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Effects of Systemic and Intra-Amygdaloid Diazepam on Long-Term Habituation of Acoustic Startle in Rats[1]

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YOUNG, B. J., F. J. HELMSTETTER, S. A. RABCHENUK AND R. N. LEATON. *Effects of systemic and intra-amygdaloid diazepam on long-term habituation of acoustic startle in rats.* PHARMACOL BIOCHEM BEHAV **39**(4) 903–909, 1991.—Two experiments were conducted to examine the effects of the anxiolytic drug, diazepam, on long-term habituation of the acoustic startle response. The experiments were based upon the hypothesis that manipulations that reduce fear should enhance long-term response decrements by reducing a fear-like sensitization process. In Experiment 1 rats given intraperitoneal injections of 0.5, 1.2, or 2.5 mg/kg showed larger decrements of startle amplitude than vehicle-injected controls both over trials within sessions and over days. In Experiment 2 rats injected with 35 μg of diazepam bilaterally into the amygdala showed larger decrements of startle amplitudes over days than vehicle-injected controls. No within-session startle effects were detected in Experiment 2. Freezing behavior was measured in Experiment 2 as an index of fear, and the amygdala injections of diazepam retarded the development of fear in the startle chamber. This index of fear was not possible in Experiment 1 because of the sedating effects of systemic diazepam. We conclude that diazepam, acting at least in part through the amygdala, attenuates the fear-like sensitization process associated with the acoustic startle stimulus. By attenuating sensitization diazepam produces larger than normal reductions in startle amplitudes over trials and days without significantly affecting initial responsiveness.

Diazepam Benzodiazepine Habituation Sensitization Startle response Amygdala

IN the potentiated startle paradigm, response amplitudes are enhanced by delivering the acoustic startle stimulus in the presence of a conditioned stimulus (CS) that has been previously paired with shock. One explanation for this effect posits that it is the energizing effects of fear elicited by the CS that augment startle responses (4). Consistent with this hypothesis, Leaton and Borszcz (14) found freezing, a behavior commonly used as an index of fear [e.g., (1)], to be positively correlated with startle amplitude in a potentiated startle paradigm.

Freezing has also been observed in the initial phases of habituation training of the acoustic startle response (3, 12, 16). Borszcz et al. (3) hypothesized that the freezing reflected fear conditioning stemming from the association of contextual cues of the startle chamber (CS) with the initially aversive startle stimulus (US). The conditioned fear was assumed to underlie a sensitization process that inflates startle amplitude and may mask the extent of the response decrements associated with habituation.

Physiological and behavioral manipulations that retard the development of fear and freezing increase the response decrements that occur across test sessions (3,15). Diazepam, a drug with marked anxiolytic properties, reduces potentiated startle (5), and attenuates freezing and other fear-related behaviors in a context associated with shock (7). In a preliminary experiment we showed that startle response amplitudes of rats treated with systemic diazepam were reduced more over habituation training than controls (24). The present study extends this finding through a dose-response analysis of the effects of systemically administered diazepam on acoustic startle decrements. In a second experiment, the anatomical basis of this effect was explored through the use of intra-amygdaloid injections of diazepam.

EXPERIMENT 1

In preliminary data (24) rats receiving intraperitoneal injections of 2.5 mg/kg of diazepam showed greater decrements in acoustic startle amplitudes across trials and sessions than vehicle-injected controls. However, this dose of diazepam significantly reduced initial response levels, complicating the interpretation of the extent of response decrements resulting from repeated stimulation. The purpose of Experiment 1 was to provide a dose-response analysis of the effects of diazepam in an

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acoustic startle paradigm. We anticipated that a dose of diazepam could be found that would induce decrements in startle response amplitudes over trials that were significantly larger than those of controls but without significantly depressing initial response levels.

METHOD

Subjects

Subjects were 38 experimentally naive male rats of the Long Evans strain reared in the Dartmouth College Psychology Department vivarium. They were approximately 120 days old at the beginning of testing, weighed between 400 and 580 g, and were kept on a 14:10-h light/dark cycle. All animals were individually housed and had ad lib access to food and water.

Apparatus

The startle apparatus has been described in detail previously (13). Animals were tested in one of two $20 \times 12 \times 14$ cm holding cages housed within separate sound attenuating chambers. The cage was constructed of 2.5-mm steel rods mounted within a Plexiglas frame and sandwiched between compression springs attached to a rigid superstructure. Vertical displacement of the chamber moved an attached magnet within a fixed coil, inducing a voltage which was digitized (10 kHz), rectified, and integrated by a microcomputer system. Startle amplitude was measured as the integrated voltage of the 200-ms epoch beginning at onset of the startle stimulus.

The startle stimulus, a 125-dB (SPL), 100-ms burst of white noise, was delivered by a 9-cm piezo-electric tweeter (Herald Electronics) centered 12-cm from the long wall of the cage. Background white noise of 68-dB was delivered continuously by a speaker mounted above the tweeter. Auditory intensities were measured with a General Radio sound-level meter (Model 1551-C, 20-kHz setting) with the microphone centered inside the startle chamber and directed towards the stimulus source.

Procedure

The animals were randomly divided into four groups. One group ($n=9$) received diazepam in the dose of 0.5 mg/kg, another ($n=9$) received 1.2 mg/kg, and a third ($n=10$) received 2.5 mg/kg. The fourth group ($n=10$) received an injection of 100 percent dimethyl sulfoxide (DMSO) which was the vehicle for all diazepam groups. Injections were given intraperitoneally in the volume of 1 ml/kg. Animals were tested every other day for a total of four test days. Each test session consisted of the presentation of 10 startle stimuli on a 60-s interstimulus interval (ISI). On the first test day, the animals were placed in the apparatus 5 min before the first stimulus presentation. On subsequent test days the animals were in the startle chambers for only 1 min before the first stimulus presentation.

RESULTS AND DISCUSSION

Figure 1 shows the mean startle response of each group on the first trial of each of the four test days. First trial points were analyzed because they are not confounded by within-session effects and are, therefore, the most sensitive index of long-term habituation. All groups showed decreased responsiveness over days, and the difference between the vehicle and drug groups appeared to increase over days. An analysis of variance (ANOVA), utilizing polynomial contrasts to determine effects of the repeated measure, yielded only marginally significant Group difference, $F(3,34)=2.93$, $p=0.074$, but significant linear, $F(1,34)=46.19$, $p<0.01$, and quadratic Day effects, $F(1,34)=19.61$, $p<0.01$. The Group $\times$ Day interaction was not significant, $F<1$. Despite the absence of an interaction, the first trial points of Day 4 were significantly different, $F(3,34)=2.94$, $p<0.05$, and the groups were not significantly different on Day 1, $F(3,34)=1.67$, $p>0.1$.

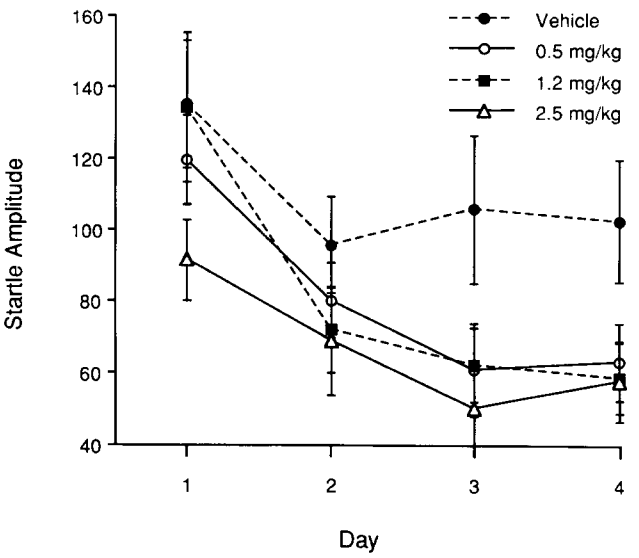

FIG. 1. Mean startle amplitudes of the three diazepam groups and the vehicle group on the first trial of each of the four test sessions.

This pattern of differences suggests that the startle response amplitudes of diazepam-treated animals were depressed more than controls by auditory stimulation. Although the initial response level of the 2.5 mg/kg group was somewhat depressed, there were no significant differences in initial responsiveness among the four groups. Group differences only emerged following stimulation suggesting that diazepam influenced the development of response decrements rather than simply suppressing motor responsiveness.

The within-session results for each of the four days of testing are shown in Fig. 2. All groups showed a pattern of habituation over trials on Day 1, linear, $F(1,34)=46.19$, $p<0.01$. The Group difference that appeared on Day 1, $F(3,34)=5.357$, $p<0.01$, increased on Day 2, $F(3,34)=12.10$, $p<0.01$, and then progressively decreased but remained significant on Day 3, $F(3,34)=7.12$, $p<0.01$, Day 4, $F(3,34)=5.184$, $p<0.01$. Analysis of Day 2 also showed a significant quadratic Group $\times$ Trial interaction, $F(3,34)=4.54$, $p<0.01$, as the 1.2 and 2.5 mg/kg diazepam groups continued to show marked within-session response decrements, while the control group showed a sensitization-like response pattern over trials. The 0.5 mg/kg diazepam group showed some response deficit followed by a sensitization-like pattern.

The pattern of within-session data suggests that diazepam attenuated the sensitizing effect of the startle stimulus. A Group difference appeared on Day 1 as the developing sensitization enhances the startle amplitudes in the control group. The Group difference was further enhanced on Day 2. The control group showed the effect of conditioned sensitization from Day 1 and a further development of sensitization with repeated trials, while the attenuation of sensitization in the diazepam groups, which appeared to be dose-related, allowed the further appearance of habituation. The Group difference decreased on subsequent days

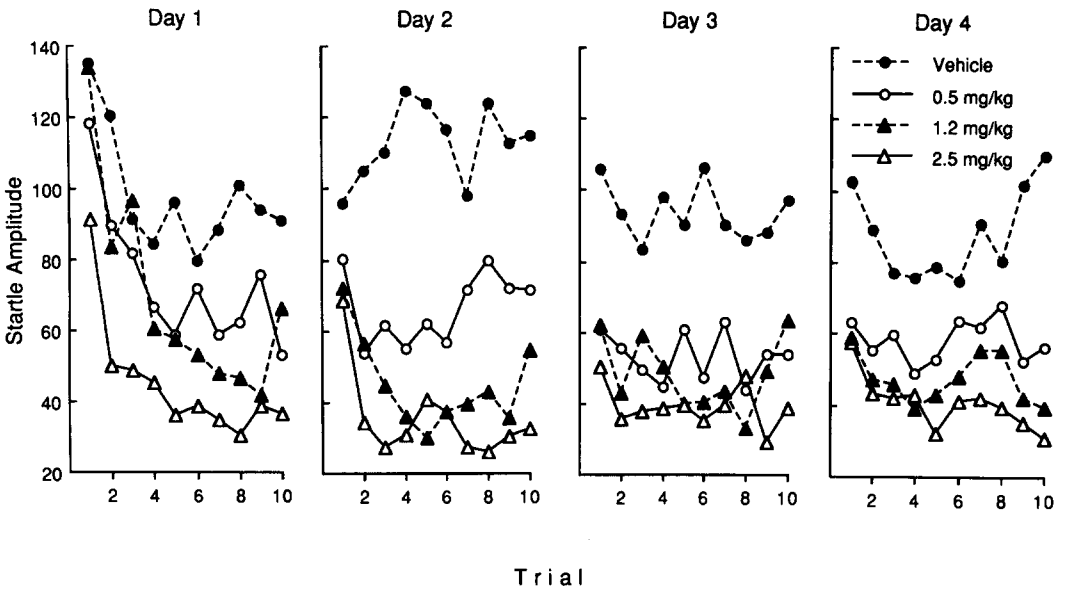

FIG. 2. Mean startle amplitudes of the three diazepam groups and the vehicle group on the 10 trials of each of the four test sessions.

as the startle stimulus began to lose its sensitization potential.

EXPERIMENT 2

Experiment 1 and our earlier data (24) suggest that diazepam produces decreased startle responding across trials by reducing fear elicited by the contextual cues of the startle apparatus. Consistent with this result is the finding of Leaton and Supple (15) that lesions of the central nucleus of the amygdala increased startle decrements and, in addition, attenuated freezing. It is well established that the amygdala plays a significant role in fear-related behaviors (2,22), and more specifically, may mediate fear-induced increases in the acoustic startle response. Central nucleus lesions block the fear potentiation of startle (11), whereas stimulation of the central, medial or intercalated nuclei increases startle responses (21).

The amygdala also appears to play an important role in mediating the anxiolytic effects of diazepam. Intra-amygdaloid injections of diazepam in rats reduces fear in a context associated with shock, as measured by suppression of barpressing (17), and by freezing (10). Consistent with these findings are reports that the amygdala, and in particular the basolateral division, contains high concentrations of benzodiazepine receptors (18,25). Thus the amygdala may be important for producing the response-attenuating effects of diazepam that were observed following repeated stimulation in Experiment 1.

In Experiment 2, we injected diazepam bilaterally into the amygdala with three expectations. First, we hoped to avoid any motor-suppressive effects the drug may have had on startle responses in the previous experiment. Second, we hoped to make possible the measurement of freezing behavior which is confounded by the motor-suppressive effects of diazepam. And third, we hoped to gain information about a possible anatomical locus for the effects seen in Experiment 1. We hypothesized that intra-amygdaloid injections of diazepam would not affect initial response amplitude but would decrease startle responding over trials and produce a concomitant reduction in freezing.

METHOD

Subjects and Surgery

The subjects were 16 experimentally naive male Long Evans rats, 170 days old and weighing between 465 g and 605 g at the beginning of testing. Housing and maintenance was identical to that of the previous experiment.

Surgery was carried out under aseptic conditions using sodium pentobarbital anesthesia (50 mg/kg, IP), supplemented with atropine (20 mg/kg) to reduce mucous secretions. Each animal was bilaterally implanted with 22-gauge stainless steel guide cannulae (Plastic Products) aimed at the basolateral division of the amygdala. The cannulae were stereotaxically placed at the coordinates 2.4 mm posterior to bregma, ±4.8 mm lateral to the sagittal suture, and 6.8 mm below dura with the skull flat. The initial coordinates were obtained from the atlas of Paxinos and Watson (19) and then adjusted from the histology of a series of preliminary animals. A nontraumatic headholder based on the design of Frommer (8) was used to prevent damage to the tympanic membranes.

Histology

At the conclusion of testing, the animals were administered a lethal dose of sodium pentobarbital (100 mg/kg) and perfused intracardially with normal saline, followed by 10 percent buffered formalin. Prior to removal of the brains, the heads were allowed to soak for at least 24 hours in the buffered formalin solution. The brains were subsequently removed from the skulls, kept overnight in a 20 percent sucrose formalin solution, and then sectioned at 40 μm on a freezing microtome. The sections were stained with cresyl violet and the injection sites localized with the aid of the Paxinos and Watson (19) brain atlas.

Apparatus and Procedure

The apparatus was identical to that used in the previous experiment except that the intensity of the white noise startle stim-

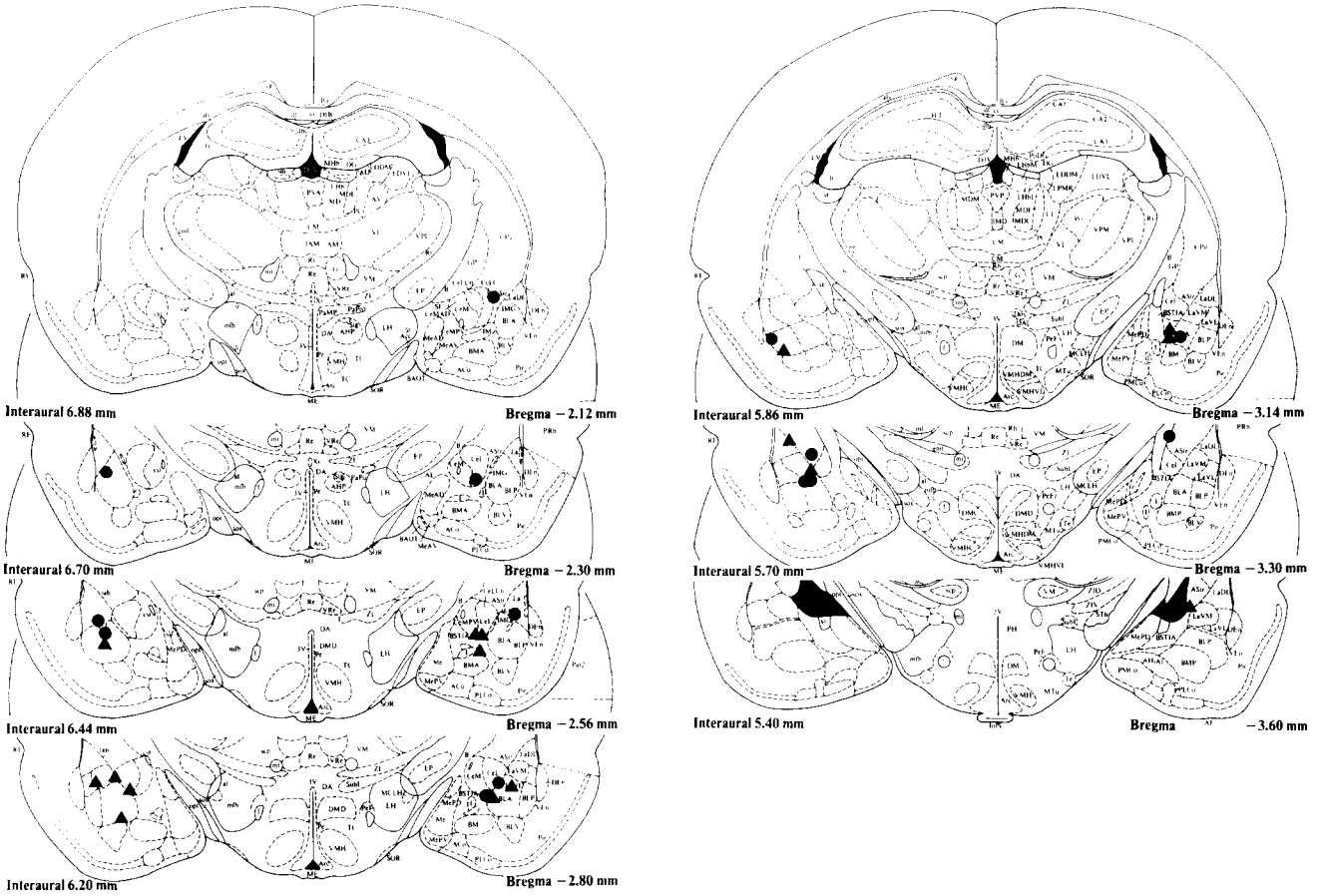

FIG. 3. Location of injection sites for 15 of the 16 animals used in Experiment 2. Injection sites are represented by triangles for the Diazepam Group, and by circles for the Vehicle Group. [Displayed on plates derived from the atlas of Paxinos and Watson (19).]

ulus was increased to 130 dB, and adjustments were made to the startle apparatus which resulted in lower overall startle amplitudes.

Two days after surgery, the cannula obturators were removed from each animal and cleaned with Povidine. During the course of this procedure, the animals were restrained in a laboratory towel wrapped firmly around the trunk and limbs. The animals were handled in this manner every day thereafter prior to testing in order to adapt them to the restraint procedure that was used during drug infusions.

Testing began 17 to 24 days after surgery. After restraining an animal in the manner described above, the cannula obturators were removed and replaced with 28-gauge injection cannulae. The injection cannulae were connected by PE 20 tubing to two Hamilton 10-μl syringes mounted in a infusion pump (Harvard Apparatus, Model 22). Eight animals received simultaneous bilateral infusions of 35 μg of diazepam, dissolved in 1 μl of 100 percent DMSO, over a period of 60 s. A further 40 s was allowed before the removal of the injection cannulae and replacement of the obturators. The eight vehicle-control animals were treated in an identical manner with the exception that only the DMSO vehicle was infused.

The animals were placed in the startle chambers twenty minutes after the infusion process was complete. Each animal was given 10 presentations of the startle stimulus on a 60-s ISI every other day for three days. Infusions (drug or vehicle) preceded each test session. During the 20-s period preceding the onset of each startle stimulus, freezing was scored at 2-s intervals. Freezing was defined as total immobility, including the vibrissae (6).

RESULTS AND DISCUSSION

Histology

The injection sites for 15 of the 16 animals are shown in Fig. 3. It was not possible to verify the cannula placement of one of the animals. All placements were located within a 1.5-mm rostral-caudal extent and a 1.5-mm medial-lateral extent of the forebrain. While the majority of injection sites were confined to the basolateral and lateral divisions of the amygdaloid complex, several sites were located in the central and basomedial divisions. Three sites were located in the amygdalostriatal transition area immediately dorsal to the amygdala, however, their proximity and the relatively large size of the cannulae probably allowed diffusion from these sites into the underlying nuclei.

Startle

Figure 4 shows the mean startle responses for the first trial of each of the three test days. While the response decrement from Day 1 to Day 2 was greater in the Diazepam group, by Day 3 the response levels of the two groups were almost identical. Polynomial contrasts yielded a significant linear effect of

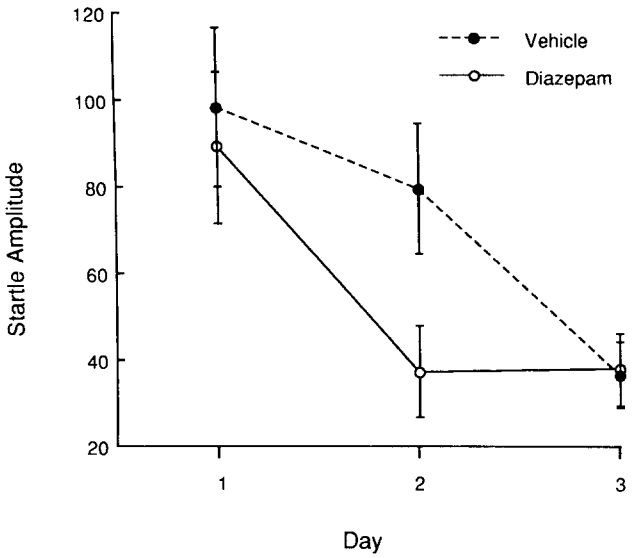

FIG. 4. Mean startle amplitudes of the Diazepam and Vehicle groups on the first trial of each of the three test sessions.

Day, $F(1,14)=20.42$, $p<0.01$, and a significant quadratic Group × Day interaction, $F(1,14)=7.33$, $p<0.05$. The Group difference was not significant, $F(1,14)=1.27$, $p>0.2$.

The greater response decrement shown by the Diazepam group from Day 1 to Day 2 parallels the larger decrement over days following systemic injections shown in Experiment 1. This result suggests that the response attenuating effect of diazepam is mediated, at least in part, by the amygdala.

The within-session results for each of the three days of testing are shown in Fig. 5. The within-session pattern of responding was similar for the two groups on Day 1. ANOVA yielded a significant linear Trial effect, $F(1,14)=13.70$, $p<0.01$, but no Group effect or interaction, $Fs<1$. On Day 2, the higher initial response level of the control group gave rise to a significant linear Group × Trial interaction, $F(1,14)=8.16$, $p<0.05$, but no effect of either Group, $F<1$ or Trial, $F(1,14)=3.14$, $p>0.05$. On Day 3, only the Trial effect was significant, linear, $F(1,14)=5.76$, $p<0.05$. The Day 2 interaction reflects the differences in long-term response decrements that were seen in the analysis of first trials. Response levels of the control group were higher than those of the Diazepam group on the first trial of Day 2, but during the course of the session decreased to approximately the same level as that of the Diazepam group.

Freezing

The percentage freezing data collapsed across the ten trials of each session are shown in Fig. 6. A log-transformation was applied to the percentage scores prior to analysis to improve the normality of their distribution. The difference between the groups on Day 1 reflects the more rapid development of freezing that occurred in the control group. On the following two days, development of freezing showed a similar course for both groups. Polynomial contrasts revealed a significant linear effect of Day, $F(1,14)=8.74$, $p<0.05$, and a significant Group × Day interaction, $F(1,14)=8.75$, $p<0.05$. The Group effect did not reach significance, $F<1$.

The reduced freezing seen on Day 1 in the Diazepam group provides an important second metric of fear conditioning in the startle apparatus. During the course of the 10 trials of Day 1, development of freezing was more rapid in the control group, indicating a greater degree of fear conditioning in this group. While the carryover of fear conditioning from Day 1 to the first trial of Day 2 is reflected in the increased startle responses of the control group, there was no difference in the freezing levels

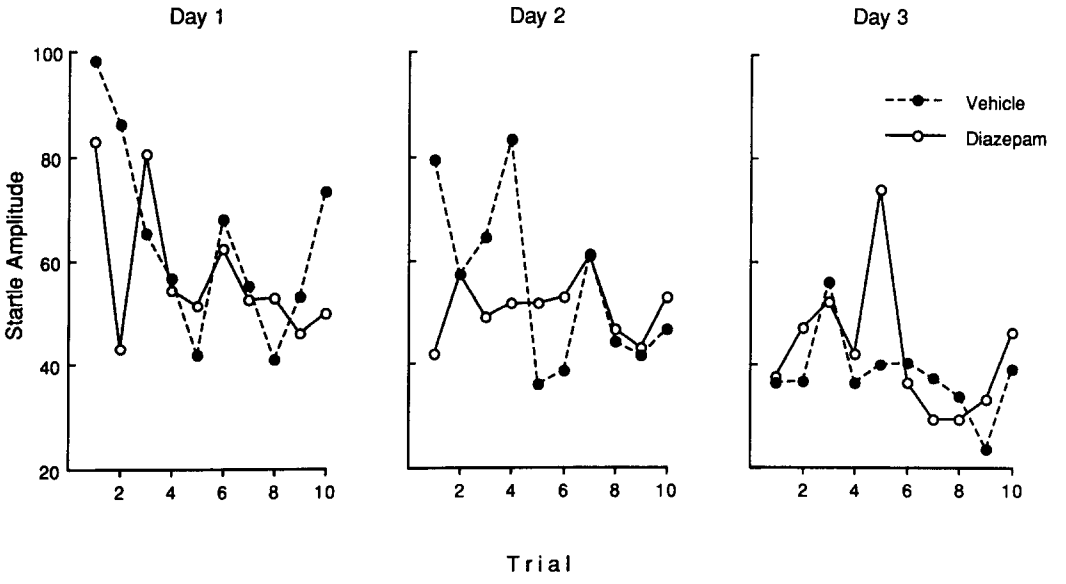

FIG. 5. Mean startle amplitudes of the Diazepam and the Vehicle groups on the 10 trials of each of the three test sessions.

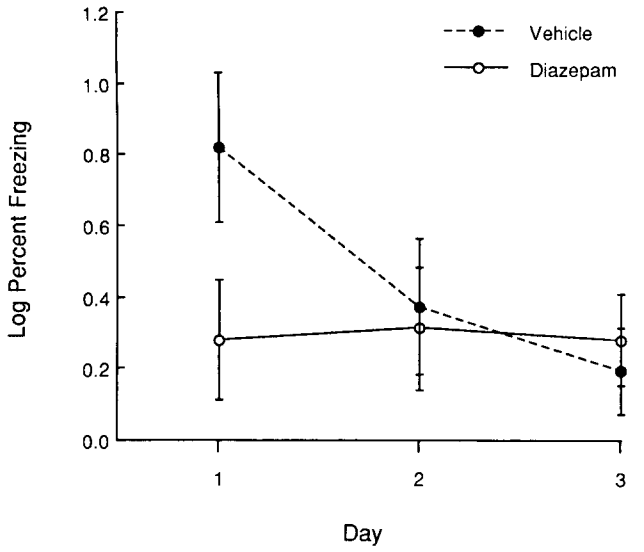

FIG. 6. Mean log percentage freezing of the Diazepam and Vehicle groups collapsed across the 10 trials of each of the three test sessions.

of the two groups on Day 2. This finding may at first appear anomalous, however, it is consistent with other data that suggest the carryover of fear conditioning from one acoustic startle habituation training session to the next may be more sensitively indexed by startle amplitude than by freezing. Freezing effects are often absent prior to the presentation of the first startle stimulus on successive test days, yet acquisition proceeds rapidly following the first presentation (15,23).

GENERAL DISCUSSION

Systemic or intra-amygdaloid administration of diazepam, a drug which is thought to reduce fear (5,7), increased long-term startle response decrements without significantly affecting response levels on initial trials. This outcome is consistent with previous studies which have shown that physiological and behavioral manipulations that reduce fear produce decreased startle responding across trials (3,15).

Response decrements in an habituation paradigm have been postulated to reflect the antagonistic action of two underlying processes: sensitization and habituation (9). While the net effect of diazepam administration in these experiments was an apparent increase in the rate of long-term habituation, we suggest the results are more consistent with an interpretation based on a decrease in sensitization. Borszcz et al. (3) have proposed that, in addition to the short-term nonassociative form of sensitization posited by Groves and Thompson (9), there exists a long-term sensitization process which is associative in nature. According to their thesis, long-term sensitization is a product of Pavlovian fear conditioning which occurs when the contextual cues of the startle chamber, as the CS, are associated with the startle stimulus, as the US. Thus, in the current series of experiments, the effect of diazepam was not to directly facilitate long-term habituation, but rather to block the fear conditioning that would otherwise mask habituation through sensitization.

Although we cannot be certain that the effects of central diazepam injections were solely related to action in the amygdala, they would appear to be related to a relatively localized brain effect. The injection of 35 μg into each amygdala was equivalent to a systemic dose of 0.12 to 0.15 mg/kg in the size of the animals used. This systemic equivalent was smaller by a factor of three than the smallest systemic dose used in Experiment 1, and yet produced a much more pronounced effect on long-term response decrements.

Importantly, diazepam produced no difference in response levels on the first trials of Day 1 in either experiment. Differences emerged between diazepam and vehicle groups only after repeated stimulation. This finding is of dual significance. First, it indicates that the drug did not simply depress responding. Second, it is consistent with an explanation of fear conditioning, which predicts that the response levels of the animals would not begin to differ until after the presentation of the first startle stimulus. Also consistent with the hypothesis of fear conditioning is the finding that the two groups in Experiment 2 had converged by Day 3. A convergence of the response levels of diazepam and vehicle groups occurs as the animals habituate to the startle stimulus and the stimulus loses its ability to support fear conditioning (3,20). This convergence was present but less marked in Experiment 1, despite the slightly less intense startle stimulus that was used. The more pronounced convergence in Experiment 2 may have resulted from incidental damage to the amygdala caused by the implanted guide cannula. Leaton and Supple (15) showed that amygdala lesions increased long-term response decrements. Consequently, response decrements in Experiment 2 may have been increased in both groups by damage to the amygdala caused by the guide cannula, and then further increased in the experimental group by the action of diazepam on undamaged parts of this structure.

In the present study, we showed that a pharmacological manipulation that reduces rear decreased startle responding across trials. This result is consistent with that produced by neurological and behavioral interventions which similarly affect fear (3,15). We suggest that by attenuating fear, diazepam blocks a sensitization process that inflates startle amplitudes and retards response decrements (3,15). It is possible that diazepam facilitates the long-term habituation process directly. However, this explanation would require two processes: fear reduction and facilitated habituation. The hypothesis outlined above predicts that both the decreased startle responding and the attenuation of the fear-related behavior, freezing, are attributable to the anxiolytic action of diazepam.

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