Pharmacology Biochemistry and Behavior, Vol. 63, No. 1, pp. 63–69, 1999

0091-3057/99/$–see front matter

PII S0091-3057(98)00251-2

Measuring Emotional Memory in the Elevated T-Maze Using a Training-to-Criterion Procedure

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Received 1 May 1998; Revised 9 September 1998; Accepted 5 October 1998

CONDE, C. A., V. COSTA AND C. TOMAZ. *Measuring emotional memory in the elevated T-maze using a training-to-criterion procedure.* PHARMACOL BIOCHEM BEHAV **63**(1) 63–69, 1999.—The elevated T-maze, an ethologically based test, has been used to investigate the effects of anxiolytic drugs on memory and the relationships between neural systems involved in such modulation. This test allows the measurement in the same rat of two kinds of aversively motivated behaviors—inhibitory avoidance and one-way escape. The apparatus consists of three arms of equal dimensions, elevated 50 cm from the floor. One arm is enclosed by walls and stands perpendicular to the two open arms. Placing the rat at the end of the enclosed arm and recording the time to withdraw from this arm during three consecutive trials assesses inhibitory avoidance. Soon afterwards, the rat is placed at the end of one of the open arms and the time to leave this arm recorded as escape response. Three days later memory is assessed by reexposing the rats to the maze. One critical question raised by these studies is whether the anterograde amnesia induced by anxiolytic drugs could be due to insufficient learning during training or to amnesia. The present work investigated whether the introduction of a multitrial training-to-criterion procedure could overcome this question. For this purpose, rats were tested as many times as needed to stay in the enclosed arm continuously for 300 s (avoidance learning to criterion). Results from Experiment 1 showed that rats trained to a learning criterion shows significantly better retention performance. Experiment 2 evaluated the effects of pretraining diazepam (DZP) treatment on this training-to-criterion protocol. The results indicate that DZP did not affect acquisition performance but induced a dose-dependent impairment of the inhibitory avoidance in the memory test. One-way escape (latency to enter the enclosed compartment from the open arms) was not affected by DZP. These results rule out the possibility that the impairment of inhibitory avoidance memory in the elevated T-maze could be due to lack of learning during training, and support the hypothesis that the disruptive effects of DZP are on processes involved in long-term storage of information. © 1999 Elsevier Science Inc.

Elevated T-maze Memory Training-to-criterion Anxiety/Fear Diazepam

RECENTLY, a new method was developed for investigating the effects of anxiolytic drugs on memory, and the relationships between neural subsystems involved in emotionally related behaviors and in processes underlying learning (5, 18,19). This experimental model allows the parallel measurement of responses related to both conditioned and innate types of fear in the same subject, and permits the simultaneous assessment of memory for these behaviors. The apparatus is a derivation of the elevated plus-maze (12,13), and is composed of two open arms disposed at right angles to one enclosed arm, elevated above the ground. It has been demonstrated that the critical motivational factor in this ethologically based model is the aversive nature of the open arms (19). Therefore, it avoids interference of pain (e.g., electric shock), the sensitivity of which can be affected by anxiolytic drugs [see (16)]. This elevated T-maze has provided a useful model to measure two kinds of aversively motivated behaviors—inhibitory avoidance and one-way escape—which may be related to anticipatory anxiety (conditioned fear) and "innate fear" (panic disorder, unconditioned fear), respectively (4, 5–7). It is known that rats have an innate fear of openness and height (10,11,13,19). Repeatedly placing the rat inside the en-

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closed arm to explore the maze allows the subject to learn inhibitory avoidance behavior of the open arms. On the other hand, the placement of the rat at the end of one of the open arms elicit an escape response towards the closed arm; performing a one-way escape response. Reexposing the animals to the situation after a time interval assesses memory of these emotionally related behaviors.

In the original validation study of the elevated T-maze (5) the effects of diazepam (DZP), a benzodiazepine anxiolytic that is know to produce anterograde amnesia (9,16), were tested. DZP (1–4 mg/kg, IP) impaired inhibitory avoidance response in a dose-dependent way when the animals were tested in the presence of the drug, an effect that may be interpreted as anxiolytic. Further retesting 3 days later, in the absence of the drug, showed an impairment of the inhibitory avoidance learning suggesting anterograde amnesia. This effect seems not be due to state-dependent learning, because in a subsequent study (18) it was demonstrated that this impairment also occurs in animals receiving DZP pretraining and pretesting. In contrast, neither escape performance nor its memory was affected by DZP treatment.

However, the results observed in the inhibitory avoidance test 3 days after training did not allow one to conclude if this effect was due to an anterograde amnesia or due to insufficient learning during training, because DZP affected inhibitory avoidance acquisition. Therefore, the purpose of the present study was first, to investigate whether the introduction of a learning-to-criterion procedure would influence the test performance, and second, to test the effects of diazepam treatment on this new protocol.

METHOD

Animals

Males Wistar rats, 250–330 g in weight, were housed in groups of four to six per cage with food and water ad lib. They were maintained under constant temperature (22 ± 1°C), and under a 12-h light/dark cycle (lights on at 0700 h).

Apparatus

The elevated T-maze was made of wood and had three arms of equal dimensions (50 × 12 cm). One arm, enclosed by walls 40 cm high, was perpendicular to two opposed arms. A Plexiglas border 1 cm high surrounded the open arms. The whole apparatus was elevated 50 cm above the floor. The experimental room had one 30-W lamp of white and indirect light above the center of the maze. The experiments were performed with a background noise and with an observer inside the room.

Procedure

Experiment 1. On the third and fourth days after their arrival in the laboratory, rats were gently handled for 5 min. On the fifth day, they were randomly assigned to two experimental groups. The first group ("noncriterion," $n = 13$), was tested using the elevated T-maze protocol described by Viana and co-workers (18). Three minutes before the test, each rat was put in an individual cage (28 × 18 cm) for habituation. Thereafter, the rat was placed at the end of the enclosed arm facing the intersection of the arms and the time taken to leave this arm with the four paws was recorded (baseline latency). The animal was them immediately removed from the open arm and the same measurement was repeated in two subsequent trials (avoidance 1 and avoidance 2) at 60-s intervals. When the rat was placed at the end of the enclosed arm, it did not see the open arms until it poked its head beyond the walls of the closed arm. Because rats have an innate fear of height and openness (10–13), reexposing the subject inside the enclosed arm to explore the maze allows the animal to learn inhibitory avoidance of the open arms. Immediately after this inhibitory avoidance training the rat was placed at the end of the right open arm, and the time taken to leave this arm with the four paws was recorded (escape 1). Placing the rat at the end of the open arm it can move towards the closed arm, performing an escape response. Three days later, avoidance (avoidance 3) and escape (escape 2) latencies were measured again. The second group ("training to criterion," $n = 41$), received similar treatment except in the number of trials where the rat was placed into the enclosed arm. In this case, the rat was placed as many times as was needed to stayed there continuously for 300 s.

Experiment 2. To assess the effect of diazepam on this training-to-criterion protocol, rats were randomly assigned to different treatment groups and were given an intraperitoneal injection of DZP (1.0, 2.0, or 4.0 mg/kg) or isotonic saline solution (SS), 1 ml/kg. After 25 min, each rat was submitted to the elevated T-maze test as above described. Three days later, in the absence of drug, memory was evaluated by reexposing the animals to the maze where avoidance and escape latencies were recorded again. In addition, 1 week later, the same animals were injected again with DZP or SS and 25 min later placed in the center of an open-field chamber (72 cm in diameter) to test crossings as index of locomotor activity.

Statistics

Within-group comparisons related to the latency to leave the enclosed arm in the training and the retention test were analyzed using a two-way ANOVA. Escape latencies, locomotor activity, and between-group comparisons within each trial were made by the unpaired *t*-test. The data related to the number of training trials to reach the criterion were analyzed using ANOVA.

RESULTS

Experiment 1

Figure 1 shows the latencies of the first inhibitory avoidance and escape response both during acquisition and 3 days later, when memory was tested. The paired *t*-test showed a significant difference ($p < 0.001$) between the avoidance latencies in the first acquisition trial and those obtained 72 h later for the criterion group. This indicates a good avoidance memory for the animals that were trained to stay 300 s in the enclosed arm. The same was not observed for the noncriterion group. That is, latency to leave the enclosed arm of the T-maze did not significantly increased ($p = 0.209$) between acquisition and retention test trials. In addition, comparison between groups of the avoidance latencies in the retention test indicated greater latencies for the animals trained to criterion (*t*-test, $p < 0.001$).

The same tendency was observed for the escape performance. As illustrated in Fig. 1B, animals from criterion group showed significantly lower ($p < 0.001$) latencies in exit from the open arm during the retention test trial than those of the noncriterion group, suggesting a better memory for the first ones.

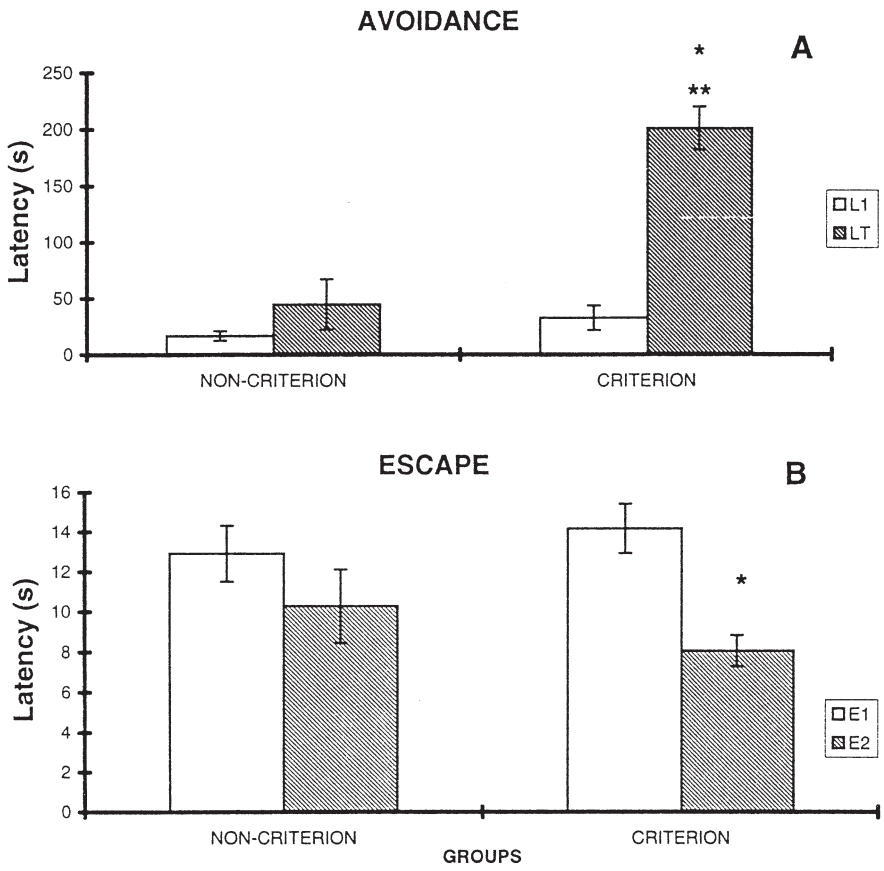

FIG. 1 Mean (±SEM) latencies to withdraw from the enclosed (A) and from the open arms (B) of the T-maze. Animals from "noncriterion" group were tested for inhibitory avoidance in three consecutive trials at a 60-s interval. After another 60 s, the latency to leave the open arm (one-way escape) was measured. Animals from the "criterion" group were trained to a learning criterion in which they were placed as often times as needed to stay in the enclosed arm continuously for 300 s. Three days later memory for these tasks was assessed by reexposing the rats to the maze. L1 = latency of the first inhibitory avoidance trial (baseline); LT = avoidance latency on the test day; E1 = escape latency on training day; and E2 = escape latency on test day. *Significantly different from test day; **significantly different from noncriterion group.

Experiment 2

After exclusion of animals that fell from the elevated T-maze, the following group sizes were obtained: $n = 9$ for saline, $n = 11$ for DZP 1 mg/kg, $n = 10$ for DZP 2 mg/kg, and $n = 10$ for the DZP 4 mg/kg group. The ANOVA of the number of training trials required to learn the task to criterion revealed no significant differences between treatment groups, $F(3, 39) = 1.107$, $p = 0.359$. This suggests that the injections of diazepam or saline did not affect the acquisition performance in the multitrial inhibitory avoidance. In the same way, DZP or saline injections did not affect the total time spent in the enclosed arm during acquisition, $F(3, 39) = 0.726$, $p = 0.543$. These results are shown in Fig. 2.

The effects of diazepam on inhibitory avoidance learning are illustrated in Fig. 3A. As can be seen, in controls as well as in rats treated with DZP, the latency significantly increased from baseline to trial 1, trial 2, and the trial where they reached the criterion (Tn), indicating good acquisition of inhibitory avoidance [test along trials, ANOVA for SS: $F(3, 34) = 21.756$, $p < 0.001$; for DZP 1, $F(3, 41) = 33.803$, $p < 0.001$; for DZP 2: $F(3, 38) = 32.546$, $p < 0.001$; for DZP 4: $F(3, 39) = 93.56$, $p < 0.001$]. The analysis of the results of the inhibitory avoidance performance tested 72 h later indicated that animals treated with 2.0 and 4.0 mg/kg DZP showed significantly lower latencies than those of the saline control group [ANOVA, $F(3, 36) = 5.784$, $p = 0.002$; Bonferroni t-test: SS vs. DZP 1, $t = 1.938$, $p > 0.05$; SS vs. DZP 2, $t = 3.017$, $p < 0.05$, SS vs. DZP 4, $t = 3.964$, $p < 0.05$]. This suggests a dose-dependent memory impairment for animals pretreated with DZP.

In contrast to inhibitory avoidance, one-way escape was not affected by DZP treatment (Fig. 3B). On the training and test days escape latencies were similar in every treatment group [ANOVA for E1: $F(3, 37) = 1.565$, $p = 0.216$; ANOVA for E2: $F(3, 39) = 1.418$, $p = 0.253$]. However, a two-way ANOVA indicates a significant effect between escape 1 and escape 2 trials, $F(1, 82) = 13.43$, $p < 0.001$; Bonferroni t-test, $t = 3.665$, $p < 0.05$, E1>E2], suggesting good memory for this component of the elevated T-maze test.

Figure 4 presents the data on locomotor activity obtained in the test performed 7 days after the retention test. It can be

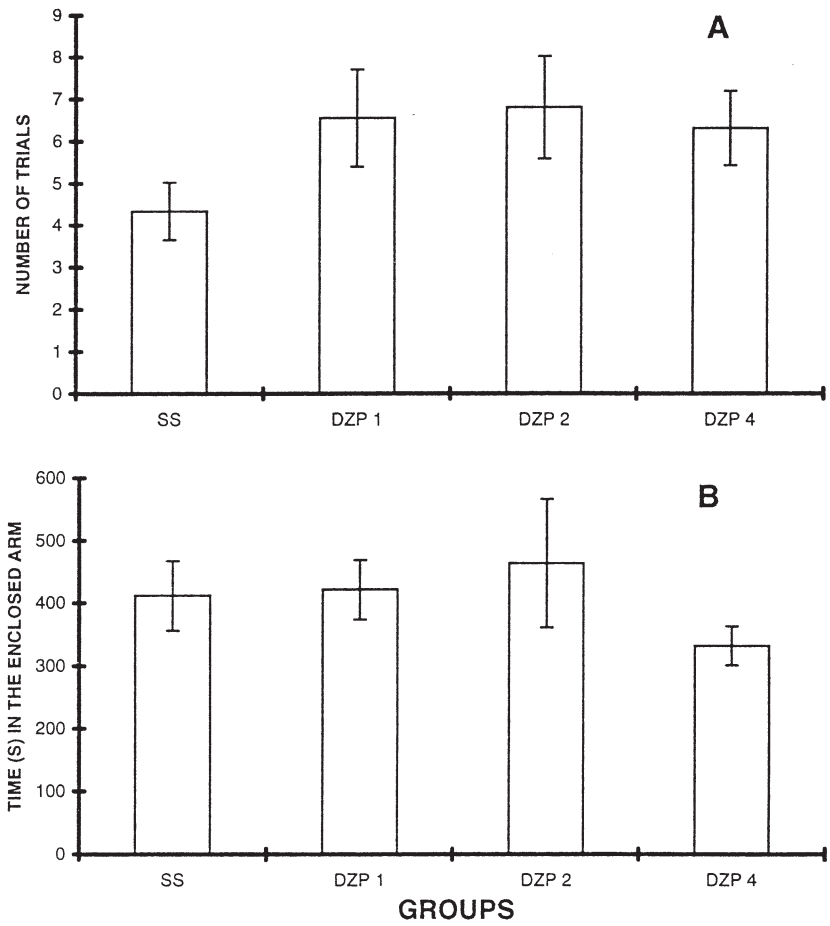

FIG. 2 Effect of diazepam (DZP) on inhibitory avoidance acquisition in rats trained to a learning criterion. Number (mean ± SEM) of training trials required to learn the task to criterion (A). Time (mean ± SEM) spent in the enclosed arm during the training (B). SS = saline; DZP 1 = diazepam 1 mg/kg; DZP 2 = diazepam 2 mg/kg; DZP 4 = diazepam 4 mg/kg. Training began 25 min after SS or DZP injection.

seen that control and animals treated with DZP 1 and 2 mg/kg spent less time exploring the center of the open field (paired t-test, $p < 0.001$). In contrast, animals treated with DZP 4 mg/kg did not show any difference between the exploratory activity at the border and the center (paired t-test, $p = 0.113$), suggesting an anxiolytic effect (Fig. 4A). Two-way ANOVA showed a significant effect of drug treatment, $F(3, 93) = 39.47$, $p < 0.001$, number of crossings recorded every minute, $F(2, 93) = 38.37$, $p < 0.001$, and drug × time interaction, $F(6, 93) = 5.044$, $p < 0.001$. The Bonferroni t-test for multiple comparisons showed the following treatment differences (all $p < 0.05$): SS>DZP4 ($t = 9.467$); SS>DZP2 ($t = 6.307$); DZP1>DZP4 ($t = 8.417$); DZP1>DZP2 ($t = 5.18$); DZP2>DZP4 ($t = 3.071$). The same test showed the following crossings differences between minutes of the session: minute 1 > minute 2 ($t = 4.887$); minute 1 > minute 3 ($t = 8.74$); minute 2 > minute 3 ($t = 3.853$). In the first minute we observed the following differences: SS>DZP2 ($t = 4.567$); SS>DZP4 ($t = 7.426$); DZP1>DZP2 ($t = 4.536$); DZP1>DZP4 ($t = 7.464$), and DZP2>DZP4 ($t = 2.778$). In the second minute the differences were: SS>DZP2 ($t = 5.88$); SS>DZP4 ($t = 7.0$); DZP1>DZP2 ($t = 3.57$); DZP1>DZP4 ($t = 4.65$). No differences were observed in the third minute. Overall, these results show that locomotor activity was significantly affected by DZP treatment. However, a significant decrement in the number of crossings between the first and the third minute of test was observed, suggesting habituation learning.

DISCUSSION

In this study, we investigate the importance of introduce a training-to-criterion procedure in the elevated T-maze test, and the effect of diazepam treatment on the emotional memory component in this new protocol.

First, our results obtained in Experiment 1 show that rats trained in the multitrial inhibitory avoidance training-to-criterion protocol have better avoidance and escape performance than those trained with three trials in the enclosed arm of the elevated T-maze. It is possible that by training the animal to an acquisition criterion, we could compensate for the data dispersion we observed with the original protocol of the elevated T-maze test (5,18).

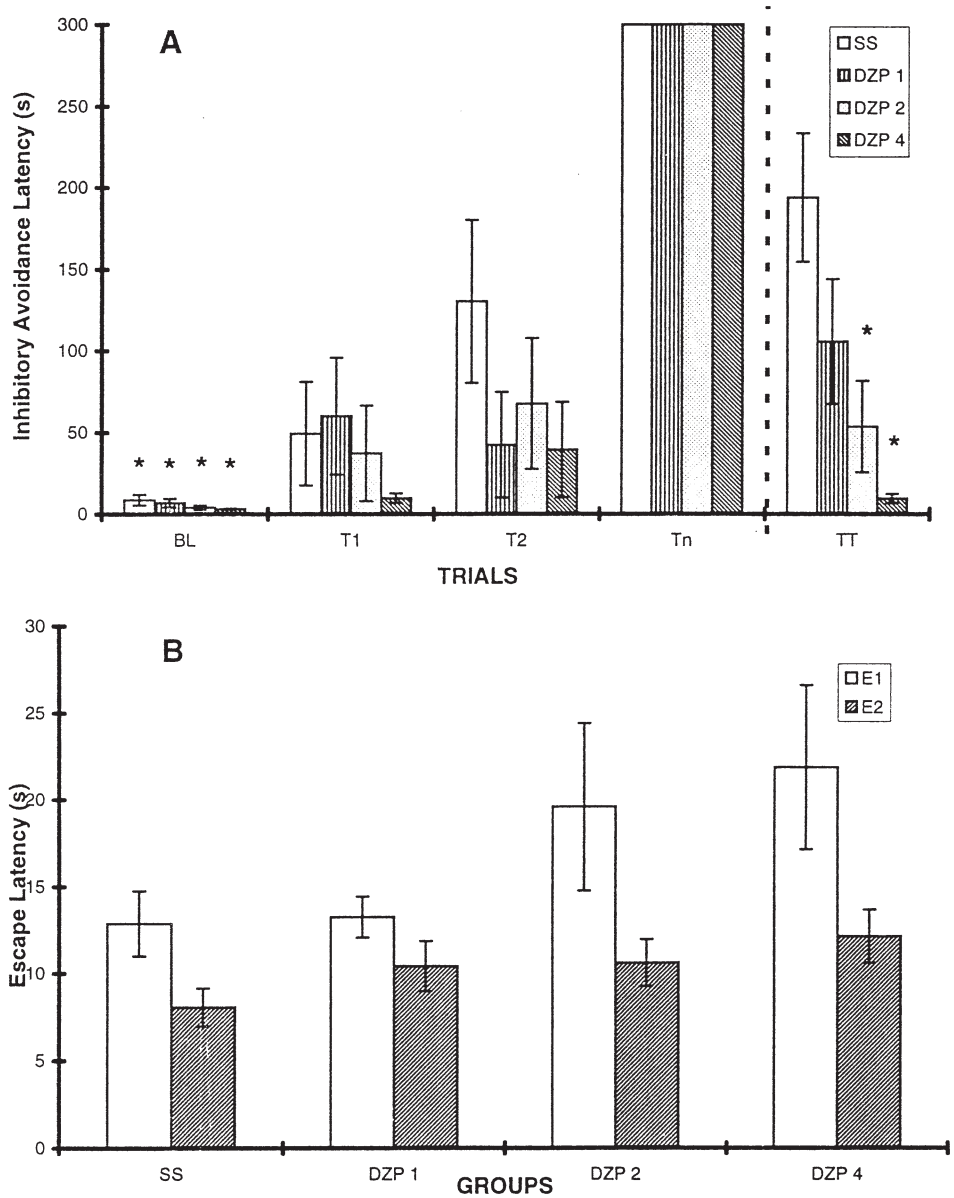

FIG. 3. Effect of diazepam on inhibitory avoidance (A) and one-way escape (B) learning. Bars represent the mean (±SEM) latencies. Rats were trained to a learning criterion as described in the legend of Fig. 1. BL = baseline; T1 = trial one of the avoidance learning; T2 = avoidance trial 2; Tn = avoidance trial where the animals reached the criterion; TT = avoidance test trial 72 h after the training, in the absence of drug. See legend of Fig. 2 for description of treatment groups. *BL, significantly different from Tn; *TT, significantly different from control group.

Second, the results of Experiment 2 using the learning criterion of 300 s in the enclosed arm showed that injection of DZP prior to training did not affect acquisition of the inhibitory avoidance task. There was no difference between diazepam-treated and control animals in the number of training trials required to learn the task to criterion and the time spent in the enclosed arm during the training (see Fig. 2). Even more, our results rule out the possibility that the impairment of inhibitory avoidance memory could be due to lack of learning during training. However, diazepam-treated animals showed a dose-dependent memory impairment in the retention test 72 h later. These results are consistent with other findings indicating that DZP produces anterograde amnesia without affecting acquisition of the conditioned response (2,4,5,15,17,18). This effect could mean that the disruptive effects of DZP are not on associative processes, but instead on processes involved either in long-term memory storage of information or in retrieval. Because DZP was administered before training, the anterograde amnesia could be interpreted in terms of state dependency. However, in a previous study (18) we demon-

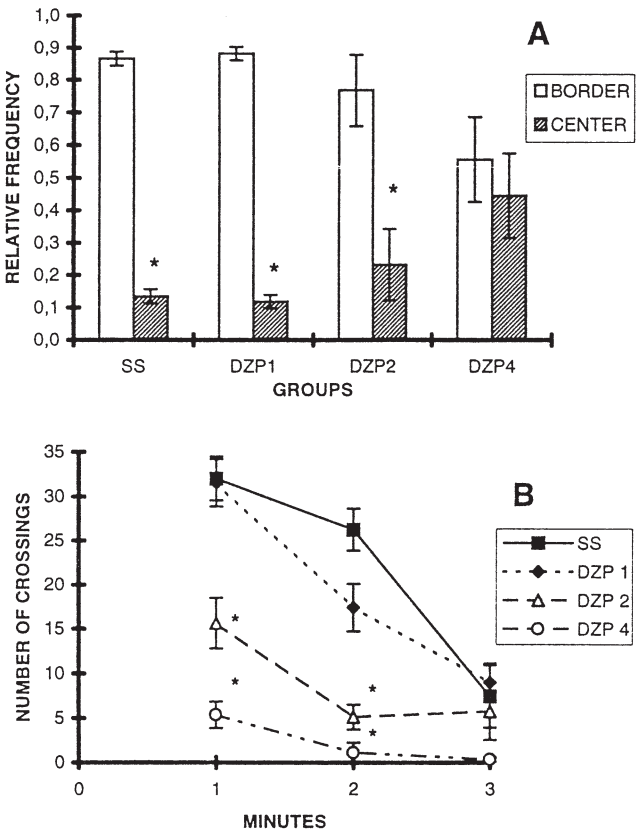

FIG 4. Effect of diazepam on locomotor activity displayed by rats in the open-field. Test was performed 7 days after the elevated T-maze testing. Bars represents the relative values (mean ± SEM) of exploring activity in the center or in the border of the open-field (A; *significantly different from border). Part B of the figure shows the time course of locomotor activity along the 3-min test. See legend of Fig. 2 for description of treatment groups. *Significantly different from control group.

strated that DZP injected both before training and before the test impaired retention of the inhibitory avoidance learning in the elevated T-maze, suggesting that the deficits observed are not due to state-dependent variables. Also in this study (18) we showed that when rats learned the inhibitory avoidance under saline, they displayed good memory even when DZP was given before the test, suggesting that once acquired, the inhibitory avoidance behavior in this test is resistant to DZP. On the other hand, it could be argued that this type of protocol using multitrials to a high criterion is not sensitive to pretrial influences of DZP. However, acquisition performance using another inhibitory avoidance task employing massed trials to a high criterion, which requires considerable overtraining, also was not sensitive to pretrial application of benzodiazepines (15,17). It is also unlikely that the amnestic effects of DZP are simply due to an attenuation of the aversiveness or a decrease in level of arousal. In this case we would have expected a deficit not only in recall 72 h later, but also in rate of acquisition to criteria.

The results obtained with the escape task in the same animals showed that this component of the elevated T-maze is not affected by the same dose of DZP that significantly impairs inhibitory avoidance memory. Our findings add to other evidence indicating that one-way escape is resistant to anxiolytic doses of DZP (5,18). One possible explanation for these results is that benzodiazepines could impair the ability of animals to withhold a highly probable response. It has been suggested that the two tasks measured in the T-maze test generate distinct types of fear/anxiety and memory that could be correlated with different subsystems in the brain mediating the mnemonic and anxiolytic effect of drugs (1,3–7,14,15,17–19). The results of the present study support this hypothesis, because the amnestic effects of DZP are mediated, at least in part, through influences involving the brain amygdaloid complex (1,14,15,17). In fact, it has been demonstrated that benzodiazepine-like immunoreactivity in the amygdala can decrease during avoidance conditioning (8), suggesting that consolidation of information is downregulated by endogenous benzodiazepines. In addition, it is well established that amygdala lesions attenuate the expression of emotional memory, whereas its integrity does not seem to be critical for other types of memory [see, e.g., (20)]. Thus, it could be possible that memory for the avoidance conditioning is mediated by DZP-sensitive brain areas, whereas memory for the escape response is mediated by other brain regions not sensitive to DZP. Although DZP did not affect criterion performance in Experiment 2, an increasing trend was observed in animals treated with high DZP doses. This was more likely due to the locomotor deficits produced by high DZP doses, because such effects were observed in the same animals when tested in the open field. However, it is important to mention that although DZP treatment affected locomotor behavior it did not impair habituation learning, as indicated by our results (see Fig. 4).

This work did not evaluate whether permanence for a continuous 300 s in the enclosed arm is the best criterion for emotional learning in this model. However, based in our data, we suggest it when measuring emotional memory in the elevated T-maze test. The present protocol also seems to be useful to compensate for possible individual and/or genetically differences related to reactivity levels and, therefore, provide homogeneous conditions for the acquisition process in the group. Further experiments using different drugs and/or brain site manipulation would be desirable to explore the potential of this protocol.

ACKNOWLEDGEMENT

This research was supported by grants from CNPq and FAPESP (Brazil). C. A. Conde was the recipient of a PhD fellowship from COLCIENCIAS and Universidad Industrial de Santander (Colombia). V. Costa was the recipient of a research fellowship from CNPq. We are grateful to J. R. Stella for technical assistance.

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