduced to the audible frequency range by a bat detector with its microphone suspended 15 cm above the cage floor. The behavior of the pup was recorded by scan sampling during which the observer noted whether or not a specific behavior pattern was occurring [27]. During the observation period, every 20 s the behavior of the pup was recorded on a checklist of behavior categories including 'locomotion'. Behaviors were expressed in rate per 10 min. Nociceptive threshold and body temperature were again measured after the 10-min isolation period. The latency of the pup to right itself after being placed supine on a flat surface was assessed twice and the average latency was calculated. The pup was brought back to the home cage. The order of testing of the three treatments was counterbalanced. All tests were conducted in the first half of the light cycle.

2.4. Testing procedure and behavioral measures on postnatal day 14

On the day of testing, pups with the same lesion treatment were marked with a nontoxic marker. Another person blind to the treatment tested the pups. Three pups were carried to the same testing room as on day 10 and put into the testing cage (46 × 25 × 21 cm). The cage was subdivided by a wire-mesh partition positioned in the middle of the cage, thereby forming two equal compartments. The three pups were placed into one compartment that was provisioned with soiled wood shavings that had been taken out of the home cage to simulate the nest area. The pups were allowed to acclimate to the testing cage for 10 min. The behavior of the pups was recorded in a 5-min baseline period again by scan sampling. During the 5-min

observation period, every 15 s the behavior of each of the three pups was recorded on a check list of behavior categories including 'immobile'. 'Immobile' was defined as any posture in which the animal did not exhibit any movement except that necessary for respiration. Afterwards, nociceptive reactivity was measured by the same thermal paw-withdrawal test used on day 10. The unfamiliar male was placed in the adjacent compartment and the behavior of the pups was recorded for 5 min. The male was then removed. Response to thermal stimulation was assessed immediately after removing of the male. The righting reflex was recorded as on day 10. The pups were put back into the home cage in the colony room. The order of testing of the three treatments was counterbalanced. All tests were conducted in the first half of the light cycle.

2.5. Histology

To verify the lesion site, all animals were euthanized by CO₂ inhalation after the testing on day 14. The brains were removed by dissection and placed in a 10% buffered formalin–30% sucrose solution until they sank. They were then sectioned coronally (50 μm) with a freezing microtome (Lipshaw, Detroit, MI), mounted on glass microscope slides, stained with cresyl violet and coverslipped. An experimenter blind to the animals' responses categorized them into either IPAG or vIPAG groups based on the location of the lesion. All lesions were reconstructed on serial coronal drawings of the rat PAG [31]. If a lesion destroyed at least 80% of the top half of the PAG in at least one coronal plane it was considered an IPAG lesion (Fig. 1A). If a lesion destroyed at least 80% of the bottom half of the PAG in at least one coronal plane it was considered a vIPAG lesion (Fig. 1B). A total of 20 animals were excluded because the lesion produced was either too small to meet these criteria or was too large and extended into the dorsal midbrain. Thirteen animals with IPAG lesions and five animals with vIPAG lesions were selected.

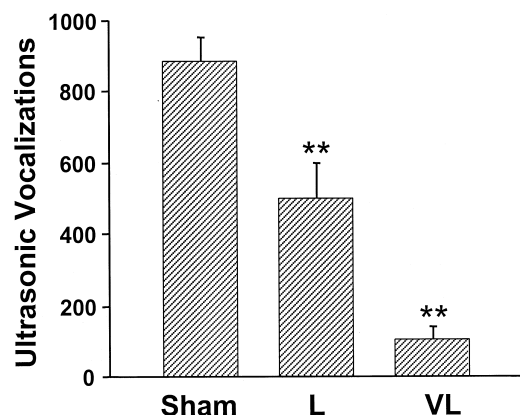


Fig. 2. Mean number (\pm S.E.) of ultrasonic vocalizations produced by PAG lesioned rats on postnatal day 10 during 10 min of social isolation. L: IPAG; V: vIPAG. ** $p < 0.01$, different from sham operated.

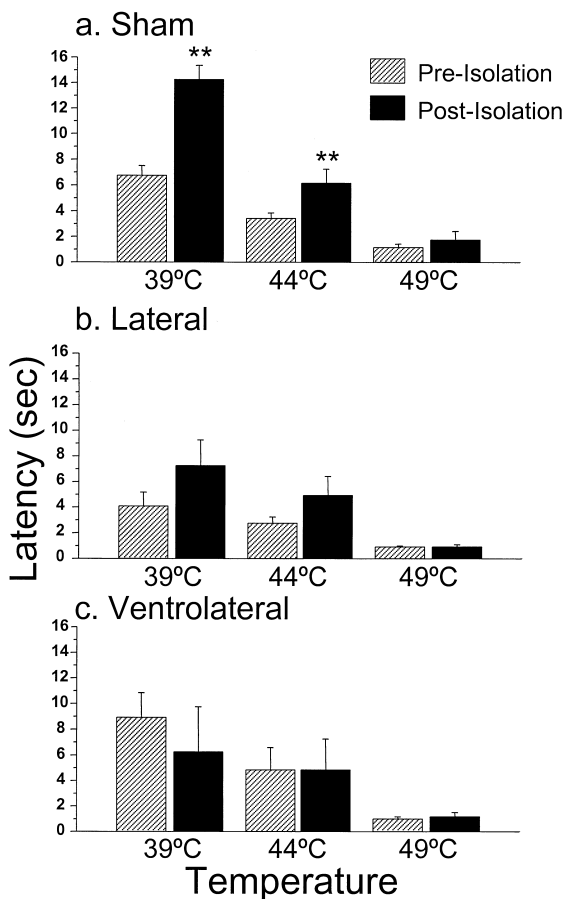


Fig. 3. Forepaw withdrawal latencies (mean ± S.E.) from three thermal stimuli of PAG lesioned rats on postnatal day 10 before and after 10 min of social isolation. ** $p < 0.01$, different from pre-isolation.

A total of 32 sham-operated animals were also derived from these litters. In all groups, no more than two animals from the same litter were used in the analysis of data.

3. Results

3.1. Ten-day-olds

Both groups of lesioned pups produced fewer ultrasonic vocalizations than did the sham-operated pups (analysis of

Table 1
Mean (S.E.) body weight, body temperature, and behaviors of PAG lesioned 10-day-old rat pups

	Sham	Lateral	Ventrolateral
Body weight (g)	23.0 (0.4)	22.7 (0.6)	22.5 (1.0)
Body temperature (°C)			
Pre	34.3 (0.1)	34.4 (0.2)	35.1 (0.2)*
Post	33.2 (0.2)	33.5 (0.2)	33.9 (0.4)
Locomotion (counts)	1.6 (0.3)	3.0 (0.6)*	4.2 (1.7)*
Righting reflex (s)	0.9 (0.1)	0.9 (0.0)	0.8 (0.1)

* $p < 0.05$, different from sham operated.

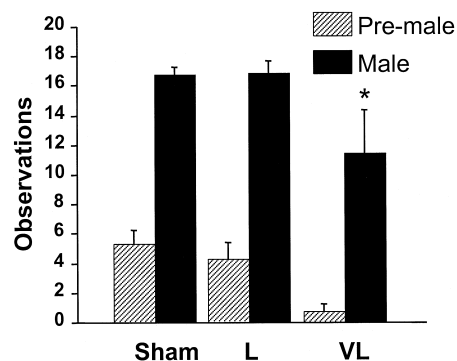


Fig. 4. Immobility (mean ± S.E.) of PAG lesioned rats on postnatal day 14 before and during 5 min of exposure to an adult male rat. L: IPAG; V: vIPAG. * $p < 0.05$, different from sham and IPAG lesioned animals.

variance ANOVA, $F_{2,47} = 12.95$, $p < 0.001$, Fig. 2). The USVs of vIPAG animals were significantly lower than of the IPAG animals (post hoc Fisher's test, $p < 0.05$) and USVs of both groups were significantly lower than USVs of sham animals ($p < 0.01$).

For nociceptive reactivity, first pre-test paw withdrawal latencies were compared. There was a trend for a difference across treatments at the low temperature ($p = 0.053$).

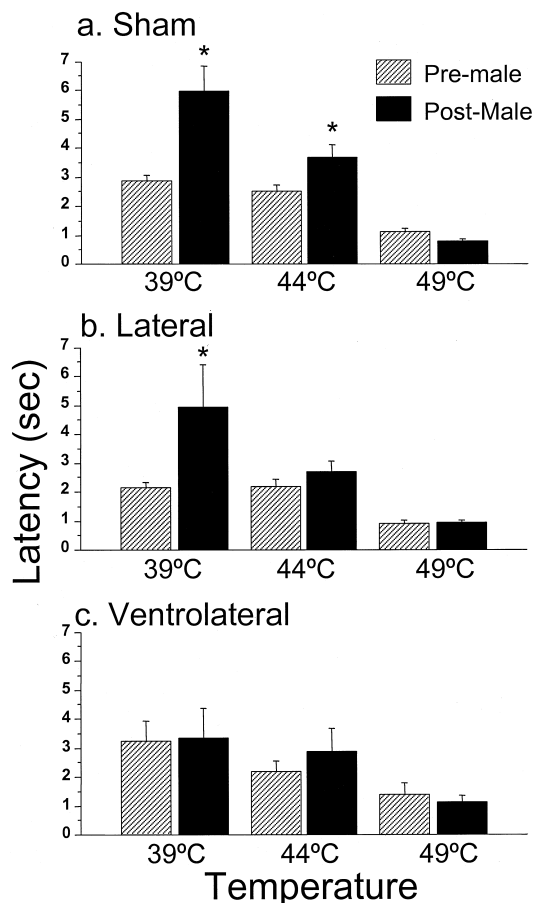


Fig. 5. Forepaw withdrawal latencies (mean ± S.E.) from three thermal stimuli of PAG lesioned rats on postnatal day 14 before and after 5 min exposure to an adult male rat. * $p < 0.05$, different from pre-male.

Table 2
Mean (S.E.) body weight and righting reflex of PAG lesioned 14-day-old rat pups

	Sham	Lateral	Ventrolateral
Body weight (g)	32.0 (0.5)	32.0 (0.9)	32.3 (1.4)
Righting reflex (s)	0.4 (0.0)	0.4 (0.0)	0.5 (0.0)

IPAG lesioned animals had shorter latencies than did vIPAG animals ($p < 0.05$). In the subsequent analyses, the data for each group were analyzed in two factor ANOVAs (time \times temperature). The analysis of the sham-operated animals indicated a significant interaction effect between time and temperature ($F_{2,62} = 10.54$, $p < 0.001$). The interaction resulted from the sham animals showing longer latencies after isolation, i.e., analgesia, at low and medium temperatures ($p < 0.01$; Fig. 3). There was no interaction effect between time and temperature for neither the IPAG nor the vIPAG lesioned animals; i.e., they did not become analgesic (Fig. 3).

Body weight did not differ across groups (Table 1). The vIPAG lesioned animals had higher body temperatures than sham animals at the beginning of the testing period ($p < 0.05$), and both IPAG and vIPAG animals showed more locomotion ($p < 0.05$) than the sham animals. There were no differences in the righting reflex across treatments.

3.2. Fourteen-day-olds

Immobility before exposure to the male did not differ across treatments. When immobility was compared during male exposure, there was a lesion effect ($F_{2,44} = 4.15$, $p < 0.05$); vIPAG animals exhibited significantly less immobility than did sham and IPAG animals ($p < 0.05$, Fig. 4).

There were no differences in pre-test paw withdrawal latencies across groups. The data for each group were analyzed in two factor ANOVAs (time \times temperature). There was a significant interaction effect between time and temperature for the sham operated animals ($F_{2,58} = 7.11$, $p < 0.01$) as they showed analgesia at low and medium temperatures after male exposure ($p < 0.05$; Fig. 5). The IPAG lesioned animals showed a marginal interaction ($F_{2,24} = 8.36$, $p < 0.07$), i.e., showed analgesia to low temperature only ($p < 0.05$), whereas there was no analgesia found in vIPAG lesioned animals (Fig. 5).

No differences in body weight or in the latency to right were observed in any of the groups (Table 2).

4. Discussion

The results of this study suggest that the PAG of the infant rat plays differential roles in the production of

age-specific responses to naturally occurring aversive situations. PAG lesions decreased vocalization during and analgesia after social isolation on postnatal day 10, and immobility and analgesia following exposure to an unfamiliar adult male on postnatal day 14.

Lesions did not affect weight gain and motor performance. Similar righting latencies of all pups on either day 10 or 14 suggest that there were no gross motoric impairments resulting from the lesions. Lesioned animals showed even more locomotion on day 10 than sham animals.

The most pronounced effect of PAG lesions was the reduction of isolation-induced ultrasonic vocalizations. Both lesion groups exhibited significantly fewer USVs than did sham animals, especially the animals with lesions to the vIPAG. This result is consistent with studies in several species, including rats, demonstrating that the PAG plays an important role in the production of vocalizations in fully mature animals [2,20,24,43]. Infant rats produce vocalizations in a higher frequency range (40 kHz) than do adults (22 kHz) and only when removed from familiar nest cues during the first three postnatal weeks [29]. Therefore, the PAG seems to control different forms of vocalization throughout the life span. Changes at the receptor level within the PAG could play a role in developmental changes in the organization of vocalizations. In support of this hypothesis, we have shown that PAG stimulation with kainic acid evoked almost no calls until day 14 [14]. In contrast, stimulation with a kappa opioid receptor agonist evoked a high rate of calls on day 7, but almost no calls by day 21 [15]. Whether decreased vocalization and increased locomotion in the PAG lesioned animals in the present study indicate that the lesioned pups perceived isolation as less threatening remains to be investigated.

On day 14, male exposure induced immobility in all groups, however to a lesser degree in vIPAG lesioned animals. In adult rats, lesions of the vIPAG decreased freezing in reaction to conditioned stimuli [12,23,25,26]. In a recent study, adult rats with lesions either to the IPAG or the vIPAG were exposed to a cat [11]. Lesions to the vIPAG decreased and to the IPAG increased unconditioned freezing. The authors suggested that the vIPAG is critical in mediating freezing, whereas during extreme risk the IPAG inhibits freezing and mediates active defensive behaviors such as escaping [11]. Predator-induced freezing in adult rats and male-induced immobility in infant rats seem thus to be mediated both by the ventral part of the PAG. However, lesioning of the IPAG, which increased freezing in the adult [11], did not increase immobility in the infant rat. It is possible that the circuits within the PAG that mediate escape responses become functional during the preweaning period. For example, kainate stimulation of the IPAG produced escape behavior in 14-day-old, but not in 7-day-old pups [14].

Lesioning of the vIPAG did not fully suppress immobility. The reasons for the discrepancy between more effective lesions in adult animals and less effective lesions in

pups in this study are not clear. It is possible that infant rats show a greater degree of functional recovery than do adult rats, resulting in compensatory processes between application of lesion and day of testing. The placement and size of the lesions in the present study are comparable to the size and placement of the lesions reported in the studies of adults; however, it is still possible that some very subtle difference in the placement of the lesions did occur. Future studies will need to be devised to distinguish between these possibilities.

The PAG seems to mediate stress-induced analgesia in infant rats, as IPAG and vIPAG lesions decreased isolation-induced analgesia on day 10, and vIPAG lesions male-induced analgesia on day 14. Also, lesioning of the IPAG produced hyperalgesia in the pre-test in 10-day-old pups. The IPAG may be involved more in basic nociception and the vIPAG more in modulation of nociception during stress. The role of the PAG in the modulation of nociception is in principle well characterized in the adult. Descending inhibitory pathways originating in the PAG modulate incoming noxious signals at various sites along the neuroaxis such as in the rostral ventral medulla and in the dorsal horn of the spinal cord [6,7,13,32]. The PAG is activated by aversive stimuli and the type of stimulus determines whether the IPAG or the vIPAG induces analgesia [8,17,18]. These neural processes seem to be similar in infant analgesia. In our study, isolation-induced analgesia was mediated by both PAG columns, whereas male-induced analgesia was mediated by the IPAG. Therefore, age-specific aversive stimuli seem to activate the PAG differentially. In the adult rat, the PAG columns also differ in the neurotransmitters involved in stress-induced analgesia. The IPAG mediates non-opioid and the vIPAG mediates opioid analgesia [2,6,12]. Stress-induced analgesia in the infant rat seems to depend on endogenous opioids as well. Isolation-induced analgesia in pups can be blocked by systemic injections of an opioid antagonist [21,34], and male-induced analgesia can be blocked by infusion of a μ opioid receptor antagonist into the vIPAG [42]. It remains to be investigated what neurotransmitter systems in the IPAG contribute to isolation-induced analgesia. Another study indicated a differential role of the IPAG and the vIPAG in antinociceptive function during early ontogeny [38]. In 10-day-old pups injection of glutamate into the IPAG but not into the vIPAG induced analgesia to thermal stimulation, whereas morphine injections had the opposite effect [38].

In conclusion, the PAG mediates responses that protect the animal from different threatening situations already at an early age. The results of the present study are consistent with adult literature that demonstrates an important role of the PAG controlling vocalization, stress-induced immobility and analgesia. The results also imply that the PAG mediates such responses throughout ontogeny in a developmentally continuous manner.

Acknowledgements

We would like to thank Ludmilla Skaredoff for her assistance as a histologist. This study was supported by MH18264, DA05712, DA00325, and DA07341.

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