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The Anxiolytic Action of Benzodiazepines is not Present in Handling-Habituated Rats

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BOIX, F., A. FERNÁNDEZ TERUEL AND A. TOBEÑA. *The anxiolytic action of benzodiazepines is not present in handling-habituated rats.* PHARMACOL BIOCHEM BEHAV 31(3) 541–546, 1988.—The acquisition of two-way shuttle avoidance (40 first trials) was used to test the anxiolytic activity of diazepam (2 and 4 mg/kg) and alprazolam (1.25 mg/kg) vs. vehicle, IP, in rats. These rats had received three different previous treatments: acute, acute with previous handling habituation for 15 days, and handling habituation combined with chronic treatment for 15 days. Results of the acute treatment showed a comparable anxiolytic action of diazepam and alprazolam, reflected by an improvement in avoidance acquisition. After handling habituation, no effect on shuttle box acquisition was obtained in rats acutely treated with diazepam, whereas the alprazolam-treated group showed a significantly impaired avoidance performance. When handling habituation was combined with chronic benzodiazepine treatment, the drugs' anxiolytic action persisted although there was a complete disappearance of their sedative effects. These behavioral results are discussed in relation to the emotional changes induced by the procedures of handling. They are tentatively linked with possible changes in the functionality of GABA neurotransmission, possibly at the level of the GABA-benzodiazepine receptor which some studies have found associated to handling habituation.

Diazepam Alprazolam Handling habituation Chronic treatment Two-way avoidance acquisition GABA Anxiety Rats

IT is well known that when laboratory animals are handled for the first time they usually react with attempts to escape and show defensive emotional responses. With repeated manipulations the resistance declines, and the intensity of the emotional reaction subsides. Classical behavioral studies have shown that repeated postnatal handling reduces emotionality lasting to adulthood. Postnatal handling produces an increase in open field exploratory behavior (13) and improves performance in two-way active avoidance (23). It has also been reported that the corticosterone response to stress may be decreased by the previous habituation to several steps of the experimental procedure (15,27).

Some workers have reported that the stress of acute handling, as well as other types of stress, modifies the functioning of the GABA-benzodiazepine receptor complex. Acute handling produces a decrease in the density of the low affinity GABA receptors (4,9), as well as a reduction of the $^{36}Cl^-$ efflux and uptake (2) in the cerebral cortex. These changes do not occur in animals previously habituated to handling, but only arise in animals nonhabituated to handling (naive). These changes can be modulated, in different ways, by agonists or antagonists of the benzodiazepine receptor (3,8). It has been postulated that those differences in GABA transmission between handling-habituated and naive rats, might reflect their particular emotional state in distinct situations (9), and also may regulate the animal's neurochemical responses to stress (4). In line with all those findings, the strains of rats derived through selective breeding to show low fearfulness (the Maudsley Non Reactive or the Roman High Avoidance) do have a higher ^3H-diazepam binding than the corresponding more emotional strains (the Maudsley Reactive and Roman Low Avoidance) (17,30). These differences have also been found in mice (29).

Overall, there seems to be a parallel between the changes in functionality of GABA-benzodiazepine receptor complex, and the behavioral responses shown by animals in stressful situations, when animal's fearfulness has been manipulated either by handling habituation or by selective breeding. Furthermore, since the GABA-benzodiazepine receptor complex has been established as the site of action of benzodiazepines (26,34), and seems to mediate the anxiolytic effect of these drugs (24), it could be predicted that the anxiolytic effect of benzodiazepines might change depending on the particular emotional state induced in animals.

In two-way shuttle active avoidance, it has been repeatedly reported that the acute administration of benzodiazepines improves performance, mainly during early acquisition (1, 5, 18, 19, 31, 32). This enhancement has been

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explained as the consequence of the reduction of freezing behavior, which appears at the early stages of acquisition in this task (19,33). The same model has also been used to derive lines of anxious and nonanxious rats through selective breeding, the Roman Low and High Avoidance strains respectively (7,14). And several studies have found that the anxiolytic drug effects on shuttle box avoidance acquisition are more prominent in highly emotional or in poor avoiding rats (11,23). If chronic habituation to handling is able to produce consistent changes in the emotional state of animals, we hypothesized that the effects of benzodiazepine treatment on shuttle box acquisition might change depending on the particular handling procedure administered to animals.

In the present study, three experiments were designed in order to ascertain the effects of two benzodiazepines, diazepam and alprazolam, on two-way active avoidance acquisition, after different manipulations of the animals and administration procedures. In Experiment 1, the anxiolytic action of acute benzodiazepines on two-way avoidance acquisition was tested in naive, nonhabituated rats. In Experiment 2, the anxiolytic effect of acute benzodiazepines in the same test was examined after exposing the animals to chronic habituation of handling. In Experiment 3, the effect of chronic benzodiazepine treatment, administered concomitantly with the handling-habituation procedure, was tested.

METHOD

Subjects

Male Sprague-Dawley rats, 8 weeks old (weight 280–290 g), derived from OFA.SD (France) were used. They were housed in groups of four per cage, with food and water freely available, with a 12 hr light-dark cycle (7:00 a.m. on), and controlled temperature (22±1°C). Before the experiments began, they passed a one-week period of adaptation to the new environment. In each one of the three experiments described below, 48 rats were randomly divided into four groups of 12 animals: control (vehicle), diazepam 2 mg/kg, diazepam 4 mg/kg and alprazolam 1.25 mg/kg.

Shuttlebox Acquisition Session

The session was performed in a two-way Lafayette Co. shuttle-avoidance box of 62×21×19 cm, divided into two equal compartments by a 8.5×8.5 cm door. Each session included 10 min of habituation, immediately followed by a series of 40 trials of avoidance acquisition. Each trial consisted of 10 seconds of CS (light and tone simultaneously) followed by 30 seconds of US (electric shock of 0.4 mA). CS or US were finished when the animal crossed to the other compartment, being considered as an avoidance response in the first case and an escape response in the other. Once the shock was over or a response had been made, a 50 second rest period was presented. Total duration of the session was around 50 min. Crossings during the habituation period were used as a measure of spontaneous activity. Rats with more than three trials without escape were discarded.

Procedure

Three different experiments were carried out.

Experiment 1. Acute: Animals were weighed daily for 15 days. On the last day, they received a single IP injection and 30 min afterwards were submitted to the shuttlebox acquisition session as described above.

Experiment 2. Acute + handling habituation: Rats were handled for 15 consecutive days. The handling-habituation procedure was similar to the one described by Corda *et al.* (10). It consisted of daily weighing and IP injection of vehicle, followed by a forced introduction of the animal's head through the blades of a guillotine for animal sacrifice, this second step lasting about 15 seconds. The animals were brought back to their home cage immediately afterwards. The guillotine step was introduced in order to have an identical situation to other biochemical experiments which are currently being performed. On the last day, rats received a final IP injection with the drug, and 30 min afterwards, animals were tested in the shuttlebox.

Experiment 3. Chronic + handling habituation: Rats were treated IP once daily with the corresponding drug or vehicle for 15 days, followed by handling habituation as explained above. On the 15th day, 30 min after the drug's administration, rats were submitted to the shuttlebox session. In order to have independent measures of the animal's emotional state during the handling-habituation procedure, vocalizations and the active attempts to remove the head from the guillotine (guillotine struggling) were measured in the control group of this experiment throughout the handling-habituation procedure. Daily presence (counted as 1) or absence (counted as 0) was scored for the two measures in each rat.

Drugs

Diazepam (Roche, S.A.) and alprazolam (Upjohn Farmoquímica, S.A.) were suspended in 1% carboxymethyl cellulose, and administered IP in 1 ml/kg of body weight. Control rats received vehicle at the same volume.

Statistics

As Kolmogorov-Smirnov tests showed that measures from the two-way active avoidance sessions were normally distributed, data were analyzed with parametric tests. One-way analysis of variance (ANOVA) was applied, and Duncan's Multiple Range tests were used for post hoc comparisons between groups. Data from the handling-habituation procedure, vocalizations and guillotine struggling, were analyzed using Friedman ANOVA nonparametric tests for repeated measures.

RESULTS

Table 1 shows that, compared to the control group, acute treatment (Experiment 1) with diazepam, at both doses (2 and 4 mg/kg), or alprazolam (1.25 mg/kg), improved performance on two-way shuttle avoidance acquisition. There were overall significant differences in the total number of avoidances, $F(3,35)=8.013$, $p<0.001$, as well as in the total mean of latencies, $F(3,35)=3.701$, $p<0.02$, reflecting the different performances between drug-treated animals and controls. The differences were found despite the clear sedation observed in the habituation crossings measure, $F(3,35)=5.935$, $p<0.003$, in drug-treated rats. Duncan's comparisons between groups are also reported in Table 1. The aforementioned enhancement of avoidance acquisition occurred mainly at the early blocks of acquisition. Pairwise Duncan's test comparisons showed significant differences in response latencies (Fig. 1), between diazepam- (at both doses) treated rats and controls at the first 10 trials. Diazepam and alprazolam-treated rats were also significantly different from control group at 20 trials. Those significant differences disappeared at the 30 trials and afterwards, although drug-

TABLE 1

TWO-WAY SHUTTLE AVOIDANCE PERFORMANCE, 30 MIN AFTER AN ACUTE TREATMENT WITH DIAZEPAM OR ALPRAZOLAM

	Habituation Crossings		Total Avoidances		Total Mean Latency		
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	n
Control	27.89	1.98	13.11	2.49	8.77	0.53	9
Diazepam 2 mg/kg	16.33	3.29*	22.78	2.50*	6.88	0.57*	9
Diazepam 4 mg/kg	6.40	3.30*	27.80	1.78*	6.23	0.56*	10
Alprazolam	9.63	5.21*	23.73	1.90*	7.04	0.53*	11

*$p<0.05$, significant difference from control group (Duncan's test).

TABLE 2

TWO-WAY SHUTTLE AVOIDANCE PERFORMANCE 30 MIN AFTER AN ACUTE TREATMENT WITH DIAZEPAM OR ALPRAZOLAM, PRECEDED BY HABITUATION TO HANDLING FOR 15 DAYS

	Habituation Crossings		Total Avoidances		Total Mean Latency		
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	n
Control	26.00	2.41	22.20	1.76‡	6.61	0.38‡	10
Diazepam 2 mg/kg	23.27	4.19	21.54	2.54	6.97	0.54‡	11
Diazepam 4 mg/kg	11.54	3.73*†	24.27	1.96‡	6.88	0.42‡	11
Alprazolam	7.80	2.15*†	14.90	2.92	8.64	0.53	10

*$p<0.05$, significant difference from control group (Duncan's test).
†$p<0.05$, significant difference from diazepam 2 group (Duncan's test).
‡$p<0.05$, significant difference from alprazolam group (Duncan's test).

treated groups continued performing better than controls. The same Duncan's comparisons for avoidances offered similar results (data not shown).

In Experiment 2 (Table 2), the anxiolytic effect of acute benzodiazepines was not observed in rats who had received handling habituation. The group treated with alprazolam presented a significantly lower performance than the other groups, both in avoidance, $F(3,38)=2.940$, $p<0.05$, and in latencies, $F(3,38)=3.602$, $p<0.025$. As in Experiment 1, a sedative action of both drugs was observed in habituation crossings, $F(3,38)=6.961$, $p<0.001$, although diazepam 2 mg/kg group did not show this sedative effect when a Duncan's test between groups was applied.

When chronic benzodiazepine treatment was combined with handling habituation (Experiment 3), between groups ANOVAs did not yield significant differences, neither in the number of avoidances nor in the total mean latency. However, as is shown in Table 3, the performance of diazepam 4 mg/kg group was improved in both measures, when compared with control group (Duncan's test, $p<0.05$). No sedative effect appeared, as measured by habituation crossings, $F(3,41)=0.896$, $p=0.4515$.

Vocalizations and guillotine strugglings were grouped in blocks of 5 days (Fig. 2). Analysis showed that at the end of the handling habituation procedure rats were less reactive. There was a significant reduction in the mean number of

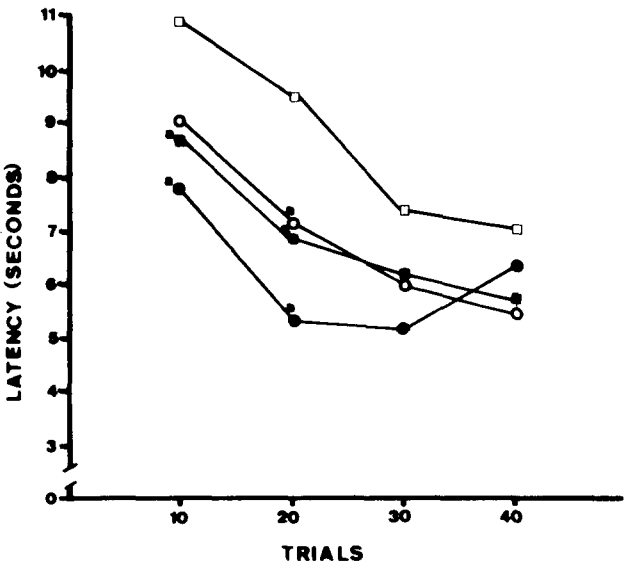

FIG. 1. Mean latencies, in seconds, of each block of 10 trials during the shuttlebox session after an acute treatment with benzodiazepines 30 min before. a $p<0.05$, significant difference from control group (Duncan test). □ Control, ■ diazepam 2 mg/kg, ● diazepam 4 mg/kg, ○ alprazolam.

TABLE 3

TWO-WAY SHUTTLE PERFORMANCE 30 MIN AFTER THE LAST ADMINISTRATION OF A CHRONIC TREATMENT WITH DIAZEPAM OR ALPRAZOLAM FOR 15 DAYS, COMBINED WITH HABITUATION TO HANDLING

	Habituation Crossings		Total Avoidances		Total Mean Latency		
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	n
Control	23.50	2.56	18.08	2.21	7.79	0.56	12
Diazepam 2 mg/kg	29.72	4.15	19.81	2.69	7.27	0.64	11
Diazepam 4 mg/kg	20.10	5.46	26.20	1.85*	5.92	0.40*	10
Alprazolam	22.64	4.80	23.08	2.38	6.73	0.59	11

*$p<0.05$, significant difference from control group (Duncan's test).

vocalizations ($p<0.01$, Friedman ANOVA) or strugglings ($p<0.02$, Friedman ANOVA), in the last five days when compared with the first five days.

DISCUSSION

The present study showed that animals can be easily and effectively habituated to daily handling. The results obtained with vocalizations and guillotine strugglings confirm that our handling-habituation procedure was effective in reducing the intensity of the animal's anxious responses induced by forced manipulation. Comparable behavioral measures have been used in other anxiety-provoking situations (unavoidable electric shocks or immobilization) in order to score the evolution and the intensity of stress-anxiety responses (36,37).

Benzodiazepines release behaviors that were previously inhibited by punishment. This has been mainly shown in conflict-punishment procedures and passive avoidance tasks, where benzodiazepines impair the suppression of ongoing behaviors (33). Such releasing effects in animals are related to the anxiolytic action in humans (19, 22, 33).

Gray (18,19) proposed that in the acquisition of two-way active avoidance, the same releasing effect of benzodiazepines should produce an enhanced performance. The improvement should be more prominent at the early stages of acquisition, when the tendency to behavioral suppression is maximal and overrides a competing tendency to actively cross between compartments (38). In this study the acute treatment with diazepam and alprazolam (without previous handling habituation) improved the overall avoidance performance, the enhancement being maximal at the initial blocks of the acquisition session. As the session advanced, differences between treated and control groups disappeared progressively until they eventually vanished. These results support Gray's hypothesis and confirm previous data (1, 5, 18, 19, 31, 32).

In addition, the benzodiazepine effect on shuttlebox acquisition can be considered an anxiolytic action for several reasons. Firstly, because it arose at relatively low doses despite a significant sedative effect. Secondly, it paralleled the differences observed in shuttlebox acquisition between selectively bred strains of fearful and nonfearful rats (6,20), and also between male and female rats (20). Finally, it mirrors the action of environmental manipulations such as early handling, which has been shown to reduce emotionality and improve shuttlebox avoidance acquisition (23).

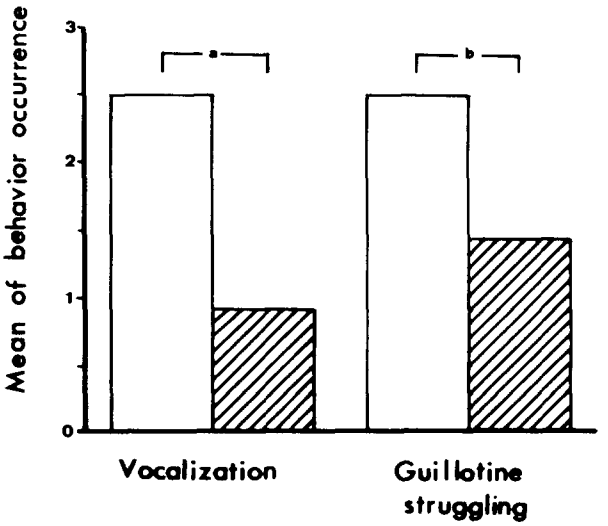

FIG. 2. Mean of occurrence of vocalization or attempt to remove the head from the guillotine (guillotine struggling) in the first or the last five days of handling habituation, in the control group of Experiment C. a $p<0.01$, Friedman two-way ANOVA. b $p<0.02$, Friedman two-way ANOVA. Open columns: first 5 days, striped column: last 5 days.

Therefore, it can be inferred from the present and previous evidence that shuttlebox two-way avoidance acquisition can be considered a valid animal model of anxiety. The unreliable results sometimes obtained with benzodiazepines in this task (35) mainly arise because either drug testing is carried out during overtraining procedures, or the drug actions are assessed over long acquisition periods. Such procedures may mask an effect which is more prominent at the start of acquisition.

The results of Experiment 2 showed that when handling habituation was followed by acute treatment with diazepam or alprazolam no antianxiety action was present on the behavioral test used. Actually, the alprazolam-treated group had a significantly lower performance than controls. Perhaps this particular action of alprazolam may be related to the

special biochemical and clinical properties which have been reported with this benzodiazepine (12). Since at the end of the handling-habituation procedure, our rats can be considered less fearful animals, these results support other evidence showing that benzodiazepines improve shuttlebox avoidance especially in poor avoiders, but not in good avoiders (11). Moreover, sodium amobarbital, administered in anxiolytic doses, improved shuttle acquisition in highly emotional rats, but not in low emotional rats (25,28). The absence of action of benzodiazepines after handling habituation needs replication in other animal models of anxiety with similar or even better face validity, such as the punishment conflict procedures, or in the social interaction test.

Several studies (2–4, 8, 10) have reported that handling-habituated rats show higher densities of $GABA_A$ receptors than nonhandling-habituated rats. Corda and Biggio (9) have explained these differences as an enhancement in GABA function probably linked with a decrease in the emotional state of habituated animals. The absence of the anxiolytic effect of benzodiazepines observed after handling habituation in Experiment 2 could be related to possible changes in the functionality of GABA neurotransmission, since the magnitude of benzodiazepines action depends on the functionality of GABA system at the moment of the drug action (21). It has been shown that the dose-response curve of GABA function presents a maximum level (21). Therefore, benzodiazepines' ability to potentiate GABA responses should be lower if GABA functionality is enhanced in handling-habituated rats. From the present behavioral data we cannot conclude whether possible changes in GABA receptor complex, after handling habituation, could account for our results. These are "post hoc" explanations which need confirmation in further studies combining behavioral experiments with direct measures of the GABA-benzodiazepine receptor function. It is necessary to establish the exact nature of the emotional changes induced by the handling-habituation procedure, as well as to clarify the associated biochemical mechanisms.

On the other hand, in Experiment 3, a complete absence of sedative action was obtained after chronic treatment with the two benzodiazepines tested. This is in agreement with many previous reports demonstrating that the sedative effect of benzodiazepines disappears when they are chronically administered [see (16) for an extensive review]. With the chronic administration used here, the higher dose of diazepam (4 mg/kg) showed a significant antianxiety action, as reflected by an improved performance in two-way avoidance acquisition. The other two drug-treated groups did not differ from controls. In this experiment, we have used a daily combination of chronic administration of drugs and handling habituation. For this reason it is difficult to ascertain the relative importance of these two procedures in the observed effects. Moreover, the repeated administration of daily effective doses of benzodiazepines may prevent any effect of handling-habituation procedures.

However, it seems worth noting that some of the effects observed in Experiment 2 (i.e., the absence of anxiolytic action after acute benzodiazepines when animals were previously habituated to handling) were not obtained when handling habituation was combined with chronic drug administration. In fact, for alprazolam and diazepam (4 mg/kg), very different actions appear in the three experimental situations studied here. This is particularly clear when these two groups are compared with their corresponding controls. Nevertheless, from the present design, it remains unclear whether the results of Experiment 3 have arisen due to chronic benzodiazepine treatment alone or handling habituation alone or from the combination of both. Perhaps underlying the usually very controversial results reported in the literature about the persistence of the antianxiety action of benzodiazepines after chronic treatments (16), there may be masked interactions between chronic administration of the drugs and handling effects.

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