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# Gabaergic–Benzodiazepine System is Involved in the Crotoxin-Induced Anxiogenic Effect

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MOREIRA, E. G., N. NASCIMENTO, J. R. ROGERO AND V. S. VASSILIEFF. *Gabaergic–benzodiazepine system is involved in the crotoxin-induced anxiogenic effect.* PHARMACOL BIOCHEM BEHAV **65**(1) 7–13, 2000.—The behavioral effects of crotoxin (CTX), the major component of *Crotalus durissus terrificus* venom, were studied in rats submitted to the open field, holeboard, and social interaction tests. CTX (100, 250, and 500 μg/kg, IP) was administered 2 h before the tests. In the open field, CTX reduced ambulation (250 μg/kg) and rearing (250 and 500 μg/kg) and increased grooming (100 and 250 μg/kg) and freezing (250 μg/kg). In the holeboard and social interaction, all the CTX doses evaluated decreased, respectively, head dip and head dipping, and social interaction time. The CTX-induced behavioral alterations could be attributed to its neuromuscular transmission blockade, but this possibility was ruled out because CTX (250 and 500 μg/kg, IP, 2 h before the rotarod test) was unable to modify the rotarod performance of rats. The involvement of the benzodiazepine receptor in the CTX-induced behavioral alterations was investigated through the pretreatment (30 min before the tests, IP) of the animals with diazepam (1.2 mg/kg), or flumazenil (4 and 10 mg/kg). Both diazepam and flumazenil antagonized the CTX-induced behavioral alterations in the open field, holeboard, and social interaction tests. This study demonstrated that: (1) CTX is an anxiogenic compound; and (2) the gabaergic–benzodiazepine system may play a role in the CTX-induced anxiogenic effect. © 1999 Elsevier Science Inc.

*Crotalus durissus terrificus* venom  Crotoxin  Anxiogenic compound  Anxiety  Exploration  Open field  Holeboard  Social interaction  Rotarod

SNAKE bites represent a serious health problem in many countries. The rattlesnake *Crotalus durissus terrificus* is responsible for about 10% of all snake bites occurring in Brazil (35), and its venom has been largely studied (2,3,27,41,48). Crotalid venom is highly toxic, and is composed of several toxins, such as crotoxin (CTX), crotamine, convulxin, and gyroxin (48). Considerable information on the biological effects of CTX is available, because this is the prevalent and most toxic component of crotalid venom (9,13,28,29,36,42,43,46,50).

CTX is a neurotoxin composed of a basic subunit, phospholipase $A_2$, and an acidic subunit, crotapotin (33). Its blockade effect on the transmission of nervous impulses at the neuromuscular junction has been extensively studied (7,12,26,47), and the proposed molecular mechanism of action involves pre- and postsynaptic mechanisms (6,11,31,48,49).

Regarding the central nervous system, the presence of high-affinity specific binding sites for CTX in the brain has been reported (15,34). Besides, CTX is able to reach the brain after being administered peripherally (10). These findings are suggestive of a CTX activity in the central nervous system.

Therefore, the aim of the present work was to investigate if CTX induced behavioral alterations in the open field, holeboard, and social-interaction tests, which are behavioral models that have been subjected to through critical appraisal to demonstrate central nervous system activity of drugs (18,20,37,40). Moreover, if CTX is able to induce behavioral alterations, it will be investigated possible mechanisms of action involved.

## EXPERIMENT 1

This first experiment was conducted to investigate if CTX acts on the central nervous system. For this purpose, CTX was

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evaluated in the open field, holeboard, and social-interaction tests.

*Method*

*Subjects.* One hundred seventy-two adult male Wistar rats were used, weighing 180–220 g. They were housed in groups of five, and maintained under a 12 L:12 D cycle (lights on: 0700 h) in a temperature-controlled environment (22 ± 2 °C) for at least 10 days prior to testing. Food and water were freely available except for the brief test sessions. Each rat was used only once.

*CTX purification.* CTX was purified from *Crotalus durissus terrificus* crude-air dried venom (Instituto Butantan, São Paulo, Brazil) by gel filtration on Sephadex G-75 (Pharmacia) followed by isoelectric pH precipitation. The Bradford method was used for protein determination, and purity was assessed by SDS-PAGE (9). CTX was lyophylized, and before using, it was dissolved in physiological saline solution.

*Behavioral tests.* The behavioral observations were blind, and carried out under low-intensity light.

*Open-field test.* The open-field apparatus was similar to that described by Broadhurst (8). Each animal was placed individually in the center of the arena and it was recorded, for 3 min, ambulation (count of floor units entered), rearing (count of times that the animal stood on its hind legs), grooming (time, in seconds, used for the animal to groom), and freezing (time, in seconds, that the animal remained immobile, often in a crouching posture, with eyes wide open and irregular respiration).

*Holeboard test.* The holeboard apparatus was an open-field arena with four equally spaced holes of 3 cm in diameter in the floor, as described by File and Wardill (23). Each rat was placed individually in the center of the arena and it was recorded, for 5 min, head dip count and head dipping duration, in seconds. A head dip was scored if both eyes disappeared into the hole.

*Social interaction test.* Rats were housed in pairs for 7 days prior to the test. The test consisted of familiarizing each pair (cagemates) of rats with the arena for a period of 8 min on 2 consecutive days. On the third day, each rat was randomly assigned to an unfamiliar partner according to weight, and each animal received the appropriate treatment. These rats were then returned to their home cage with their original cagemate until testing. After an appropriate pretreatment time, each pair of unfamiliar rats was placed in the arena and observed for social interaction behavior for 5 min. Social interaction time (in seconds) per pair of rats was measured as the time spent sniffing the partner, climbing over and crawling under the partner, mutual grooming, genital investigation, and following and walking around the partner (18). Aggressive behavior was not considered to be a social interaction behavior (18,21).

*Procedure.* CTX was administered intraperitoneally in the doses of 100, 250, or 500 μg/kg, which were chosen according to its $LD_{50}$ (1300 μg/kg). Control group received saline. The behavioral evaluation was carried out after a pretreatment time of 2 h.

*Results*

Data are summarized in Table 1. ANOVA demonstrated that CTX reduced ambulation, $F(3, 49) = 5.3$, $p < 0.01$, and rearing, $F(3, 49) = 8.91$, $p < 0.001$, whereas increased grooming, $F(3, 48) = 4.3$, $p < 0.01$, and freezing, $F(3, 48) = 3.49$, $p < 0.05$, in the open field test; reduced head dip, $F(3, 31) = 3.14$, $p < 0.05$, and head dipping, $F(3, 32) = 4.7$, $p < 0.01$, in the holeboard test, and decreased social-interaction time, $F(3, 43) = 6.97$, $p < 0.001$, in the social interaction test. Post hoc comparisons (Dunnett) revealed that CTX significantly modified the behaviors at the following doses: ambulation (250 μg/kg), rearing (250 and 500 μg/kg), grooming (100 and 250 μg/kg), freezing (250 μg/kg), head dip (100, 250, and 500 μg/kg), head dipping (100, 250, and 500 μg/kg), and social-interaction time (100, 250, and 500 μg/kg). These CTX-induced behavioral alterations are characteristics of anxiogenic drugs.

Because the dose of 250 μg/kg was efficient to alter all the behaviors evaluated, it was chosen to continue the study of possible mechanisms involved in the CTX-induced behavioral effects.

## EXPERIMENT 2

Considering that CTX is a neurotoxin that blocks the neuromuscular junction, one could suppose that the CTX-

TABLE 1

EFFECT OF CTX ON THE BEHAVIOR OF RATS IN THE OPEN FIELD, HOLEBOARD, AND SOCIAL-INTERACTION TESTS

| | CTX (μg/kg, IP) | | | |
|---|---|---|---|---|
| | Saline | 100 | 250 | 500 |
| Open field | | | | |
| Ambulation (count) | 65.8 ± 2.8 (12) | 58.0 ± 3.1 (12) | 49.3 ± 2.7* (12) | 59.6 ± 2.9 (14) |
| Rearing (count) | 24.9 ± 1.5 (12) | 19.8 ± 1.7 (12) | 16.2 ± 1.5* (12) | 14.9 ± 1.4* (14) |
| Grooming (s) | 12.3 ± 0.9 (12) | 20.8 ± 1.9* (11) | 18.3 ± 1.2† (13) | 16.8 ± 2.3 (13) |
| Freezing (s) | 2.8 ± 0.8 (12) | 4.4 ± 1.2 (12) | 7.4 ± 1.5† (11) | 2.9 ± 0.9 (14) |
| Holeboard | | | | |
| Head dip (count) | 10.4 ± 1.9 (8) | 5.4 ± 1.3† (7) | 6.3 ± 0.9† (9) | 5.6 ± 0.8† (8) |
| Head dipping (seconds) | 12.4 ± 1.9 (8) | 5.5 ± 0.9† (8) | 6.8 ± 1.3† (9) | 6.5 ± 1.2† (8) |
| Social interaction | | | | |
| Social-interaction time (s) | 51.6 ± 4.4 (11) | 32.6 ± 3.7* (10) | 28.0 ± 3.6* (12) | 31.5 ± 4.4* (11) |

Data are presented as means ± SE. Pretreatment time was 2 h.
*$p < 0.01$ and †$p < 0.05$ compared with saline.

induced behavioral effects could be attributed, at least in part, to its neuromuscular effect. To test this hypothesis, CTX was evaluated in the rotarod test, which is a sensitive test to detect motor deficits (17,38).

*Method*

*Subjects.* The fifty-five animals used in this experiment had the same characteristics as those described for Experiment 1.

*Procedure.* Rats were trained on the rotarod (Ugo Basile, Italy) at 5 rpm for 2 min, three times on the day prior to the experiment. On the test day, rats that could not retain their balance on the bar at 5 rpm for 2 min were discarded to establish a more reproducible baseline (38). Rats were administered intraperitoneally with CTX (250: $n = 8$; 500: $n = 7$; 1000: $n = 8$; 1250 $n = 8$; 1500: $n = 7$ and 1600 μg/kg: $n = 10$) or saline ($n = 7$), and after 2 h they were placed on the bar and the times that the rats remained in the rotating rod were recorded. The time limit was fixed at 2 min.

*Results*

Data obtained from the rotarod test were analyzed with two different objectives. First, it was evaluated if the doses of 250 and 500 μg/kg of CTX would impair the rotarod performance of rats. As can be seen in Fig. 1, neither of these doses altered the rotarod performance of rats [ANOVA, $F(2, 22) = 0.19$, $p > 0.05$]. Second, the $ED_{50}$ of CTX to impair the performance of rats evaluated in this test was determined, using the probits analysis. The $ED_{50}$ was 1101.8 μg/kg, with the 95% confidence limits ranging from 736.9 to 1447.9 μg/kg. The correlation coefficient was 0.92. These data showed that the doses of CTX able to induce behavioral alterations did not cause motor deficits.

EXPERIMENT 3

Considering that CTX-induced behavioral alterations cannot be attributed to a motor deficit, and that they are typical of anxiogenic drugs, Experiment 3 was designed to evaluate if the GABAergic–benzodiazepine system, which plays an important role in the anxiety mechanisms, would be involved in the CTX behavioral effects. For this purpose, the influence of a benzodiazepine receptor agonist (diazepam: DZP) and an antagonist (flumazenil: FLU) on the CTX-induced behavioral alterations was investigated. However, prior to this investigation, DZP and FLU were evaluated by themselves in the open field, holeboard, and social-interaction tests to detect these drug effects under the used protocol and laboratory conditions.

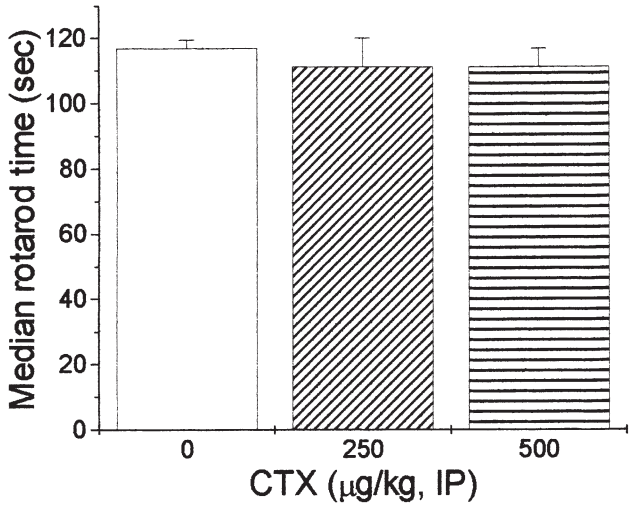


FIG. 1. Evaluation of CTX in the rotarod test. Rats were injected with saline ($n = 7$) or CTX in the doses of 250 ($n = 8$) or 500 μg/kg ($n = 7$), IP, 2 h before the rotarod test. Data are means ± SE. Dose zero corresponds to control. (ANOVA, $p > 0.05$).

*Method*

*Subjects.* The animals used in this experiment had the same characteristics as those described in Experiment 1.

*Drugs.* CTX was prepared as described in Experiment 1. DZP (Sanofi, Brazil) was diluted in propyleneglycol (Sigma, St. Louis, MO) 40% v/v. FLU (Roche, Brazil) was ultrasonically dispersed in distilled water to which two drops of Tween 80/10 ml had been added.

*Behavioral tests.* The behavioral tests had the same characteristics as those described for Experiment 1.

*Procedure.* Intraperitoneal route was used for all treatments. CTX was administered in the dose of 250 μg/kg; DZP in the dose of 1.2 mg/kg, and FLU in the doses of 4 and 10 mg/kg. Control groups received the appropriate vehicle, which were: saline for CTX, propyleneglycol 40% for DZP and distilled water with drops of Tween 80 for FLU.

*Behavioral evaluation of DZP.* Fifty-five rats were injected with saline, and after 1.5 h, with DZP or its vehicle. The behavioral evaluation was performed after a further 30 min.

*Behavioral evaluation of FLU.* Seventy-one rats were injected with saline, and after 1.5 h, with FLU or its vehicle. The behavioral evaluation was performed after a further 30 min.

*Effects of DZP on the CTX-induced behavioral alterations.* One hundred fifteen rats were injected with CTX or saline, and after 1.5 h, with DZP or its vehicle. The behavioral evaluation was performed after a further 30 min.

*Effects of FLU on the CTX-induced behavioral alterations.* One hundred sixteen rats were injected with CTX or saline, and after 1.5 h, with FLU or its vehicle. The behavioral evaluation was performed after a further 30 min.

*Results*

*Behavioral evaluation of DZP.* Student's *t*-test demonstrated that DZP increased ambulation, $t(16) = 2.84$, $p < 0.05$, head dip, $t(11) = 2.31$, $p < 0.05$, head dipping, $t(11) = 2.9$, $p < 0.05$, and social-interaction time, $t(10) = 2.53$, $p < 0.05$. Rearing, $t(16) = 1.24$, $p > 0.05$, grooming, $t(16) = 0.51$, $p > 0.05$, and freezing, $t(16) = 1.47$, $p > 0.05$, were not altered (Table 2).

*Behavioral evaluation of FLU.* FLU did not induce alterations in ambulation, $F(2, 18) = 0.72$, $p > 0.05$, rearing, $F(2, 18) = 2.74$, $p > 0.05$, grooming, $F(2, 18) = 0.72$, $p > 0.05$, freezing, $F(2, 18) = 2.28$, $p > 0.05$, head dip, $F(2, 17) = 1.8$, $p > 0.05$, head dipping, $F(2, 17) = 0.5$, $p > 0.05$, and social-interaction time, $F(2, 12) = 0.53$, $p > 0.05$ (Table 3).

*Effects of DZP on the CTX-induced behavioral alterations.* Data are summarized in Figs. 2A, 3A, and 4A. ANOVA demonstrated an overall effect of the treatments in the ambulation, $F(2, 26) = 10.1$, $p < 0.001$, rearing, $F(2, 25) = 8.05$, $p < 0.01$, grooming, $F(2, 26) = 29.59$, $p < 0.001$, freezing, $F(2, 24) = 8.42$, $p < 0.01$, head dip, $F(2, 24) = 4.06$, $p < 0.05$, head dipping, $F(2, 24) = 4.56$, $p < 0.05$, and in the social-interaction time, $F(2, 28) = 15.75$, $p < 0.001$. Post hoc comparisons

TABLE 2

EFFECT OF DIAZEPAM ON THE BEHAVIOR OF RATS IN THE OPEN FIELD, HOLEBOARD, AND SOCIAL-INTERACTION TESTS

| | SAL + VEH | SAL + DZP |
|---|---|---|
| Open field | ($n$ = 8) | ($n$ = 10) |
| Ambulation (count) | 66.9 ± 4.8 | 99.3 ± 9.4* |
| Rearing (count) | 23.9 ± 2.7 | 29.2 ± 3.2 |
| Grooming (s) | 8.6 ± 1.7 | 7.3 ± 1.8 |
| Freezing (s) | 1.9 ± 1.0 | 0.5 ± 0.2 |
| Holeboard | ($n$ = 6) | ($n$ = 7) |
| Head dip (count) | 6.3 ± 0.5 | 10.9 ± 1.8* |
| Head dipping (s) | 8.3 ± 1.6 | 17.5 ± 2.6* |
| Social interaction | | |
| Social-interaction time (s) | 68.4 ± 12.6 | 115.2 ± 12.8* |

Data are means ± SE. SAL: saline; VEH: propyleneglycol 40%; DZP: diazepam 1.2 mg/kg. All drugs were administered IP. Pretreatment time for SAL was 2 h and for VEH and DZP 30 min.

*$p < 0.05$ compared with SAL + VEH.

(Tukey) revealed that CTX (250 μg/kg) reduced ambulation, rearing (Fig. 2A), head dip, head dipping (Fig. 3A), and social-interaction time (Fig. 4A), whereas it increased grooming and freezing (Fig. 2A). An anxiolytic dose of DZP (1.2 mg/kg) was able to reverse CTX-induced alterations of the ambulation, rearing, grooming, freezing (Fig. 2A), head dipping (Fig. 3A), and social-interaction time (Fig. 4A), but it was unable to reverse the head dip alteration (Fig. 3A).

*Effects of FLU on the CTX-induced behavioral alterations.* The effects of FLU on the CTX-induced behavioral alterations are summarized in Figs. 2B, 3B, and 4B. ANOVA demonstrated an overall effect of the treatments in the ambulation, $F(3, 37) = 7.3$, $p < 0.001$, rearing, $F(3, 36) = 7.37$, $p < 0.001$, freezing, $F(3, 34) = 11.62$, $p < 0.001$, head dip, $F(3, 29) = 10.04$, $p < 0.001$, head dipping, $F(3, 29) = 7.08$, $p < 0.01$,and in the social-interaction time, $F(3, 23) = 7.12$, $p < 0.01$. Grooming was not altered, $F(3, 33) = 1.60$, $p > 0.05$. Post hoc comparisons (Tukey) revealed that CTX (250 μg/kg) reduced ambulation, rearing (Fig. 2B), head dip, head dipping (Fig. 3B), and social-interaction time (Fig. 4B), whereas it increased freezing (Fig. 2B). Both 4 and 10 mg/kg of FLU reversed the effects of CTX on ambulation, freezing (Fig. 2B), head dip, head dipping (Fig. 3B), and social-interaction time (Fig. 4B), but both of them were unable to reverse the rearing alteration (Fig. 2B).

## GENERAL DISCUSSION

The present findings demonstrated that CTX induced behavioral alterations in the open field, holeboard, and social-interaction tests.

The open field test is considered as an indicator of the emotional state of the animal (1), and is currently used in the screening of drugs that act on the central nervous system. CTX decreased ambulation and rearing and increased freezing and grooming. Ambulation and rearing can be respectively regarded as indicators of locomotor activity and exploratory behavior, whereas grooming and freezing are positively correlated with fear or emotionality (1,4,5,16,19,32). Thus, it is suggested that CTX increases the emotionality and decreases the exploratory activity of rats. Moreover, CTX also diminished the head dip and head dipping in the holeboard test, demonstrating its negative influence on the exploratory behavior (23). Because it has been reported that high emotionality inhibits exploration (1), the diminished exploratory behavior can be a consequence of the CTX-increased emotionality state.

CTX reduced the social-interaction time in the social interaction test, an animal model of anxiety (22). This behavioral alteration is consistent with an anxiogenic effect. Considering that anxiety is seen as a component of the emotional state (14), the CTX-induced anxiogenic effect is consistent with the increased emotionality state detected in the open field test.

A relevant aspect to be considered is that the CTX-induced behavioral alterations could be attributed to its blockade effect on the neuromuscular transmission. However, the evaluation of CTX on the rotarod test showed that the doses of CTX that induced behavioral alterations did not impair the motor coordination of the rats. Moreover, it was also shown that the $ED_{50}$ of CTX in the rotarod was almost 10-fold higher than the lowest dose of CTX able to induce behavioral alterations (100 μg/kg). These results rule out the possibility of the behavioral effects of CTX be attributed to its peripheral effect, and support the above

TABLE 3

EFFECT OF FLUMAZENIL ON THE BEHAVIOR OF RATS IN THE OPEN FIELD, HOLEBOARD, AND SOCIAL-INTERACTION TESTS

| | SAL + VEH* | SAL + $FLU_4$ | SAL + $FLU_{10}$ |
|---|---|---|---|
| Open field | ($n$ = 6) | ($n$ = 8) | ($n$ = 7) |
| Ambulation (count) | 75.0 ± 2.7 | 66.0 ± 4.5 | 74.9 ± 9.2 |
| Rearing (count) | 27.5 ± 1.5 | 18.4 ± 3.1 | 24.1 ± 3.1 |
| Grooming (s) | 11.1 ± 1.9 | 8.2 ± 1.4 | 9.1 ± 2.0 |
| Freezing (s) | 0.3 ± 0.3 | 1.7 ± 0.6 | 0.9 ± 0.5 |
| Holeboard | ($n$ = 6) | ($n$ = 7) | ($n$ = 7) |
| Head dip (count) | 9.7 ± 1.7 | 11.7 ± 1.7 | 7.9 ± 1.0 |
| Head dipping (s) | 13.8 ± 1.6 | 13.8 ± 2.4 | 11.5 ± 1.3 |
| Social interaction | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) |
| Social-interaction time (s) | 89.4 ± 10.3 | 80.4 ± 17.5 | 100.5 ± 12.9 |

Data are means ± SE. SAL: saline; VEH: propyleneglycol 40%; DZP: diazepam 1.2 mg/kg. All drugs were administered IP. Pretreatment time for SAL was 2 h and for VEH and DZP 30 min.

*$p < 0.05$ compared with SAL + VEH.

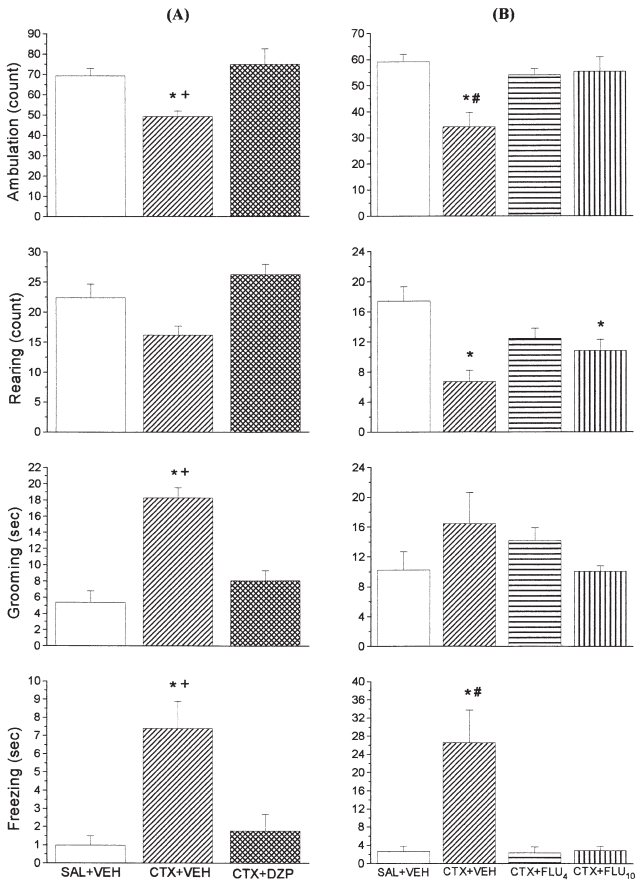


FIG. 2. Influence of diazepam (A) and flumazenil (B) on the behavioral effects induced by CTX in rats submitted to the open field test. Data are means ± SE. SAL: saline; VEH: propyleneglycol 40% or distilled water with drops of Tween 80; CTX: crotoxin 250 μg/kg; DZP: diazepam 1.2 mg/kg; $FLU_4$: flumazenil 4 mg/kg; $FLU_{10}$: flumazenil 10 mg/kg. All drugs were administered IP. Pretreatment time for CTX and SAL was 2 h and for VEH, DZP, and FLU was 30 min. $*p < 0.05$ compared with SAL+VEH; $+p < 0.05$ compared with CTX+DZP; $\#p < 0.05$ compared with CTX+$FLU_4$ and CTX+$FLU_{10}$.

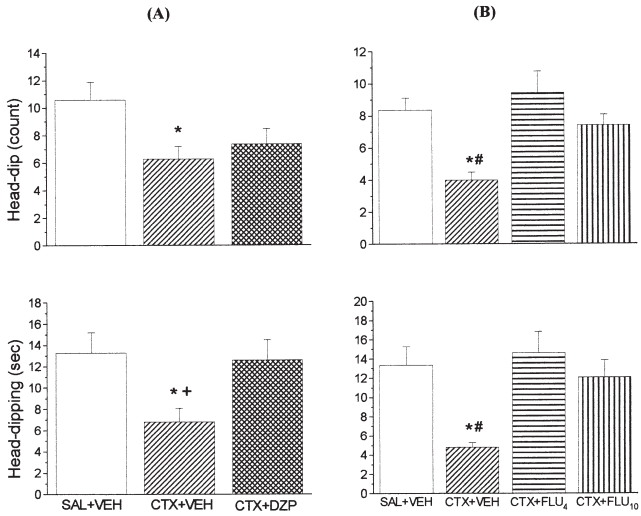


FIG. 3. Influence of diazepam (A) and flumazenil (B) on the behavioral effects induced by CTX in rats submitted to the holeboard test. Data are means ± SE. SAL: saline; VEH: propyleneglycol 40% or distilled water with drops of Tween 80; CTX: crotoxin 250 μg/kg; DZP: diazepam 1.2 mg/kg; $FLU_4$: flumazenil 4 mg/kg; $FLU_{10}$: flumazenil 10 mg/kg. All drugs were administered IP. Pretreatment time for CTX and SAL was 2 h and for VEH, DZP, and FLU was 30 min. $*p < 0.05$ compared with SAL+VEH; $+p < 0.05$ compared with CTX+DZP; $\#p < 0.05$ compared with CTX+$FLU_4$ and CTX+$FLU_{10}$.

suggestion that the CTX-induced behavioral alterations are due to an anxiogenic effect of this toxin.

It is important to point out that CTX presents a high molecular weight, which could prevent it from crossing the blood–brain barrier. However, biodistribution studies have demonstrated recovery of labeled CTX in brain tissue after intravenous administration (10). Moreover, the present results point out the ability of CTX to reach the central nervous system because neurobehavioral alterations are regarded as sensitive end points of drug-induced central effects. It is conceivable that the obtained neurobehavioral alterations may result from the binding of CTX to specific binding sites for CTX in the brain that have been already described with binding studies (15,34).

The CTX behavior profile was similar to that reported for benzodiazepine receptor inverse agonist (25,37,39). Inverse agonist binds to the benzodiazepine receptor, but have the opposite effect of the benzodiazepine agonist. Inverse agonist reduces the frequency of chloride ionophore opening, decreasing the inhibitory effects of γ-aminobutyrate (40). The effects of inverse agonist can be antagonized by both benzodiazepine receptor agonist and antagonist (24). The present findings have shown that diazepam treatment, a benzodiazepine agonist, reversed the CTX-induced behavioral alterations, suggesting an involvement of the GABAergic–benzodiazepine system in the behavioral effects of CTX. Nevertheless, because it has been shown that diazepam by itself induces behavioral effects as an anxiolytic drug (21,30,51), one cannot rule out a physiological antagonism between CTX and diazepam.

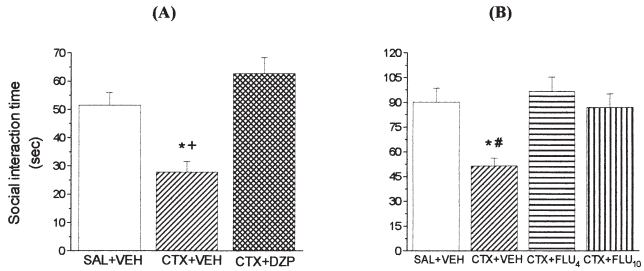


FIG. 4. Influence of diazepam (A) and flumazenil (B) on the behavioral effects induced by CTX in rats submitted to the social-interaction test. Data are means ± SE. SAL: saline; VEH: propyleneglycol 40% or distilled water with drops of Tween 80; CTX: crotoxin 250 μg/kg; DZP: diazepam 1.2 mg/kg; $FLU_4$: flumazenil 4 mg/kg; $FLU_{10}$: flumazenil 10 mg/kg. All drugs were administered IP. Pretreatment time for CTX and SAL was 2 h and for VEH, DZP, and FLU was 30 min. $*p < 0.05$ compared with SAL+VEH; $+p < 0.05$ compared with CTX+DZP; $\#p < 0.05$ compared with CTX+$FLU_4$ and CTX+$FLU_{10}$.

To determine if the benzodiazepine receptor participates in the CTX behavioral effects, flumazenil, a benzodiazepine selective antagonist (24), was used. Flumazenil lacked behavioral effects by itself, in accordance with several studies in the literature (21,44,45,51), but it was able to reverse the CTX-induced behavioral effects. It is widely accepted that the reversion of a drug effect by flumazenil supports its selective action on the benzodiazepine receptor (44,45,51). Therefore, the flumazenil result coupled with the diazepam result further supports the fact that CTX may be acting as a benzodiazepine inverse agonist.

In conclusion, this study demonstrated that: (1) CTX is an anxiogenic compound; and (2) the GABAergic–benzodiazepine system may play a role in the CTX-induced anxiogenic effect.

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