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Stress-Induced Hypoalgesia and Defensive Freezing Are Attenuated by Application of Diazepam to the Amygdala

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HELMSTETTER, F. J. *Stress-induced hypoalgesia and defensive freezing are attenuated by application of diazepam to the amygdala.* PHARMACOL BIOCHEM BEHAV **44**(2) 433–438, 1993.—Recent studies have shown that lesions of the amygdala, as well as systemic administration of benzodiazepine receptor agonists, block the hypoalgesia and defensive behavior normally observed when rats are exposed to stimuli associated with shock. The present study was conducted to determine if the direct application of a small quantity of the benzodiazepine diazepam (DZP) to the amygdala would affect defensive freezing and hypoalgesia as measured by the formalin test. Independent groups of rats were prepared with chronic cannulae aimed at the basolateral or central regions of the amygdala. Bilateral injection of DZP (30 μg) into the basolateral amygdala attenuated both the defensive freezing behavior and the hypoalgesia seen during an 8-min period following a series of three brief foot-shocks. The same dose of DZP applied to the central amygdala attenuated the freezing response, although this effect may have been due to limited diffusion of the drug into the basolateral region. Baseline levels of formalin-induced behavior were not affected by DZP in either group. These results support the idea that hypoalgesia is one component of an integrated defensive response shown by rats in anxiety- or fear-related situations and that the amygdala represents an important forebrain component of a neural circuit that subserves the expression of this response.

Stress-induced analgesia Pavlovian conditioning Fear Anxiety Benzodiazepines
Antinociception Defensive behavior Learning Memory

NEURAL systems exist within the mammalian brainstem and spinal cord that are capable of modifying the transmission of noxious information from the periphery into the CNS (1). These systems may be activated by a wide range of environmental stressors including the presence of predators or aggressive conspecifics (5,15), as well as electric shock (14,24,27). Importantly, these endogenous antinociceptive systems may also be activated by once "neutral" stimuli that have been paired with a noxious or stressful event during Pavlovian conditioning (4–6,8,10,11).

There is a growing body of evidence to support the idea that hypoalgesia in the behaving animal is part of an integrated defensive response to fear-provoking stimuli (5). Experimental manipulations designed to influence motivational processes such as fear or anxiety often modulate the expression of stress-induced hypoalgesia. For example, the suppression of stereotyped nociceptive reactions to an SC injection of dilute formalin displayed by rats after exposure to stimuli that have been paired with electric shock is blocked by systemic injections of benzodiazepine receptor agonists (6). Similarly, the time-dependent elevation of rat tail-flick latencies seen after shock is also blocked by diazepam (DZP) (14). The same treatment simultaneously attenuates defensive freezing behavior, which can serve as an independent index of anxiety or fear (6). Conversely, systemic and ICV administration of anxiogenic compounds, such as certain benzodiazepine receptor "inverse agonists," produces a dose-related hypoalgesia (7,9).

Recent studies have shown that lesions of the amygdala, a forebrain structure known to be critical for the expression of fear and anxiety in the rat (2,13), eliminate the hypoalgesia and freezing displayed by animals exposed to shock-associated cues (8). The central nucleus of the amygdala projects directly onto the population of opioid-sensitive cells in the ventrolateral region of the periaqueductal gray, which appear to be critical for the expression of this form of hypoalgesia (11,21). Thus, the amygdala may represent a critical forebrain structure within the neural system responsible for the performance of hypoalgesia as a Pavlovian conditional response [see (8) for discussion].

The basolateral subdivision of the amygdala, which includes the lateral and basolateral nuclei and parts of the

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basomedial nucleus (3), possesses one of the highest concentrations of radiolabled benzodiazepine binding in the rat brain (18,25,29). Prior work indicated that local application of small amounts of benzodiazepine agonists in the amygdala results in highly selective anxiolytic effects in the rat, leading some authors to conclude that the amygdala may represent a critical CNS site mediating the anxiolytic effects of systemically administered benzodiazepines (12,16,17).

There is some controversy, however, with respect to the relative amount of sensitivity to benzodiazepines at sites within the amygdala. Several studies indicated that the most sensitive area for selective anxiolytic effects is the basolateral group of nuclei, which also show the greatest amount of [^{3}H]DZP binding (20,22,25). Many of these studies reported weak or nonexistent effects of benzodiazepines applied to the central nucleus. On the other hand, at least one study (23) reported that the central nucleus is highly sensitive to benzodiazepine treatment while the same pharmacological treatment applied to the basolateral amygdala was without effect.

This experiment was designed to determine if either the basolateral or central subdivision of the amygdala is a potential locus of action for the attenuation of freezing and hypoalgesia by DZP.

METHOD

Subjects, Surgery, and Histology

Thirty-four adult, female Long-Evans rats (240–355 g) bred locally from stock obtained from Blue Spruce Farms (Altamont, NY) were housed individually in hanging stainless steel cages with free access to rat chow and water. The colony was maintained on a 14 L : 10 D cycle and all test procedures were conducted during the light portion of the cycle. Subjects were adapted to handling and transportation procedures for 5 days prior to surgery.

Rats were anesthetized with ketamine HCl (100 mg/ml/kg, IP) and sodium pentobarbital (2.5 mg/rat, IP) and mounted in a standard stereotaxic frame. A group of 14 animals was prepared with chronic bilateral stainless steel guide cannulae (26 gauge, Plastic Products C315G, Plastic Products, Roanoke, VA) aimed at the basolateral subdivision of the amygdala as defined by deOlmos et al. (3). Cannulae were positioned using the following coordinates: AP, 0.0; ML, ±5.0 relative to bregma; V, −7.0 with the incisor bar raised 5.0 mm from the interaural line. Twenty animals were prepared with similar cannulae aimed at the central amygdala using the following coordinates: AP, +0.2; ML ±4.25; V, −6.3. All subjects were allowed at least 1 week to recover prior to testing.

Following behavioral testing, all subjects were overdosed with sodium pentobarbital (75–100 mg/kg, IP) and perfused transcardially with isotonic saline followed by phosphate-buffered 10% formalin solution. Brains were removed after the perfused head had soaked in buffered formalin with cannulae in place for at least 24 h. A series of 40-μm frozen section were collected throughout the cannulae tracks and stained with cresyl violet. The locations of injection sites were determined with the aid of a rat brain atlas (19).

Apparatus and Procedure

Beginning on the fourth day following surgery, each animal was handled by the experimenter once each day. On days 6 and 7, animals were adapted to the restraint procedure to be used during drug infusions. Subjects were lightly anesthetized by brief exposure to ether and wrapped in a clean cotton towel such that only the head protruded. While the animal was restrained in the towel, cannula obturators were removed and cleaned with a 50% Betadine solution. Wound edges were also treated with 50% Betadine.

On the eighth day following surgery, animals were tested. All subjects were restrained as described above and given a bilateral infusion of DZP or vehicle via 33-ga injection cannulae (Plastic Products C315I) prepared so as to extend 0.5 mm past the end of the guide and connected to a pair of Hamilton microsyringes (Hamilton Co., Reno, NV) via PE20 tubing. An infusion pump allowed simultaneous bilateral delivery of solutions. One half the animals in the basolateral group received 30 μg diazepam HCl (Sigma Chemical Co., St. Louis, MO) dissolved in 1.0 μl DMSO (Sigma) that was infused at a constant rate over a 40-s period. This dose of DZP was chosen because a similar amount injected into the amygdala was found to be approximately as effective in attenuating conditioned suppression as 1 mg/kg given systemically (17). The remaining animals in the basolateral group received an equal volume of the DMSO vehicle. One half the animals with cannulae aimed at the central amygdala received 30 μg DZP dissolved in 0.5 μl DMSO infused over a 40-s period. The injection volume was reduced for this group because the central nucleus is smaller than the lateral and basolateral nuclei. The remaining animals with central cannulae received 0.5 μl DMSO. The injection cannulae were left in place for approximately 30 s following infusion for all animals to maximize diffusion of solutions into surrounding tissue.

All behavioral testing took place in a pair of rodent observation chambers (23.5 × 29 × 19.5 cm) constructed of Plexiglas and stainless steel. The floor of each chamber consisted of 18 stainless steel rods spaced 1.25 cm apart, through which scrambled AC foot-shock could be delivered via a Grason-Stadler shock generator/scrambler. Each chamber was enclosed in a sound- and light-attenuating chest with a Plexiglas window through which the experimenter could observe the rat. Illumination was provided by a 7.5-W white lightbulb mounted directly over each observation chamber. Ventilation fans provided constant background noise at 70–72 dB.

Immediately following drug administration, each rat was given a 0.05-ml SC injection of 15% formalin in saline into the dorsal surface of a hindpaw and returned to the home cage. Animals were removed from the home cage and placed in observation chambers 25 min later. All subjects remained in the observation chambers for a total of 13 min. The amount of time each animal spent engaged in defensive freezing behavior and stereotyped behavioral reactions to the formalin injection was recorded using a time-sampling procedure (4,6, 8,10,11) in which an observer blind to treatment conditions scored animals' behavior as belonging to one of the following categories:

1. *Freezing*—the absence of all body movement except that required for respiration. Rats normally freeze in a species-typical crouching posture.
2. *Paw-licking*—any contact between the formalin-injected hindpaw and the animal's mouth.
3. *Paw-lifting*—the sustained flexion of the injected hindlimb such that the paw remains elevated and close to the body.
4. *Other*—any behavior not meeting the definition of freezing, paw-licking, or paw-lifting. If an animal remained mo-

FIG. 1. Injection sites for animals included in the analysis. (■), basolateral/vehicle; (●), basolateral/diazepam; (⬢), central/vehicle; (▲), central.

tionless while holding its paw off the grid floor, it was scored as paw-lifting [see (5) for photographs of the behavior].

Each subject's behavior was recorded once every 4 s. Samples scored as paw-licking and paw-lifting were added to form a single category called formalin-induced behavior.

During the first 4 min after being placed in the chamber, no stimuli were presented and postdrug baseline levels of freezing and formalin-induced behavior were recorded. After this 4-min period, a series of three foot-shocks (1.3 mA/0.5 s) were presented at 20-s intervals. No behavior was recorded from the instance of the first shock until 20 s after the final shock. At this time, observation resumed and behavior was recorded for an additional 8-min period following shock. It is important to note that even though behavior was scored immediately following foot-shock the freezing and hypoalgesia observed during this period are considered primarily Pavlovian conditional responses to stimuli present at the time of shock delivery rather than unconditional responses to the shock itself (4,5,14).

RESULTS

Histology

The locations of injection sites for all animals from the central and basolateral groups whose data were included in the analysis are presented in Fig. 1. Animals in group basolateral were accepted for analysis only if the ends of their guide cannulae were located in or directly above the lateral or basolateral nuclei on both sides of the brain. Four animals were rejected from the basolateral group because they did not meet these criteria. For group central, injection sites for 11 animals were determined to lie within or border on the central nucleus bilaterally. A total of nine animals in this group whose cannulae were misplaced on one or both sides were eliminated from the behavioral analysis.

Because the groups of animals implanted with cannulae in the basolateral and central amygdala were run at different points in time, the data were not pooled and a separate analysis of variance (ANOVA) was conducted for each group.

Basolateral Amygdala

Freezing scores were subject to a repeated-measures ANOVA treating observation period (pre- vs. postshock) as a within-subjects variable. During the 4-min period prior to shock, animals engaged in little freezing (VEH: $n = 5$, mean = 2.68%, SEM = 1.64; DZP: $n = 5$, mean = 5.33%, SEM = 3.88). Presentation of foot-shock substantially increased the amount of freezing and DZP attenuated this response during the postshock period. This observation was supported by a reliable observation period × drug interaction, $F(1, 8) = 7.41$, $p < 0.03$. Planned comparisons indicated that groups differed significantly during the postshock period, $F(1, 8) = 12.14$, $p < 0.008$, but not during the preshock period, $F(1, 8) < 1.0$. The mean percentages of time spent freezing during the postshock period for the basolateral groups are presented in Fig. 2A.

Hypoalgesia is inferred from the suppression of behavioral responses to the formalin injection. Because the percentage of time animals spend engaged in these behaviors tends to follow a Poisson distribution, the raw formalin-induced behavior scores were subject to a square-root transformation prior to analysis so that they would better conform to the assumptions

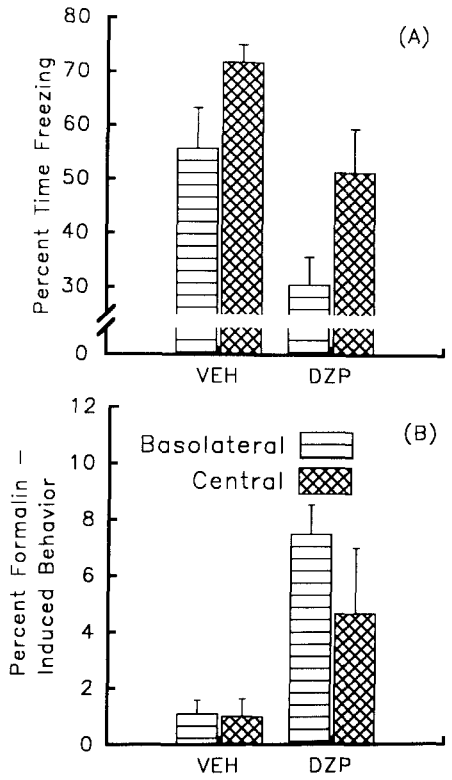

FIG. 2. (A). Percentage of time rats spent freezing during the postshock observation period as a function of cannulae placement and treatment. Diazepam (DZP) (30 μg) significantly reduced the amount of postshock freezing when applied to either the basolateral or central amygdala. (B). Percentage of the postshock observation period rats spent engaged in formalin-induced behavior. Hypoalgesia, as indicated by the suppression of the stereotyped behavioral reaction to formalin injection, was significantly attenuated in rats with basolateral cannulae given DZP.

of ANOVA (10). The pattern of results for hypoalgesia was somewhat similar to that of freezing. The mean raw scores for formalin-induced behavior are shown in Fig. 2B. An ANOVA on transformed scores yielded a significant main effect for pre- vs. postshock observation period, $F(1, 8) = 43.89$, $p < 0.001$. The observation period × drug interaction, however, was not significant. Subsequent planned comparisons indicated that while the drug had no effect on baseline formalin-induced behavior (VEH: mean = 31.98%, SEM = 10.21; DZP: mean = 41.34%, SEM = 10.60) during the preshock period, $F(1, 8) < 1.0$, the attenuation of hypoalgesia (increase in net amount of formalin-induced behavior) by DZP following foot-shock was statistically reliable, $F(1, 8) = 5.20$, $p < 0.05$.

Central Amygdala

The effects of DZP applied to the central amygdala on freezing behavior are depicted in Fig. 2A. The general pattern of results was somewhat similar to that seen in the basolateral group, although the magnitude of DZP's effects was smaller. ANOVA indicated a reliable main effect for observation period, $F(1, 9) = 164.71$, $p < 0.001$. The main effect for drug, $F(1, 9) = 4.94$, $p < 0.053$, and the observation period × drug interaction, $F(1, 9) = 3.90$, $p < 0.08$, were not signifi-

cant. Planned comparisons indicated that DZP did not affect freezing during the period prior to shock (VEH: $n = 5$, mean = 2.0, SEM = 2.21; DZP: $n = 6$, mean = 0.0, SEM = 0.0) while, as can be seen in Fig. 2A, DZP significantly reduced the amount of freezing observed after shock, $F(1, 9) = 9.593$, $p < 0.02$. Foot-shock significantly suppressed formalin-induced behavior, $F(1, 9) = 32.47$, $p < 0.001$. However, when DZP was applied to the central amygdala it did not attenuate this suppression, as demonstrated by planned comparisons that showed DZP did not significantly affect formalin-induced behavior during the baseline (VEH: mean = 17.3, SEM = 0.99; DZP: mean = 17.0, SEM = 4.53), $F(1, 9) < 1.0$, or postshock period, $F(1, 9) = 1.41$, $p > 0.25$. Thus, while presentation of foot-shock resulted in hypoalgesia, as indicated by a significant suppression of formalin-induced behavior, the application of DZP to the central amygdala did not affect this suppression. These results are presented in Fig. 2B.

Of the nine animals rejected from group central based upon misplaced cannulae, subjects with one of the two cannulae placed lateral to the target so that they received a unilateral injection of DZP in the basolateral amygdala ($n = 3$) tended to show less freezing and more formalin-induced behavior than animals in which DZP had been applied to the central amygdala. Injections of DZP made outside the central or basolateral amygdala did not appear to affect these measures.

DISCUSSION

The basolateral amygdala appears to be an important CNS site for benzodiazepine modulation of aversive conditional responding. DZP applied to the basolateral amygdala attenuated both the freezing and hypoalgesia displayed by rats in the presence of apparatus cues paired with shock. Benzodiazepine receptors in this area of the forebrain may also mediate selective benzodiazepine effects on other aversively motivated behaviors in rats (25). While the effects of peripherally administered benzodiazepines are sometimes disrupted (26) and other times not affected (28) by destruction of cells within the amygdala, the results obtained with the present paradigm are consistent with the amygdala serving as a critical central site of action for DZP. Intraamygdaloid injection of benzodiazepines may operate via the same local modulatory mechanisms disrupted after ibotenic or electrolytic lesions of the amygdala because the pattern of effects on hypoalgesia and freezing following lesions and local DZP administration is similar (8).

As described previously, studies that compared the effects of benzodiazepine agonists applied to the basolateral vs. central amygdala provided conflicting data. The present results do not provide an unequivocal resolution of this discrepancy. In the present study, DZP after application in or near the central nucleus reduced the amount of freezing observed but was ineffective in attenuating hypoalgesia in the same animals. The reduction in freezing was smaller than that seen following administration of the same dose of DZP into the basolateral amygdala, which supports the idea that the lateral and basolateral nuclei are more sensitive to this compound. It is also possible that this dissociation reflects some differential sensitivity of these two dependent measures; DZP may have some relatively weak effect in the central nucleus that is better reflected through freezing behavior. Because the central nucleus does not appear to have a relatively high degree of benzodiazepine binding, this weak effect may more likely be the product of limited diffusion of drug solutions from the injection site to more sensitive areas within the basolateral amygdala. A similar result was reported by Scheel-Kruger and Petersen (22), who found a weak anxiolytic effect in two animals whose cannulae were located on the lateral border of the central nucleus; other central nucleus placements were completely ineffective. It is also possible, although rather unlikely given the results of previous studies on the effects of amygdala lesions in this preparation (8), that the contrasting pattern of results found with equal doses of DZP at these two sites represents a partial anatomic dissociation of the two responses.

No effect of DZP on baseline levels of formalin-induced behavior was observed in either group. This result is consistent with a similar lack of effect of intraamygdaloid benzodiazepines on tail-flick thresholds (25) and systemically administered benzodiazepines (6) or lesions of the amygdala (8) on formalin-induced behavior. It is therefore unlikely that DZP altered levels of formalin behavior in the present experiment by any direct influence on nociception.

In conclusion, the present results support the idea that the hypoalgesia seen in rats exposed to certain classes of environmental stressors is one component of an integrated defensive response, the expression of which critically depends upon benzodiazepine-sensitive processes within the amygdala.

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