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Do Dorsolateral Periaqueductal Gray (dlPAG) Lesions Affect Learning or Increase Performance?

By

Jake Jasch Klassen

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Master of Arts

Department of Psychology

University of Manitoba

Winnipeg, Manitoba

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Affect Learning of Increase Performance?

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Jake Jasch Klassen

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

Master of Arts

JAKE JASCH KLASSEN©1999

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Jake Klassen

August, 1999

Abstract

Much of what is known about the brain mechanisms of fear comes from tracing neural pathways of individual component behaviors. The particular component behavior examined in this thesis is conditioned defensive freezing in the rat (Rattus norvegicus). When a rat is placed in a context in which an aversive shock has occurred, it freezes. Two adjacent areas in the midbrain periaqueductal gray (PAG) have been found to affect the expression of freezing: (a) the ventral periaqueductal gray (vPAG), which evokes freezing, and (b) the dorsolateral PAG (dlPAG), which attenuates freezing and evokes unconditioned responses such as analgesia and scrambling. Importantly, it has also been suggested that, during dlPAG activated scrambling, the rat cannot learn to associate environmental cues with shock (Fanselow, DeCola, De Oca, & Landeira-Fernandez, 1995). Evidence for this learning deficit includes the observation that freezing is attenuated if rats receive a series of shocks in close succession rather than in a more distributed fashion (e.g., Fanselow & Tighe, 1988). If the dlPAG is lesioned, however, similar levels of freezing are found with massed and distributed shock, presumably because dlPAG activation cannot interfere with learning (Fanselow et al., 1995). The three experiments described in this thesis found support for the increased learning hypothesis against the alternative that dlPAG lesions simply alter the balance of freezing and scrambling (performance hypothesis). Experiment 1 (lesions before conditioning) looked for differences in extinction after dlPAG lesions in rats that received context conditioning with either massed or distributed shock. Experiment 2 (lesions after conditioning) determined the effects of dlPAG after the rats had already experienced the context-shock pairings. Finally, Experiment 3 examined

freezing and analgesia in dlPAG lesioned and sham rats that had received either 1 or 3 (massed) shocks. The results of these experiments support Fanselow et al.'s (1995) increased learning hypothesis. Specifically, the results showed that (a) dlPAG lesions placed before, but not after, conditioning facilitated fear conditioning (b) dlPAG lesions did not influence the level of conditioned fear supported by a single shock, and (c) these effects do not appear to be mediated by differences in pain sensitivity or in the magnitude of the unconditioned response to the shock reinforcer.

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Do Dorsolateral Periaqueductal Gray (dlPAG) Lesions

Affect Learning or Increase Performance?

Introduction

Experience tells us that fear is a powerful emotion, and although we know fear well from our own experience, it is only recently that important advances have occurred in our understanding of the neural basis of fear. One reason that research has progressed so slowly is a lack of sophisticated experimental techniques. Another factor is a poor understanding of fear itself. Is fear a coordinated set of physiological responses, or is it a central emotional state that motivates behavior? Perhaps the most important impediment has been the search for a single place in the brain that is responsible for coordinating all aspects of the processing of emotionally laden stimuli (for a review, see LeDoux, 1996). In particular, research in this area was guided for many years by the idea that there might be a unified brain center for emotion in the limbic system (MacLean, 1949). More recently, researchers have begun to make better progress by pursuing a less ambitious research agenda that involves tracing the neural basis of particular component behaviors, such as freezing (e.g., Fanselow, 1991; LeDoux, 1996) or potentiated startle (Davis, 1992).

Most of what is known about the neural basis for fear comes from the study of defensive conditioning in the rat. According to Bolles (1970), animals possess a limited number of prepackaged reactions, or species-specific defense reactions (SSDRs), which have evolved as a defense against predators. Initially, Bolles argued that defensive behaviors were organized hierarchically and behaviors that were ineffective in a given situation were suppressed by punishment (instrumental conditioning). Later, Bolles (1975)

suggested that SSDRs are not selected through punishment, but that features of the environment determine the topography of elicited defensive reactions (e.g., Blanchard, Fukunaga, & Blanchard, 1976a).

One currently popular account of how the environment determines the nature of the response is described by predatory imminence theory (e.g., Fanselow & Lester, 1988). According to this theory, defensive behavior is divided into three categories: (a) pre-encounter, (b) post-encounter, and (c) circa-strike. Casually speaking, these categories correspond in the rat to avoidance, freezing, and fighting. Pre-encounter behavioral changes include alterations in foraging patterns in rats at risk for predatory contact (Fanselow, Lester, & Helmstetter, 1988). The next level of predatory imminence involves situations in which a predator is actually detected (post-encounter). Here, the rat engages in post-encounter defensive behaviors, such as freezing in the presence of a cat (Blanchard, Fukunaga, & Blanchard, 1976a). Freezing presumably reduces the chance of detection by minimizing any releasing stimuli that might otherwise trigger the predator to attack. If the predator makes physical contact, the rat will rapidly switch from post-encounter defensive behavior to circa-strike defensive behavior. It vigorously defends itself by leaping, biting, and calling (e.g, Depaulis, Keay, & Bandler, 1992). The central point of the theory is that environmental factors (the probability of predatory contact) determine the level of fear, and the level of fear then selects the appropriate defensive behavior, either pre-encounter, post-encounter, or circa-strike (Fanselow & Lester, 1988).

In the laboratory, fear and its underlying neurophysiology have been most

intensively studied in conditioned defensive freezing (e.g., Blanchard, Fukunaga, & Blanchard, 1976b). Such studies involve exposing rats to a conditioned stimulus (CS), usually a tone or light, which is then followed by an aversive unconditioned stimulus (US), usually a brief shock. Contextual stimuli present at the time of the CS-US pairing may also become conditioned. After a sufficient number of CS-US pairings, the CS and the context each come to be associated with the US. The effect of these newly formed associations is often assessed directly by measuring freezing, but it can also be measured indirectly by potentiated startle (e.g., Davis, 1992) or through the suppression of appetite appetitively motivated behavior (e.g., Estes & Skinner, 1941). Fanselow (1994) and LeDoux (1996) have independently examined the neural pathways responsible for the elicitation of freezing. The tactic has been to lesion a particular neural center and determine its subsequent impact on fear learning. Through a systematic investigation of neural connections, they have delineated the beginnings of an emotional network for fear.

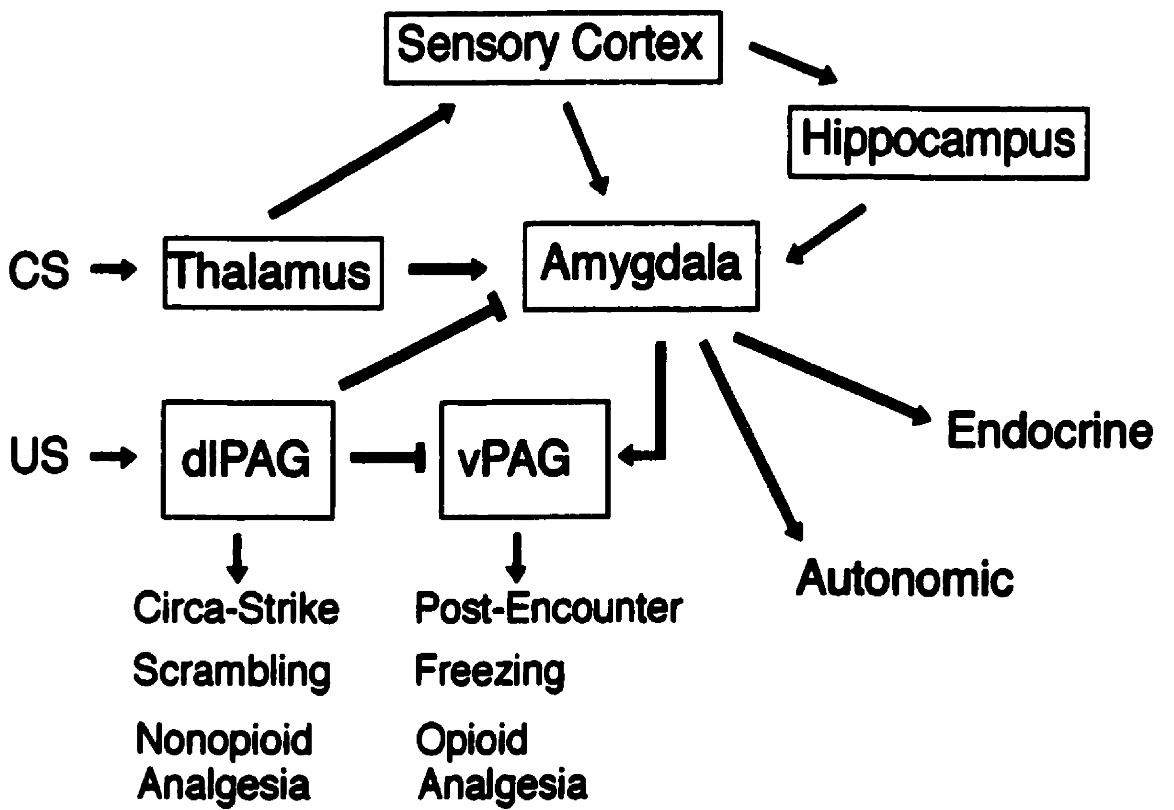
Neural Pathways for Fear

Fear conditioning is thought to involve the strengthening of synaptic connections resulting from the intersection in the brain of pathways transmitting information about the CS and the US. The US must intersect a variety of CS pathways, which originate in different sensory systems. The major goal of previous research has been to trace the processing of the CS, through its sensory system, to the motor system controlling the conditioned response (CRs), thus revealing the circuitry responsible for fear conditioning. As mentioned, the strategy that has worked best uses the classical lesion method with

modern neuroanatomical tracing techniques. If the CS is an auditory stimulus, then the CS pathway begins in the auditory system. Because the auditory system is linearly organized with relays from lower to higher centers, one can easily determine the degree to which the auditory CS must be processed in different types of conditioning procedures. Thus, neuroanatomical tracing techniques allow a determination of the highest auditory station required. If the key connections to the US input are traced in a similar manner, we can learn, using the classical lesion technique, where the CS and the US intersect.

Figure 1 summarizes the neuroanatomical pathways thought to be involved in fear conditioning to a simple auditory CS. LeDoux, Sakaguchi, and Reis (1984) demonstrated that lesions of the midbrain and thalamic stations of the auditory pathway prevent conditioning, but that lesions of the auditory cortex have no effect. These results suggest that the CS pathway leaves the auditory system at the level of the thalamus. Subsequently, LeDoux, Ruggiero, and Reis (1985) confirmed, using anatomical tracing techniques, that the auditory thalamus projections extend to the amygdala as well as to the auditory cortex. Further investigations showed that destruction of the connections between the auditory thalamus and the amygdala impedes conditioning (Iwata, LeDoux, Meeley, Arneric, & Reis, 1986) and that the lateral nucleus of the amygdala is the area critical for the reception of the auditory stimulus (LeDoux, Cicchetti, Xagoraris, & Romanski, 1990). Although the auditory cortex is not required for conditioning, projections from the auditory thalamus via the auditory cortex to the amygdala are sufficient to mediate conditioning with a single auditory CS paired with the US. Both the thalamo-amygdaloid and the thalamo-cortico-

Figure 1. A neural circuit diagram showing both CS and US processing in defensive fear conditioning. Also indicated are the behaviors known to be evoked by the dlPAG and the vPAG.


Sensory Cortex
Hippocampus
CS
Thalamus
Amygdala
US
dlPAG
vPAG
Endocrine
Autonomic
Circa-Strike
Scrambling
Nonopioid
Analgesia
Post-Encounter
Freezing
Opioid
Analgesia

amygdaloid pathways can mediate simple conditioning (Romanski & LeDoux, 1992). Auditory cortical areas are required for the learning of subtle discriminations, such as learning that one auditory stimulus is paired with the US and another is not (called differential conditioning; Jarrell, Gentile, Romanski, McCabe, & Schneiderman, 1987).

The thalamic pathway to the amygdala is direct, short, and provides fast transmission; however, its ability to represent the auditory stimulus is limited (Bordi & LeDoux, 1994). The thalamo-cortico-amygdaloid pathway, on the other hand, is indirect, long and transmission is slower; however, it has a greater capacity to represent the auditory stimulus (Romanski & LeDoux, 1992). Thus, the thalamo-amygdaloid pathway provides quick fear activation in simple conditioning (by simple stimulus features); whereas, the cortical pathway is required for emotional responses tied to perceptually complex stimulus objects (e.g., differential conditioning). The thalamo-amygdaloid inputs and cortical inputs meet in the lateral nucleus of the amygdala (LeDoux et al., 1991). Within the amygdala, the lateral nucleus is the sensory interface and likely critical site of information consolidation from parallel auditory projections during fear conditioning (LeDoux, 1990).

During CS-US pairings, contextual stimuli are inevitably present and should also become associated with the US. Contextual conditioning, like conditioning to a CS, relies on the amygdala; however, unlike CS conditioning, it also relies on the hippocampus (Phillips & LeDoux, 1994). The hippocampus, traditionally linked to complex information processing functions, is also thought to be involved as a higher-order sensory structure for encoding context. Contexts are not defined by the presence or absence of any single

feature; they are defined by the simultaneous presence of a number of different features. The role of the hippocampus may be to link these individual stimulus elements together to form a coherent stimulus representation of the entire stimulus complex (e.g., Gluck & Myers, 1993). Lesions of the hippocampus abolish later contextual conditioning, but if the hippocampus is lesioned some time after the conditioning episode (28 days), the context-US association can be retrieved and evoke freezing behavior (Kim &Fanselow, 1992). Remotely acquired trace conditioning is also unaffected by hippocampal lesions, although recently acquired trace conditioning is abolished (e.g., Kim, Clark, & Thompson, 1995). Although the direction of information flow between hippocampus and amygdala is not certain, the hippocampus (by way of the subiculum) projects to the lateral nucleus (Philips & LeDoux, 1992). Thus, the hippocampus may transfer contextual inputs concerning the conditioning environment to the amygdala, where, just as with the thalamic and cortical information, emotional content is added to context. Contextual fear conditioning would allow the organism to distinguish dangerous contexts from safe ones.

Response Selection

Whereas the lateral nucleus of the amygdala receives projections from various sensory areas, the central nucleus projects to several motor areas (LeDoux, 1993). Lesions of the central nucleus disrupt conditioned responses expressed through various motor modalities including sympathetic and parasympathetic responses, neuroendocrine responses, and freezing behavior (for a review, see LeDoux. 1996). Lesions of centers receiving central nucleus projections disrupt those specific responses. For example, the

lateral hypothalamus (LH) is essential in the circuitry controlling the cardiovascular response elicited by a conditioned fear stimulus. Lesions in this region disrupt conditioned arterial pressure responses, but do not impact conditioned freezing behavior or suppression of operant responses (LeDoux, Iwata, Cicchetti, & Reis, 1988). Thus, it seems that lesions of the LH interfere with cardiovascular responses to a CS without affecting other behavioral responses to that CS in the same animal (Iwata, Chida, & LeDoux, 1987).

The path from the central nucleus of the amygdala to the bed nucleus of the stria terminalis (BST) controls neuroendocrine responses, the release of ACTH from the pituitary gland, and the release of corticosterone from the adrenal cortex (Gray, Piechowski, Yracheta, Rittenhouse, Betha, & van der Kar, 1993). Such control is accomplished by way of projections from the BST to the paraventricular hypothalamus (PVN), which controls pituitary secretions. Again, lesions of BST have no effect on freezing behavior (Iwata et al., 1987).

The focus of the present thesis is the projection of the central nucleus to the midbrain periaqueductal gray (PAG). The midbrain PAG mediates several of the species-specific defensive responses including post-encounter and circa-strike defensive behaviors. Lesions of the ventral periaqueductal gray (vPAG) abolish freezing and reduce the suppression of food-related behaviors that normally occur in aversive situations (Kim, Rison, & Fanselow, 1993). Chemical destruction of cell bodies in the caudal region of the vPAG attenuates freezing (Kiernan & Cranney, 1992); thus, the effect is not due to the destruction of fibers of passage. The vPAG is also critical in opioid-based analgesia that

accompanies fear (Helmstetter & Landeira-Fernandez, 1991). Morgan and Liebeskind (1987), for example, found that electrical stimulation of the vPAG produced a short-lasting anti-aversive effect characteristic of opioid mediation. Elsewhere, microinjections of morphine have been shown to affect the vPAG but not the dlPAG (Yaksh, Yeung & Rudy, 1976). By contrast, the dorsolateral region of the periaqueductal gray (dlPAG) controls circa-strike defensive behaviors (Fanselow, 1991). Lesions of the dlPAG block the circa-strike responses to electric shock, but do not reduce post-encounter responses such as freezing to contextual cues associated with shock (Fanselow, 1991). Autonomic changes (e.g., heart rate) in support of overt circa-strike behavior are also controlled by the dlPAG (Carrive, 1991). The analgesic effects produced by electrical stimulation of the dlPAG, in contrast to vPAG stimulation, are long lasting (Morgan & Liebeskind, 1987) and likely involve the neurotransmitter serotonin or 5-hydroxtryptamine (5-HT; Nogueira & Graeff, 1995).

In summary, the vPAG and dlPAG control different modes of defensive behavior. The former mediates the post-encounter defenses, while the latter mediates circa-strike defenses. Because survival often requires the rat to switch rapidly from freezing to circa-strike defenses, Fanselow (1994) has maintained an inhibitory influence of the dlPAG on the neuroanatomical loci supporting freezing. Such influence would involve inhibition at the level of both the vPAG (freezing) and, more importantly perhaps, the amygdala (conditioned fear; for a review of the neuroanatomical basis for the dlPAG's inhibition of the vPAG see Fanselow, 1994).

If correct, this view suggests that lesions of the dlPAG should attenuate inhibition of fear conditioning. Such loss of inhibitory control by the dlPAG should be revealed if shocks are presented closely together, the so-called massed shock condition (Fanselow & Tighe, 1988). When a rat receives three shocks spaced only 3 s apart (massed shock), it does not engage in high levels of post-encounter defensive freezing when it is later tested in the conditioning context. If, however, the shocks are distributed 60 s apart (distributed shock), there is marked freezing when the rat is later exposed to the conditioning context (Figure 2). According to Fanselow (Fanselow, DeCola, & Young, 1993), the massed shock deficit is caused by interference, or inhibition, from the shock UR on association formation. Nociceptive input (shock) actives dlPAG-evoked scrambling, and scrambling, in turn, briefly inhibits fear conditioning in the amygdala. If a subsequent shock is presented soon after the first shock, it will not serve as a reinforcer for contextual conditioning. Essentially, this hypothesis holds that rats given massed shock only receive a single effective shock reinforcer.

Consistent with this hypothesis, Fanselow, DeCola, De Oca, and Landeira-Fernandez (1995) reported that lesions of the dlPAG attenuate the massed shock deficit. Their experiment used a 3 x 2 factorial design, which included lesion (sham, dlPAG, or vPAG) and conditioning (massed vs. distributed) as between-subject factors. The four most relevant groups were the sham-massed, dlPAG-massed, sham-distributed, and dlPAG-distributed groups. In test, rats with dlPAG lesions that had received massed shock (dlPAG-massed) showed significantly more contextually evoked freezing during a single test session

Figure 2. A diagram which indicates the times of shock delivery in massed (3 s) and distributed (60 s) shock groups. The black arrows indicate time and the red bars indicate shock

Conditioning Manipulations

3 s (massed shock)

60 s (distributed shock)

❚ = shock

than sham controls (sham-massed). They also froze at levels comparable to the distributed groups (sham-distributed and dlPAG-distributed). These differences produced a statistical interaction between lesion and conditioning. This interaction is remarkable because one would not readily expect lesions to facilitate conditioning. Removal of the inhibitory influence of the dlPAG on the amygdala presumably allowed better learning of the context-US association.

There is, however, an alternative interpretation that would predict more freezing after dlPAG lesions. Given that mutually incompatible behaviors are controlled by the vPAG and the dlPAG, as mentioned above, lesions of the dlPAG could simply abolish any inhibitory input from the dlPAG to vPAG. In its simplest form, this hypothesis predicts that dlPAG lesions simply increase freezing in general. Unlike the interpretation provided by Fanselow et al. (1995), this response probability account denies that conditioning increased at all in the dlPAG massed group. The freezing response could have just been more easily evoked at a low level of fear. Freezing may not have increased commensurately in the dlPAG-distributed group because of a ceiling effect. There was little room for freezing in the dlPAG-distributed groups to increase beyond the approximately 80% level seen in the sham-distributed group. Thus, ceiling effects in the only test session administered combined with a main effect of the lesion could have been responsible for the statistical interaction between lesion (dlPAG or sham) and conditioning (massed vs. distributed).

The purpose of these experiments was to assess the viability of the learning and response probability interpretations. Experiment 1 assessed the effects of dlPAG lesions on

the acquisition and extinction of contextually evoked freezing. Experiment 2 examined whether dlPAG lesions occurring after the learning experience would also affect the magnitude of freezing, as might be anticipated by a response probability account but not by an account based on facilitated learning. Experiment 3 compared contextual conditioning with either a single or massed shock in rats that had dlPAG or sham lesions. The rats of this experiment also received a hot-plate test for unconditioned analgesia.

Experiment 1

Experiment 1 was modeled after Fanselow et al. (1995; Experiment 1), but included a large number of extinction test sessions (repeated unreinforced exposures to the context) to reveal any differences in the level of freezing between the dlPAG-distributed and sham-distributed groups. The experiment was a 2 x 2 factorial design, which included lesion (sham vs. dlPAG) and conditioning (massed vs. distributed) manipulations. If dlPAG lesions increase response probability, freezing should decline more rapidly in the sham-distributed group than the dlPAG- distributed group, although initially the levels of freezing might be comparable.

Method

Subjects. The animals were 48 naive male Sprague-Dawley rats (Rattus norvegicus) obtained from Charles River Inc., St. Constant, QC, Canada. The rats weighed approximately 250 g at the time of arrival. They were individually housed with continuous access to food (Prolab, PMI Feeds, St. Louis, MO) and water in a vivarium maintained on a 14:10-hr light:dark cycle. Handling occurred daily from their arrival at the vivarium. The

experimental procedures occurred during the light portion of the light:dark cycle.

Surgery. All rats (350 – 400 g) received an intramuscular injection of antibiotic (.03 cc; Penlong XL, Pfizer Inc.) 1-2 hr before surgery. They were then anesthetized with an intramuscular injection of a ketamine (.36 cc; Ketaset, Wyeth-Ayerst Inc.) and xylazine (.09 cc; Rompun, Bayer Inc.) combination at a 4:1 ratio. Once anesthetized, the rats received a subcutaneous injection of atropine (.02 cc; atropine sulfate, M. T. C. Pharmaceuticals). Next, the head fur was shaved from the interocular region to approximately 10 mm posterior of the interaural point. The rats were then placed in a stereotaxic instrument, with their heads level according to Paxinos and Watson's (1986) criteria. The shaved area was then cleaned using a surgical cleaning solution and all surgical instruments and equipment were bathed in 70% alcohol solution prior to the surgery. An incision was made to expose the skull and rats were then randomly given one of two procedures. For half of the rats, the dlPAG groups, a hole (2 mm diameter) was made in each hemisphere of the skull using a dental drill. A stainless-steel electrode (00 insect pin, insulated except for .5 mm of exposed tip) was lowered to the lesion site at a 10° angle toward the midline. Lowering the electrode in a method of triangulation at a 10° angle toward the midline allowed holes to be drilled at 1.5 mm away from the midline (instead of .6 mm), thereby avoiding rupturing the midsaginal sinus, and the accompanying excessive bleeding, associated with a straight vertical path to the dlPAG. The lesions were produced by passing current (1.0 mA anodal, 10 s) through the electrode (Grass, DC Constant Current Lesion Maker, Model DC LM5A, Quincy, MA, USA). Four lesions (two electrode entries per hole) were created with the

stereotaxic coordinates 7.2 and 8.0 mm posterior to bregma, -1.5 and +1.5 mm lateral to the midline, and 5.5 mm ventral to the skull (Paxinos & Watson, 1986). For the remaining groups (the sham groups), skull holes were made, but the electrode was not lowered into the brain (per Fanselow, DeCola, De Oca, & Landeira-Fernandez, 1995). Electrodes were cleaned, retested in a sodium solution, and bathed in alcohol before reusing. The incisions were then sutured (2-0 Suturamid, Ethicon, Peterborough, Ont.).

After the surgery the rats were housed in opaque plastic recovery boxes with solid floors covered in wood shavings. During recovery, the rats were monitored on a daily bases by a trained animal caretaker. A topical antibacterial agent was administered to the wounds as required. The rats had continuous access to food and water. Upon recovery (about 3-5 days after surgery), with incision wounds healed and behavioral responses (e.g., feeding behavior, motor coordination, reflexes) normal, the rats were taken from the plastic boxes and returned to their home cages in the colony room.

Apparatus. Pavlovian conditioning was conducted in a single observation chamber, which was a standard operant box measuring 30 x 30 x 27.5 cm with clear Plexiglas ceiling and sides and aluminum front and back panels. The front panel contained an inoperable lever, a recessed food tray, and jeweled stimulus lights on either side of the food tray (none of these devices were used in the experiment). The floor consisted of 6-mm stainless steel rods, spaced 1.6 cm apart center to center, which could be electrified by a Coulbourn constant-current shocker and scrambler (Coulbourn Instruments, Lehigh Valley, USA). The operant box was placed in an opened door, sound-attenuated cube measuring 60 x 40 x 40

cm. Background noise was provided by a ventilation fan mounted in the experimental room housing the sound-attenuated cube. The fluorescent ceiling lights were turned off, and the room was lit by a 60 W red floodlight. A video camera placed 1 m in front of the operant chamber allowed videotaping of the session.

Procedure. On Day 7 and 8 after the surgery, the rats were taken from their home cages in the colony room and brought to the experimental room. They were given the opportunity to explore the conditioning box for 5 min in the absence of CSs and USs. On Day 9 and 10 following surgery, the rats were placed in the conditioning box and given either massed or distributed shocks (1.0 mA). Half of the dlPAG and half of the sham groups received a massed shock procedure. A single bout of massed shock (three, 1 s footshocks separated by 3 s intervals) was presented 90 s after placement in the box. The remaining dlPAG and sham rats received distributed training in which three, 1sec footshocks were presented at 60 s intervals, 90 s after placement in the box. Rats were returned to their home cages 90 s after the last shock[1]. The conditioning box was cleaned with a water and vinegar solution between trials. Thus, there were four groups at the end of this stage: dlPAG-massed, dlPAG-distributed, sham-massed, and sham-distributed.

Then each rat was tested for freezing during 12 daily sessions. In each test session, the rats were placed in the conditioning box for a 5 min period. No other stimuli were presented. In an adjacent room, video monitoring and recording equipment allowed

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[1] Thus, during the conditioning trial the massed group spent approximately 180 s in the conditioning box and the distributed group spent approximately 300 s in the conditioning box.

freezing to be scored by a trained observer blind to the lesion condition of the animal. Freezing was defined as the absence of movement except that needed for respiration. An auditory signal presented to the observer, and only audible to the observer, paced the scoring of freezing using a time-sampling procedure. Every 5 s, the rat was scored as freezing or not freezing. After the 5-min test session, the rats were returned to their home cages. Videotaped sessions were kept for later review for determining interrater reliability.

Histology. On the day of the last behavioral test the rats were sacrificed via carbon dioxide inhalation. They were perfused intracardially with 0.9% saline solution followed by 10% formalin solution. Their brains were removed and stored in 10% formalin for 5 to 15 days. The brains were then mounted and, using carbon dioxide gas, frozen onto a microtome platform (Model 860, American Optical Company, Buffalo, USA). The frozen brains were sectioned, at 75µm thickness, from approximately 6 mm to 9 mm posterior to bregma (Paxinos & Watson, 1986). Every other section was mounted on a gelatinized slide and stained with cresyl violet. A trained neuropsychologist, blind to the experimental conditions of the rats, recorded the lesion damage on diagrams taken from a rat brain stereotaxic atlas (Paxinos & Watson, 1986). PAG damage extended to regions lateral and dorsolateral of the aqueduct (see Figure 3).

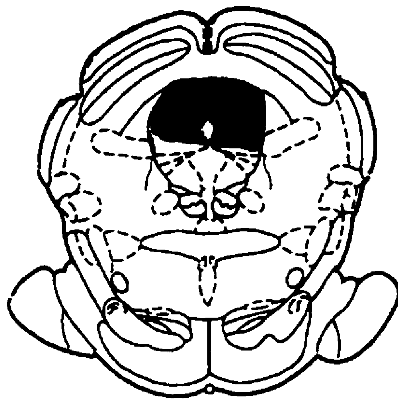
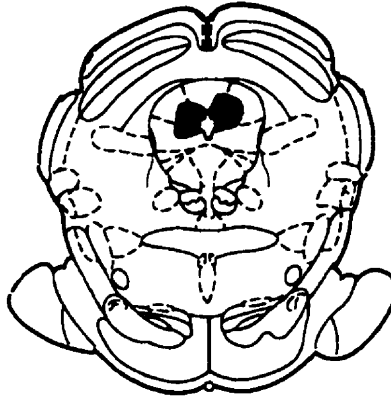
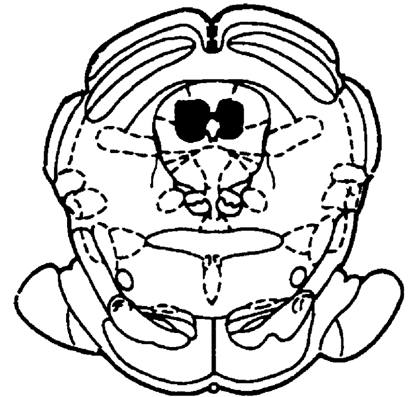
Results

Nine rats were excluded due to surgical difficulties or misplaced lesions, leaving the following group sizes for analysis: 11 sham-massed, 10 dlPAG-massed, 9 sham-distributed, and 9 dlPAG-distributed rats. The results of Experiment 1 (Figure 4) are consistent with

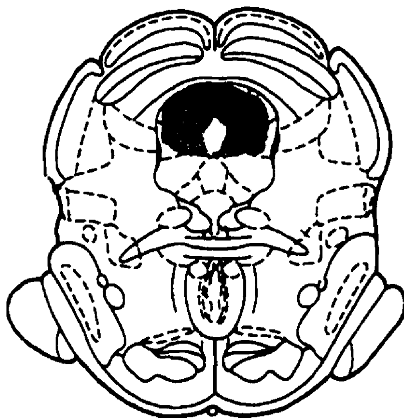
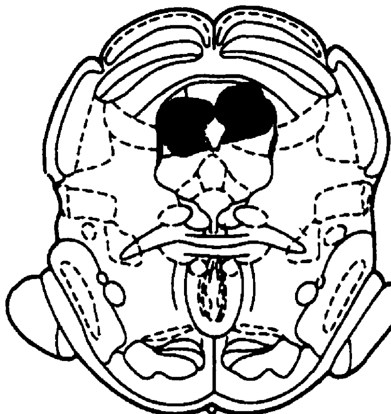
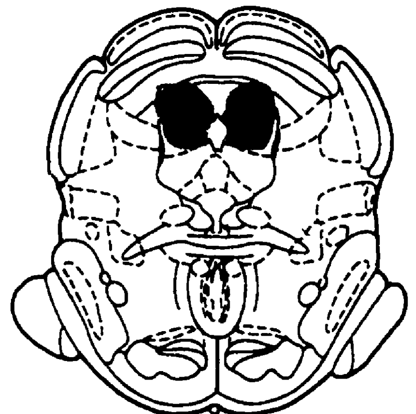
Figure 3. The extent of the electrolytic lesions for all three experiments are recorded on diagrams taken from a rat brain stereotaxic atlas (Paxinos & Watson, 1986). Three anterior to posterior (top to bottom) coronal brain sections (Bregma -7.30 mm to -8.00 mm) are shown for each experiment. The red area indicates the maximum region affected by the lesions whereas the grey indicates the minimum region effected by the lesions. As can be seen, there was minimal collateral damage to brain areas other than the dlPAG (e.g., superior colliculus). Additionally, lesion damage was greater in the lateral PAG than in the dorsal PAG. No visible damage was evident in the vPAG.

Experiment 1 **Experiment 2** **Experiment 3**

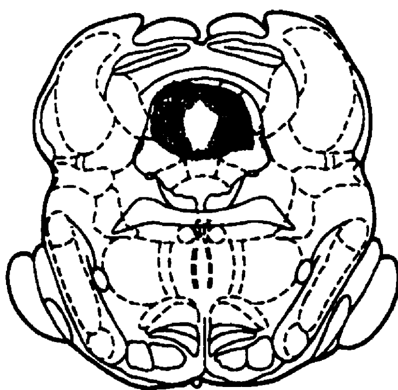
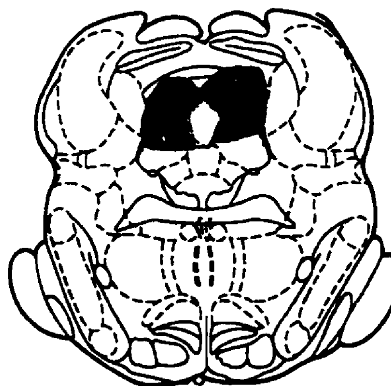
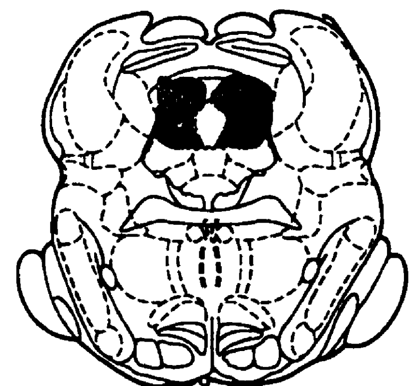
Bregma -7.30 mm

Bregma -7.64 mm

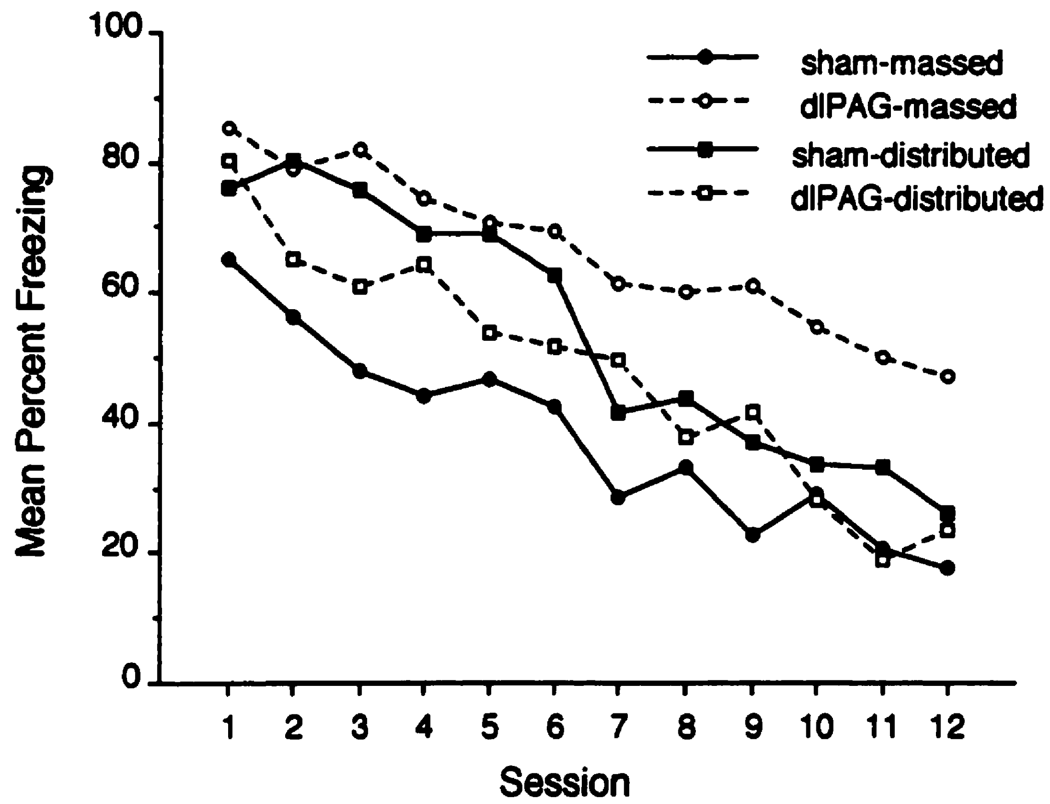




Bregma -8.00 mm

■ Maximum Lesion ■ Minimum Lesion

Figure 4. Mean percent freezing across 12 days of testing (extinction trials) is shown for each group in Experiment 1. The dlPAG-massed group froze more than its unlesioned control group (sham-massed). No effect of lesion was found in distributed groups.



100
80
60
40
20
0
Mean Percent Freezing
1
2
3
4
5
6
7
8
9
10
11
12
Session
sham-massed
dlPAG-massed
sham-distributed
dlPAG-distributed

those of Fanselow et al. (1995, Experiment 1). Overall, there were no significant differences in mean percent freezing between the dlPAG-distributed (M=48%) and sham-distributed (M=54%) groups. In accordance with the learning interpretation, the dlPAG-massed group froze (M=66%) more than the sham-massed (M=38%). This pattern of responding produced a lesion x conditioning interaction, $F(1,35) = 5.13$, but no main effects for either lesion or conditioning. There was a main effect of session, $F(11,385) = 45.98$, which was due to extinction of context fear.

How are these findings to be interpreted? If dlPAG lesions had increased the probability of freezing, freezing should have declined more rapidly in the sham-distributed group than in the dlPAG-distributed group. Since this result was not found, the results of Experiment 1 support the learning interpretation (Fanselow, DeCola, De Oca, & Landeira-Fernandez , 1995).

Experiment 2

Experiment 2 was identical to Experiment 1 except that dlPAG lesions were placed after the rats received conditioning. If dlPAG lesions merely increased the probability of freezing, lesions after conditioning should have the same effect as lesions placed before conditioning (Experiment 1). By contrast, if dlPAG lesions affect learning and not performance, lesions placed after conditioning (learning) should be without consequence.

Method

Subjects, Surgery, and Apparatus. The number and type of subjects, the surgical procedure, and the apparatus were the same as in Experiment 1.

Procedure. Experiment 2 differed from Experiment 1 in that the surgery-conditioning order was reversed, and there were 10 instead of 12 test sessions. On Day 1 and 2, the rats were taken from their home cages in the colony room and brought to the experimental room. They were given the opportunity to explore the conditioning box for 5 min. On Day 3 and 4, the rats were placed in the conditioning box and given either massed or distributed shocks. On the day following the last conditioning trial, Day 5, half of each of the massed and distributed groups received dlPAG lesions. The other half of each group received a sham procedure. Again, there were four groups at the end of this stage: dlPAG-massed, dlPAG-distributed, sham-massed,
and sham-distributed. Following surgery, the animals were allowed to recover.

On the seventh day following surgery, Day 12, the rats were returned to the original conditioning box and tested for freezing during 10 daily sessions. In each test session, the rats were placed in the conditioning box for 5 min and scored for freezing, as in Experiment 1.

Histology. On the day following the last test day, Day 23, the rats were sacrificed and the histology was performed using the procedures described in Experiment 1.

Results

As in Experiment 1, rats with surgical difficulties or misplaced lesions were excluded from the study, leaving the following group sizes: 8 sham-massed, 9 dlPAG-massed, 9 sham-distributed, and 8 dlPAG-distributed rats. The main finding was that dlPAG lesions had no effect on the level of freezing observed in the test, as expected by the

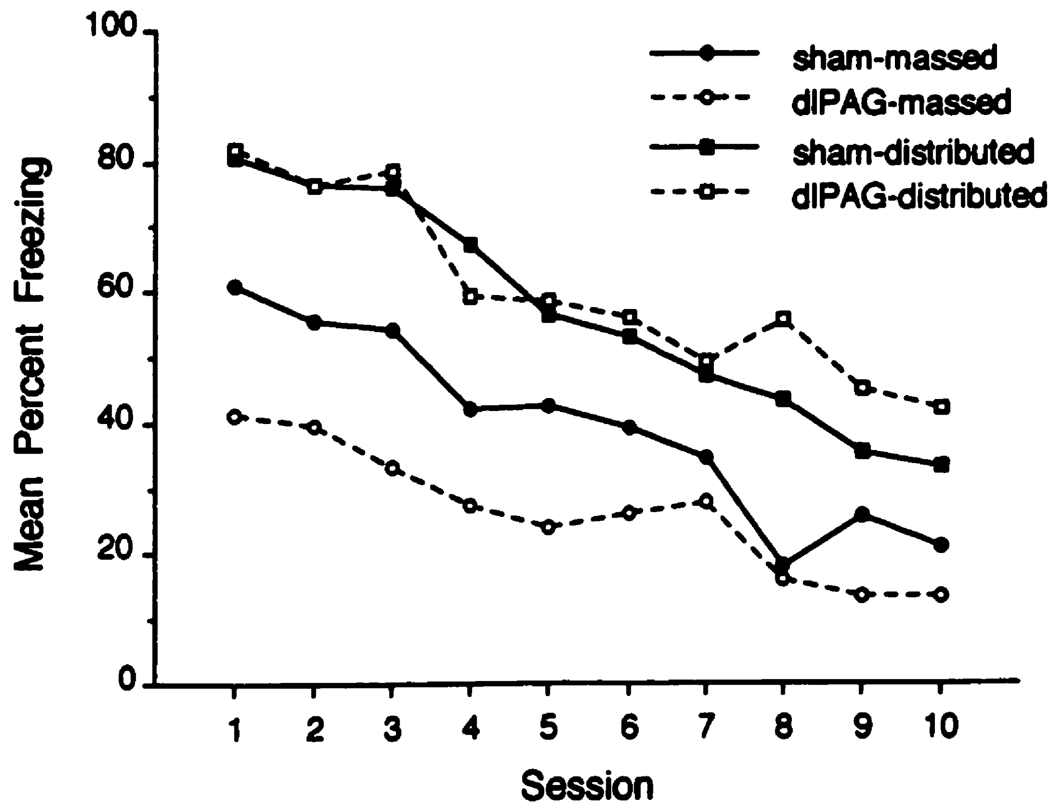
learning account (Figure 5). The rats of the distributed groups ($\underline{M}$=58%) froze more than the rats of the massed groups ($\underline{M}$=32%), regardless of lesion condition. ANOVA revealed only main effects of conditioning, $\underline{F}(1,30) = 10.77$, and sessions, $\underline{F}(9,270) = 25.92$. If dlPAG lesions had increased the probability of freezing, they should have produced the same effect as lesions placed before conditioning (Experiment 1). By contrast, if dlPAG lesions affect learning rather than performance, lesions placed after conditioning (learning) should have had no consequence. Again, the results of Experiment 2 supported the learning account.

Experiment 3

The purpose of Experiment 3 was to examine the conditions under which fear conditioning is enhanced by dlPAG lesions. In particular, do dlPAG lesions enhance fear conditioning with procedures other than massed shock that also produce low levels of contextually-evoked freezing? According to the neural pathways in Figure 1, dlPAG lesions should enhance fear conditioning only if one shock closely follows another shock. Only if shocks are massed can dlPAG activation from Trial N interfere with learning on Trial N+1.

Experiment 3 then was similar to Experiment 1 except for the following changes. First, the conditioning manipulations now consisted of either 3 (massed) shocks or 1 shock instead of massed versus distributed shocks as in Experiments 1 and 2. Second, the unconditioned response (UR) to the shock US was measured. Here, individual rats were rank ordered in terms of their unconditioned reaction to shock in Session 1. Third, a post-lesion analgesic test to a hot-plate was administered to examine group differences in pain perception. This was done to test the possibility that dlPAG-lesioned animals might simply

Figure 5. Mean percent freezing across 10 days of testing (extinction trials) is shown for each group in Experiment 2. This time, however, with lesions occurring after conditioning, there is a marked attenuation in mean percent freezing for the dlPAG-massed group, indicating the massed shock deficit.



100
80
60
40
20
0
Mean Percent Freezing
sham-massed
dlPAG-massed
sham-distributed
dlPAG-distributed
1
2
3
4
5
6
7
8
9
10
Session

be more sensitive to painful stimuli than unlesioned animals. Among other things, PAG activation produces analgesia (Mayer, Wolfle, Akil, Carder, & Liebeskind, 1971). Therefore, it is possible that the increased freezing by the dlPAG-massed group in Experiment 1 is a result of the increased aversiveness of the shock US after destruction of the dlPAG. If this were true, dlPAG lesions should facilitate conditioning in both the massed and 1 shock conditions, and this difference in pain sensitivity should be evident in a hot-plate test. Finally, the sham control groups in Experiment 1 and 2 had their skulls exposed and a hole drilled but the electrode was not lowered. True shams were created for Experiment 3 by lowering the electrode to the appropriate midbrain sites but not administering electric current.

Method

Subjects. The number and type of subjects were the same as in Experiment 1.

Surgery. The surgical procedure was identical to Experiment 1 except that instead of only drilling a hole in the skull of the sham groups, the electrode was lowered to the appropriate lesion site but no current was administered. The electrode was extracted after 10 sec and thereafter the rats followed the same surgical procedure as in Experiment 1.

Apparatus. The apparatus was the same as in Experiment 1 with the addition of a hot plate for testing analgesia. A Forma Scientific model 2095 heated bath circulator was used to maintain a constant hot plate temperature. The hot plate consisted of a brass plate having hot water circulating through its core. Water temperature was maintained, via thermometer and heater, at a constant temperature of 52° C. A clear Plexiglas cylinder with

open ends (30 cm in diameter, 50 cm in length), resting upright on the surface of the hotplate, prevented the animal from fleeing.

Procedure. After surgery, the rats were randomly assigned to either the massed or single shock conditions. For the massed groups, the conditioning procedures were identical to Experiment 1. The single groups were treated identically to the massed groups except for the deletion of the second and third shocks in each of the two conditioning sessions. These conditioning sessions were videotaped for later evaluation by a trained rater blind to the experimental condition of the rats. Individual rats were rank ordered in terms of their unconditioned reaction to shock in Session 1. Only the response to the first shock was measured because these data would not be contaminated by differences in freezing. A three point Lickert scale, where 1 was low responding and 3 was high responding, allowed for judging degree of behavioral responding. Behaviors taken into consideration in this ranking included flinching, scrambling, and vocalization. The rats were then tested in extinction for contextually evoked freezing for 10 sessions. The day after the last test for contextual conditioning, the animals were given 1 session of preexposure on the hot-plate. Each rat was brought from its home cage and placed on the surface of the hot-plate which was at room temperature (20°C). The rat was allowed to explore the hot-plate apparatus for 2 min and was then returned to its home cage. The following day the rats were tested on the hot-plate, now maintained at a temperature of 52°C. The time between placement on hotplate surface until retraction and licking of one of the hind paws was recorded manually with a stopwatch. After the hind paw lick, the rat was immediately removed from the hotplate and

returned to its home cage. Following the hotplate test, the rats were sacrificed and the histology performed.

Histology. The histological procedure for Experiment 3 was identical to Experiment 1.

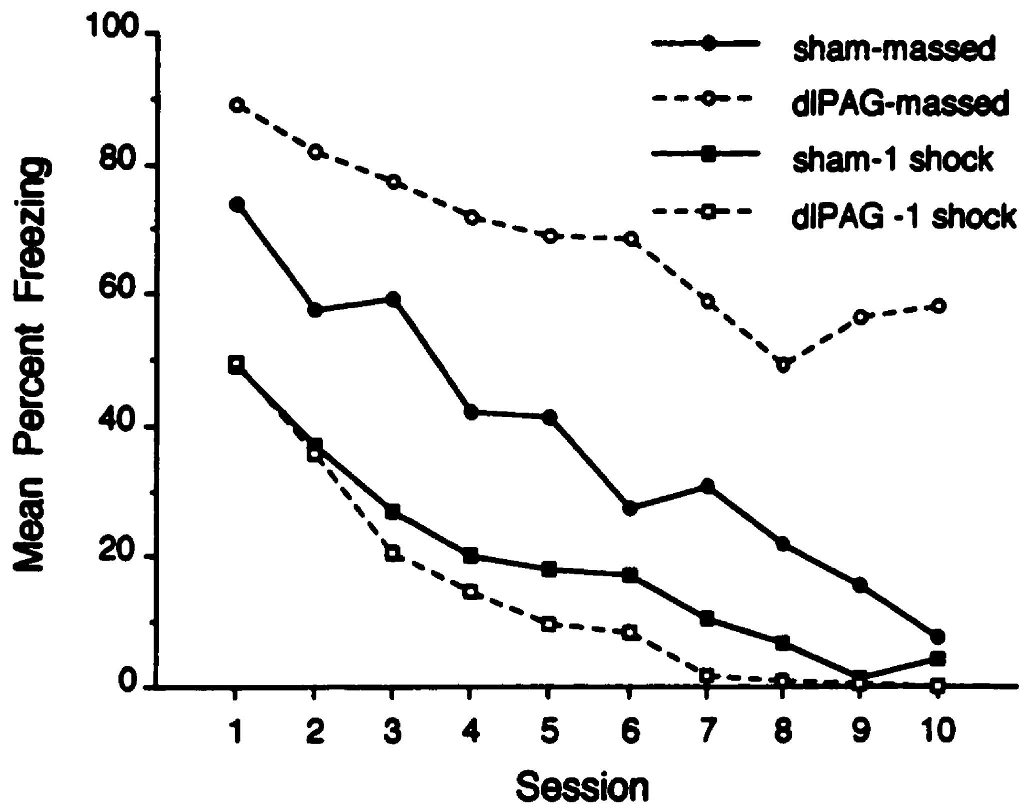
Results

Again, rats with surgical difficulties or misplaced lesions were excluded (see Figure 3 for acceptable lesions), leaving the following group sizes: 8 sham-massed, 5 dlPAG-massed, 8 sham-single, and 8 dlPAG-single rats. Results for Experiment 3 (Figure 6) revealed no lesion effect in the rank order test for group differences in the unconditioned scrambling response (UR; Kruskal-Wallis $p>.05$). The test for freezing revealed that the single shock groups froze less than the massed groups (M=11.6%, M=49.3%, respectively). This difference was confirmed by an ANOVA, which revealed main effects of conditioning, $F(1,25) = 46.65$, and sessions, $F(9,225) = 36.04$. ANOVA also revealed a conditioning x lesion interaction, $F(1,25) = 11.07$. The interaction was due to more freezing in the dlPAG-massed group (M=68%) than in the sham-massed group (M=38%). There were no differences between dlPAG-single (M=14%) and sham-single (M=19%). The hotplate test for analgesia produced no significant difference ($F(1, 27) = 2.51$) in paw-lick latency in the dlPAG lesion (M=21 sec, SD=11.23) and sham (M=15.56 sec, SD=7.16) groups. Thus, the present study found no evidence that the dlPAG lesions affected pain sensivity.

General Discussion

The major findings of the present experiments were that (a) dlPAG lesions placed

Figure 6. The results of Experiment 3 reveal that, as in Experiment 1, the dlPAG-massed group spent significantly more time freezing than any of the three other groups.



Mean Percent Freezing
Session
0
20
40
60
80
100
1
2
3
4
5
6
7
8
9
10
sham-massed
dlPAG-massed
sham-1 shock
dlPAG -1 shock

before, but not after, conditioning facilitated fear conditioning as measured by freezing (b) dlPAG lesions did not influence the level of conditioned fear supported by a single shock, and (c) these effects do not appear to be mediated by differences in pain sensitivity or in the magnitude of the unconditioned response to the shock reinforcer. These results are consistent with Fanselow et al.'s (1995) suggestion that dlPAG activation inhibits association formation in
the amygdala.

There are several points in the present results which show that lesions of the dlPAG do not simply increase conditioned freezing. Experiment 1 found no evidence that freezing declined especially slowly in dlPAG lesioned rats after distributed shock. Experiment 3 also found that dlPAG lesions did not enhance contextually evoked freezing in general. In that experiment, dlPAG lesion facilitated conditioning with massed shock but had no such effect with a single shock. Importantly, if the dlPAG lesions were placed after conditioning (Experiment 2), they had no detectable consequence on the level of conditioning with either massed or distributed shock.

Likewise, the dlPAG lesions did not simply increase the effectiveness of shock to serve as a reinforcer. Lovick (1993) showed that brain regions such as the nucleus raphe obscurus may inhibit cells of the dlPAG, an effect attributed to the brain neurotransmitter serotonin (5-HT). Consistent with this interpretation, Nogueira and Graeff (1995) found that 5-HT1a agonists raised the threshold of aversive stimulation in the dlPAG in a dose-dependent fashion. Elsewhere, Tive and Barr (1992) found the excitatory neurotransmitter

glutamate to be an effective analgesic in the dlPAG in rat pups. Thus, it is conceivable that the dlPAG lesions modulated the aversion generated in the dlPAG, through a variety of neurotransmitter mechanisms, and made the rats more sensitive to painful stimuli like shock, enhancing contextually evoked freezing.

The results of the present experiments are inconsistent with such an account. First, Experiment 1 did not find a slower decline of freezing in the dlPAG-distributed group than in the sham-distributed group, which suggests that shock was not simply a more powerful reinforcer after dlPAG lesions. Only in the dlPAG-massed groups was enhanced freezing observed. Likewise, Experiment 3 found lesions of the dlPAG had facilitative effects on contextually evoked freezing with massed shock but not with a single shock. Hot-plate tests for analgesia were also unable to distinguish dlPAG lesioned rats from sham controls.

The unconditioned scrambling response to shock is another component of the aversive reaction that might be affected by dlPAG lesions (Fanselow, 1991; Fanselow, DeCola, & Young, 1993). In particular, dlPAG lesions might reduce the unconditioned scrambling reaction to shock, which under normal circumstances interferes with the processing of contextual stimuli. Thus, dlPAG lesions might facilitate contextual conditioning with massed shock because the context is better processed and not because dlPAG lesions eliminate an inhibitory influence of the dlPAG on association formation in the amygdala. However, unlike Fanselow (1991), there were no group differences in the unconditioned scrambling response to shock in Experiment 3. Thus, the effects seen in the present experiments are not open to this alternative interpretation. Another implication of

these results is that the unconditioned scrambling response should not be used as an analog of the dlPAG's inhibitory input on the amygdala during massed shock.

What then explains the differences in the effects of dlPAG lesions on the unconditioned scrambling response to shock in the present experiments and those of Fanselow (1991)? Visual comparisons suggest that Fanselow's lesions (Fanselow et al., 1995) were larger and more dorsal than the lesions in the present experiments. Thus, lesion size might be an important factor. It might be interesting to know if the present results, and those of Fanselow et al. (1995), would also be obtained with chemical lesions, which do not disrupt fibres of passage. In any case, the results of the present experiments and those of Fanselow et al (1995) support the notion that association formation is modulated by the dlPAG.

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