Pharmacology Biochemistry and Behavior, Vol. 43, pp. 677–681, 1992
Printed in the U.S.A.

0091-3057/92 $5.00 + .00

Effect of Various Training Procedures on Performance in an Elevated Plus-Maze: Possible Relation With Brain Regional Levels of Benzodiazepine-Like Molecules

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Received 2 December 1991

DA CUNHA, C., M. LEVI DE STEIN, C. WOLFMAN, R. KOYA, I. IZQUIERDO AND J. H. MEDINA. *Effect of various training procedures on performance in an elevated plus-maze: Possible relation with brain regional levels of benzodiazepine-like molecules.* PHARMACOL BIOCHEM BEHAV 43(3) 677–681, 1992.—Rats submitted to one, two, or seven sessions of exploration to a new environment (habituation) or exposed to an inhibitory avoidance training showed different degrees of anxiety, evaluated by the elevated plus-maze test. Also, the brain regional levels of benzodiazepine (BDZ)-like molecules in rats submitted to one, two, or seven sessions of habituation were differentially decreased with respect to nontrained rats. The percentage of time spent in the open arms of the elevated plus-maze for each group correlates with the data of decrease in the BDZ-like immunoreactivity in amygdala ($r = 0.77$, $p < 0.0005$), hippocampus ($r = 0.68$, $p < 0.0005$), and septum ($r = 0.57$, $p < 0.005$). These results suggest that the limbic system responds to anxiogenic experiences by changing the BDZ-like molecule levels in relation to the degree of anxiety and/or stress that accompany these experiences.

Benzodiazepines Anxiety Stress Elevated plus-maze Benzodiazepine-$GABA_A$ receptor complex

THE presence of benzodiazepines (BDZs) and BDZ-like molecules in brain have been recently well documented (1,21, 25,28). IN a previous article, we reported on the rapid decrease of BDZ-like molecules in various brain structures of the rat brain following habituation and inhibitory avoidance training procedures, which suggested a release of these substances (29). The results suggested a relation between the stressful or anxiogenic content of the tasks and the apparent release of BDZ-like molecules in different brain regions (13,29). Also, previous work by Skolnick and their associates suggested that acute stress induces the release of an endogenous modulator with BDZ-like properties in cerebral cortex (27). Very recent evidence of Primus and Kellogg (23) on changes of properties of the BDZ–GABA–chloride channel complex caused by exposure to unfamiliar environments has also been interpreted as suggesting an endogenous release of BDZ receptor agonists.

The present article studies the effect of various behavioral tasks on performance in an elevated plus-maze. This performance can be taken as a measure of anxiety (10,22). An attempt was made to correlate these measures with levels of BDZ-like molecules in various regions of the brain after the behavioral tasks.

METHOD

Subjects and Behavioral Procedures

Male Wistar rats (261 ± 17 g) from our own breeding stock were used. They were divided into five groups.

Group 1. Animals were killed immediately after being withdrawn from their home cages.

Groups 2–5. Animals were exposed to a 50 × 25 × 25-cm acrylic box, with a frontal glass panel and a floor made of parallel 1-mm-caliber bronze bars spaced 0.8 cm apart. A 5-cm high, 8-cm wide formica platform was placed on the left

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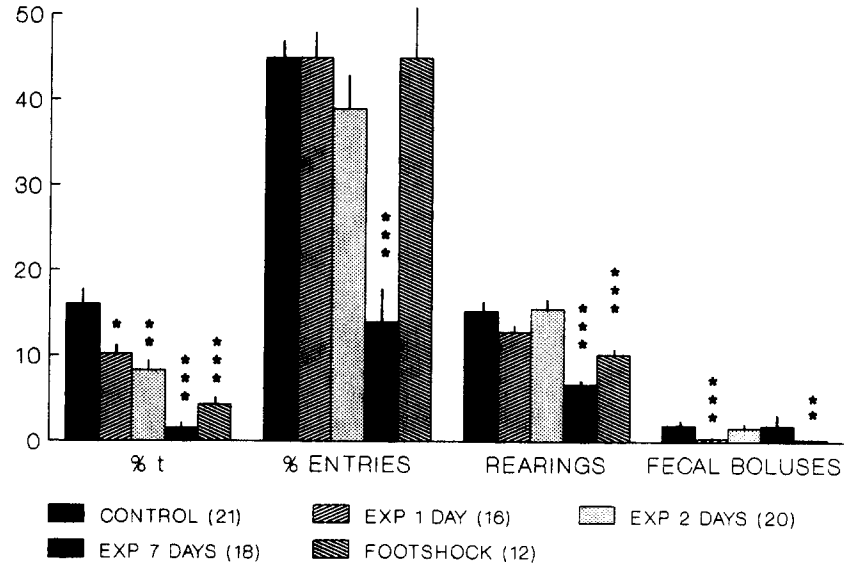

FIG. 1. Effect of various behavioral tasks on performance in an elevated plus-maze. Bars represent, respectively, means (± SEM) percentage of open arms entries, percentage of time spent in the open arms, and number of rearings and fecal boluses made by rats given a 5-min test in the elevated plus-maze immediately after being exposed to an unfamiliar environment for the first, second, or seventh daily session or after receiving a 0.35-mA foot-shock (inhibitory avoidance training). The number of experiments is indicated in parentheses. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$, with respect to control group, Duncan's test after one-way analysis of variance (ANOVA).

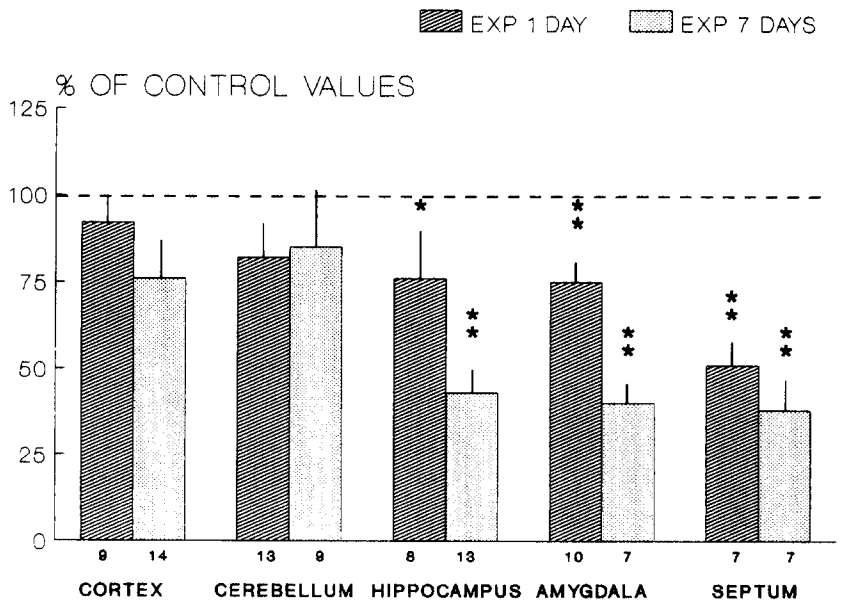

FIG. 2. Effect of one or seven daily sessions of exploration in an unfamiliar environment on rat brain regional levels of BDZ-like molecules. Results are expressed as percentage of control values (dotted line). Each bar represents the means ± SEM of 6–10 pools of brain tissues from 2–5 animals each. Control values for diazepam equivalents/g protein are: cerebral cortex, 54 ± 5; cerebellum, 134 ± 25; hippocampus, 158 ± 18; amygdala, 355 ± 45; septum, 756 ± 176. $*p < 0.01$; $**p < 0.05$, with respect to control (nontrained rats), Duncan's test after one-way ANOVA.

extreme of the box (29). These groups were submitted to different behavioral procedures, as follows.

Group 2. Animals were placed on the platform and left to explore freely the box during 30 s.

Group 3. This procedure was repeated twice, with a 24-h interval between sessions.

Group 4. Animals were submitted first to six daily 1-min handling sessions and subsequently to seven daily sessions of exploration of the box, as above.

Group 5. Animals were placed in the box and received a 0.35-mA, 2-s foot-shock immediately after they stepped down from the platform.

The procedure used in groups 2, 3, and 4 corresponds to a habituation training and that used in group 5 corresponds to an inhibitory (passive) avoidance training (29).

Elevated Plus-Maze Measurements

Immediately after completing the tasks described above, animals were transferred to an elevated plus-maze (22). The apparatus consisted of four perpendicularly disposed wood arms (50 × 10 cm; two had 40-cm high wood walls and two were open) linked by a central 10 × 10-cm square. The maze was suspended 50 cm from the room floor. Animals were placed on the central part of the maze facing a closed arm. The time spent and number of entries into the open arms and the number of rearings and fecal boluses were counted during 5 min. Based upon previous validations of the elevated plus-maze test of anxiety (22), in the present study we used the term "anxiogenic" to define the behavioral procedure that causes a selective decrease in the time spent and/or number of entries in the open arms.

Biochemical Assays

Rats were killed by decapitation 26 ± 2 s after completing the behavioral tasks described above, and membranes were prepared for binding assays and supernatants for BDZ-like immunoreactivity determinations, as described previously (20,29). Briefly, the cerebral cortex, cerebellum, amygdala, hippocampus, and septum were dissected out on ice, homogenized in 10 vol ice-cold sucrose 0.32 M, and centrifuged at 1,000× *g* for 10 min. The supernatants were centrifuged at 40,000× *g* for 30 min to give a pellet corresponding to a crude synaptosomal membrane fraction. This fraction was homogenized in 20 vol 25 mM Tris-HCL buffer, pH 7.3, centrifuged at 40,000× *g* for 30 min and stored at −70°C until used. Central type BDZ receptors were measured using [^{3}H]flunitrazepam ([^{3}H]FNZ, 87 Ci/mmol, (New England Nuclear, Newton, MA) as previously described (18,20).

To determine BDZ-like immunoreactivity, supernatants of the last two centrifugations were pooled and passed through Sep-Pak (Waters Assoc., Milford, MA) C-18 cartridges and concentrated, as described elsewhere (21). The BDZ-like immunoreactivity was determined by using a specific monoclonal antibody (MAb 21-7F9) against BDZs (25) as described previously (21,29). Data are expressed as diazepam equivalents (DE) based upon extrapolation from standard displacement curves generated using diazepam (Logit plot) (24). Protein determinations were carried out by Lowry's method (15).

RESULTS

Performance of the five groups in the elevated plus-maze is shown in Fig. 1. As can be seen, the time spent in the open arms was lower both in animals submitted to a simple exploration (habituation) and in those exposed to the inhibitory avoidance training procedure. In the former, the reduction was proportional to the number of sessions. Entries into the open arms were lower in the group exposed to seven sessions of exploration than in all other groups.

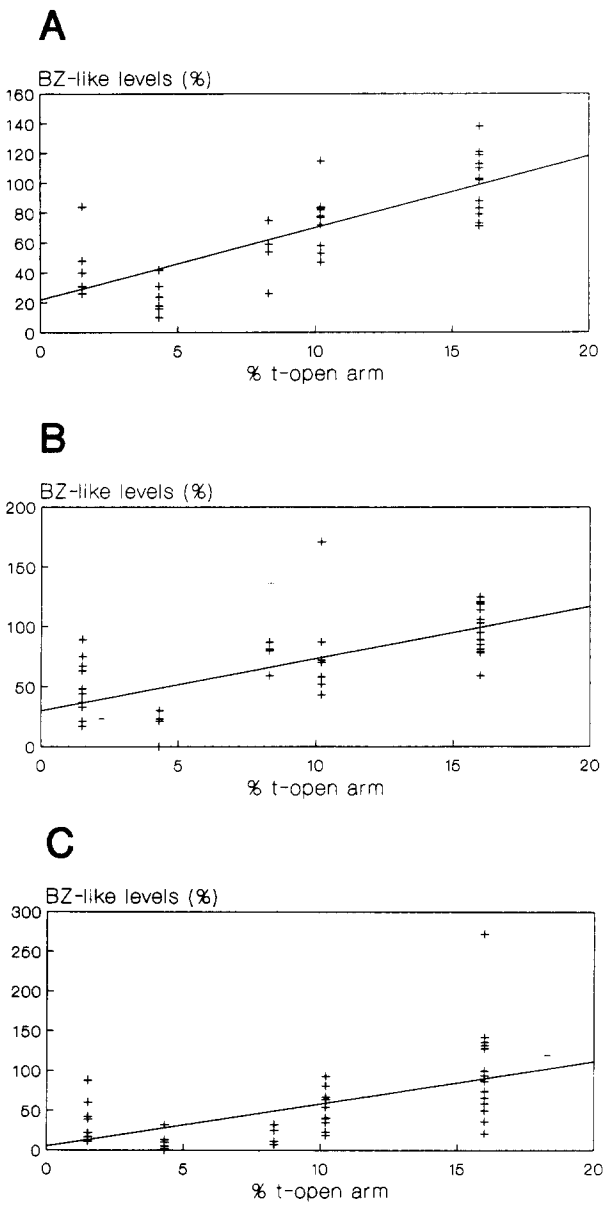

FIG. 3. Correlation between levels of BDZ-like molecules in amygdala, hippocampus, and septum of rats submitted to various anxiogenic tasks and a behavioral measure of anxiety. The graphics plot the mean percentage of time spent in the open arms by rats given a 5-min test in the elevated plus-maze against the percentage of BDZ-like immunoreactivities levels with respect to the control. Immediately after being tested in the plus-maze or killed for biochemical determinations, rats were submitted to the same task described in Fig. 1. (A). Amygdala; $n = 38$, $r = 0.77$, $p < 0.0005$. (B). Hippocampus $n = 44$, $r = 0.68$, $p < 0.0005$. (C). Septum; $n = 43$, $r = 0.57$, $p < 0.005$.

Total arm entries are reduced both in the group exposed to the inhibitory avoidance training procedure and in rats submitted to seven sessions of exploration, $F(4, 82) = 18$, $p < 0.001$. Analysis of covariance (ANCOVA) showed that the reduction in open arm entries observed in the group exposed to seven sessions of exploration was quite independent of any reduction in closed arm entries, $F(1, 35) = 79$, $p < 0.001$. In addition, ANCOVA showed that the reduction in time spent in the open arms in both groups was quite independent of any reduction in the total arm entries, $F(2, 27) = 7.1$, $p < 0.005$. The number of rearings was lower both in the group of seven sessions of exploration and in the one exposed to foot-shock (inhibitory avoidance) than in all others. The number of fecal boluses decreased only in the two "acute" groups: group 2 (one session of exploration) and group 5 (one session of inhibitory avoidance training).

The effect of one or seven daily exploration sessions on brain regional BDZ-like immunoreactivity is shown in Fig. 2. Values were significantly lower than those of controls in hippocampus, amygdala, and medial septum. The decrease of BDZ-like immunoreactivity in these structures was higher after seven than after one exploration sessions. No significant changes were observed in cerebral cortex and cerebellum. The results from the other two experimental groups have been recently published [(29), see the Discussion Section]. No changes were observed in BDZ-receptor binding in groups 2, 3, or 4 with respect to control (data not shown).

DISCUSSION

The results show that all behavioral treatments studied were anxiogenic to some degree. Further, in the habituation task the time spent in the open arms decreased with the number of sessions. In addition, in the group exposed to seven exploration sessions the number of entries into the open arms was lower than in any other group. This measure is considered less sensitive to anxiogenic variables than the time spent in the open arms (22). All this suggests that, for this task, anxiogenicity increases with repetition, indeed contrary to what is generally assumed (12). In fact, measures of anxiogenicity (entries and time spent in the open arms) were higher in the group exposed to seven sessions of exploration than in animals exposed to the inhibitory training procedure, which involves a foot-shock. It is likely that, for a rat, being repeatedly handled by a human may constitute a highly anxiogenic or even stressful procedure. Furthermore, recent studies demonstrated that ten days of handling produces opposite effects compared to BDZs in an active avoidance paradigm (8), thus indicating that handling may be seen as a kind of daily restraint stress. On the other hand, it has been repeatedly shown that gentle handling has anxiolytic effects [see (2)]. It should be taken into account that differences in handling procedures, individual emotionality or reactivity to the behavioral paradigms, and baseline anxiety levels of animals could explain these discrepancies (2,8,9,16). In this context, it is important to mention that repeated exposure to mild aversive stimuli can lead to a reduction or increase of the animal's anxious responses depending upon the parameters of the procedure (16). Then, it is tempting to suggest that our results could be due to these parametric factors. Nevertheless, only parametrical research using different procedures of handling, as well as different time intervals between sessions of exploration, will clarify this point.

No changes were observed in BDZ-receptor binding in any of the groups studied except a mild increase in B_{max} observed in the inhibitory avoidance-trained rats [(29) and unpublished observations]. On the other hand, one or seven daily sessions of habituation procedures caused a decrease of BDZ-like immunoreactivity in medial septum, amygdala, and hippocampus. These findings are similar to those reported elsewhere (29) for the 2-day habituation and the inhibitory avoidance procedure (i.e., in groups similar to groups 3 and 5 of the present study). Taking this into consideration, we pooled the BDZ-like immunoreactivity data of these two groups with the data of Wolfman et al. (29) and studied their correlation with the percentage of time spent in the open arm of the elevated plus-maze described above. The results of this analysis are shown in Fig. 3. If one considers the decreases of BDZ-like immunoreactivity described here and by Wolfman et al. (29) as reflecting the release of BDZ-like molecules (13), the correlation data support the hypothesis that BDZ-like molecules are released in the brain (23,27), specifically in medial septum, hippocampus, and amygdala (29), in proportion to the degree of anxiety associated with each behavioral procedure (13).

The low amounts of BDZ-like molecules found in selected brain regions precluded their chemical identification. Although we and others have tested the crossreactivity of MAb 21-7F9 with a large series of compounds [(5) and unpublished observations], it cannot be totally ruled out that compounds recognized by this monoclonal antibody correspond to molecules not structurally related to authentic BDZs.

The findings further support the hypothesis that the BDZ-$GABA_A$ receptor complex plays a pivotal role in the biochemistry and physiology of stress and anxiety (2,6,7,14,19,26, 27,29).

The behavioral paradigms studied here, in addition to generating a level of anxiety, are also learning paradigms used in the study of habituation and inhibitory avoidance (13, 29). The BDZ-$GABA_A$ receptor complex in the amygdala, medial septum, and hippocampus plays a role in the modulation of the memory for habituation and inhibitory avoidance, as ascertained by the effect of the local infusion of diverse agonists and antagonists at these receptor sites and by ligands of the GABA-gated Cl^- channel (3,4,11-13,17,29). Microinjection of the BDZ receptor antagonist, flumazenil, into the amygdala, medial septum, and hippocampus immediately after training in the habituation and inhibitory avoidance task used here enhances retention of these two tasks (29), which strengthens the suggestion that these behaviors do release BDZ-like substances in the three brain structures (13).

In conclusion, the decrease in regional brain levels of BDZ-like molecules seen after the various behavioral procedures probably represents a general response of the limbic system to anxiety and/or stress in relation to the level of anxiety and/or stress that accompany these tasks.

ACKNOWLEDGEMENTS

The authors especially thank Dr. A. De Blas for kindly providing the monoclonal antibody against BDZ. They also thank M. G. Burstein and E. Kornisiuk for their technical assistance. This research was supported by grants from CONICET (Argentina), FAPERGS and CNPq (Brazil), and IFS (Sweden).

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