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Crotoxin-induced behavioral effects in rats

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Abstract

Crotoxin is the major component of *Crotalus durissus terrificus* venom. In view of the presence of high-affinity specific binding sites for crotoxin in the brain, the objective of this work was to investigate whether crotoxin induces behavioral effects in the open-field and hole-board tests. Adult male Wistar rats (180-220 g) treated with crotoxin, 100, 250 and 500 µg/kg, *ip*, administered 2 h before the test, presented statistically significant behavioral alterations (ANOVA for one-way classification complemented with Dunnet test, $P<0.05$). In the open-field test, 250 and 500 µg/kg of crotoxin increased freezing (from 3.22 sec to 10.75 sec and 11.2 sec) and grooming (from 13.44 sec to 22.75 sec and 21.22 sec) and decreased ambulation (from 64.8 to 39.38 and 45.8). The dose of 500 µg/kg also decreased rearing (from 24.9 to 17.5). In the hole-board test, 500 µg/kg of crotoxin decreased head-dip count (from 6.33 to 4.00). All the crotoxin-induced behavioral effects were antagonized by an anxiolytic dose of diazepam (1.5 mg/kg, *ip*, 30 min before the tests). These results show that crotoxin reduced open-field activity and exploratory behavior as well. We suggest that these effects express an increased emotional state induced by this toxin.

Key words
- Crotoxin
- *Crotalus durissus terrificus* venom
- Emotionality
- Open-field
- Hole-board

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Crotoxin (CTX) is the major toxic component of *Crotalus durissus terrificus* venom. It is composed of a basic subunit with weak toxicity but with high enzymatic activity and an acidic subunit, which is devoid of both enzymatic and toxic activities. When the subunits are associated, the complex presents 10-fold higher toxicity compared to the basic subunit (1).

The pharmacological blockade effect of CTX on the transmission of nervous impulses at the neuromuscular junction level has been extensively studied, and its proposed molecular mechanism of action involves pre- (2) and post-synaptic activities (3,4).

Toxicological effects have been reported after direct CTX administration into the rat's hippocampus which are characterized by behavioral and neuropathological alterations accompanied by electrographic patterns of epileptic activity closely related to the behavioral alterations (5). However, pharmacological effects on the central nervous system may also be expected since the presence of high-affinity specific binding sites for

Table 1 - Behavioral effects induced by crotoxin in rats submitted to the open-field and hole-board tests.

Rats were injected intraperitoneally with crotoxin (100, 250, 500 µg/kg) or saline 2 h before the open-field or hole-board test. Data are reported as the means ± SEM for 8-10 animals in each group. *P<0.05 compared to control (dose 0) (Dunnet test).

	Dose (µg/kg)			
	0	100	250	500
Open-field				
Freezing (sec)	3.22 ±0.94	7.70 ±1.35	10.75* ±3.16	11.20* ±2.77
Grooming (sec)	13.44 ±2.04	17.50 ±3.23	22.75* ±1.74	21.22* ±2.02
Ambulation (count)	64.80 ±3.33	55.20 ±3.56	39.38* ±2.92	45.80* ±4.69
Rearing (count)	24.90 ±2.77	20.10 ±2.28	17.88 ±2.00	17.50* ±2.31
Hole-board				
Head-dip (count)	6.33 ±0.74	5.00 ±0.93	4.78 ±0.36	4.00* ±0.99
Head-dipping (sec)	7.52 ±1.11	6.89 ±1.56	4.28 ±0.54	5.04 ±1.85

CTX in the brain has been reported (6). Although biodistribution studies demonstrated a low recovery of labeled CTX in brain tissue after intravenous administration (7), it is likely that the peripheral injection of this compound may lead to brain concentrations sufficient to induce behavioral effects.

The objective of the present study was to determine whether intraperitoneal CTX administration induces behavioral effects in rats. The experimental models chosen for behavioral evaluation were the open-field and hole-board tests because they are used to demonstrate drug-induced central nervous system effects (8,9).

Male Wistar rats weighing 180-220 g were housed 5 per cage at room temperature (21 ± 2°C) under a 12-h light-dark cycle with food and water available *ad libitum*. Behavioral evaluation was conducted in a dark sound-proof room with dim red lights. To minimize the possible influences of circadian changes during the tests, different treatments were alternated. Before introducing each animal, the apparatus was washed with 5% (v/v) ethanol solution to avoid the possible bias due to odor trails left by previous animals.

The open-field apparatus was similar to that described by Broadhurst (10). The hole-board was an open-field arena with four equally spaced holes (3 cm in diameter) in the floor, as described by File and Wardill (8). In the open-field, we recorded for 3 min ambulation (number of floor units entered) and rearing (number of times that the animal stood on its hind legs) and the duration, in seconds, of grooming (time used for the animal to groom) and freezing (time that the animal remained completely immobile, often in a crouching posture, with eyes wide open and irregular respiration). In the hole-board, head-dip count and head-dipping duration, in seconds, were recorded for 5 min, and a head-dip was scored if both eyes disappeared into the hole (8).

All drugs were injected intraperitoneally. Rats were randomly divided into two experimental groups. Group 1 received CTX (100, 250, 500 µg/kg) or saline 2 h before the open-field or hole-board test. Group 2 received CTX or saline as described for group 1, plus diazepam (1.5 mg/kg) or its vehicle (40% propylene glycol in saline) 30 min before the test.

The LD_{50} of crotoxin for rats was calculated by the method of Thompson and Weil (11). Rats were injected intraperitoneally with crotoxin (667, 1000, 1500 and 2250 µg/kg) and deaths were recorded for 7 days.

Crotoxin was purified from *Crotalus durissus terrificus* crude venom 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 (12). Diazepam was purchased from Sanofi.

Data were analyzed by ANOVA for one-way classification (13) and subsequently

post-hoc tests were performed using the Dunnet test, with the level of significance set at $P<0.05$.

Table 1 shows the effect of CTX on rats submitted to the open-field and hole-board tests. ANOVA indicated a significant overall drug effect on the count of ambulation [$F_{(3,38)} = 7.99$, $P<0.01$], rearing [$F_{(3,39)} = 2.96$, $P<0.05$] and head-dip [$F_{(3,34)} = 2.99$, $P<0.05$]; and on the duration of grooming [$F_{(3,33)} = 3.33$, $P<0.05$] and freezing [$F_{(3,39)} = 3.29$, $P<0.05$]. Head-dip duration was not significantly modified by crotoxin ($P>0.05$). Post-hoc comparisons (Dunnet) revealed that ambulation, grooming and freezing were significantlly modified by 250 and 500 µg/kg, whereas rearing and head-dip were significantly modified by 500 µg/kg ($P<0.05$).

Table 2 shows that diazepam reversed the effects of CTX on the behaviors evaluated (ANOVA, $P>0.05$).

The rats did not present any other alterations. There was no death during a 24-h period after CTX treatment. The calculated LD_{50} was 1333 µg/kg.

The present findings demonstrate that the intraperitoneal injection of CTX induced behavioral alterations in rats such as increased freezing and grooming accompanied by decreased ambulation, rearing and head-dips. These behavioral alterations can be regarded as a pharmacological effect of CTX on the brain, since the toxic behavioral effects on this organ are characterized by wet dog shakes, myoclonic paw movements, contralateral walking in circles and abrupt wild running, alone or followed by generalized tonic-clonic convulsions (5).

Since the 1950's, freezing behavior has been considered a direct measure of emotionality (14) and this premise has been supported by later studies showing increased freezing in emotional animals (15). Moreover, emotionality can also be expressed by grooming since it has been reported that intensification of this behavior is related to fear or an increased emotional response (16).

Table 2 - Effect of diazepam on behavioral effects induced by crotoxin in rats submitted to the open-field and hole-board tests.

The experimental rats were injected intraperitoneally with crotoxin (100, 250, 500 µg/kg) 2 h before the open-field or hole-board test, plus diazepam (1.5 mg/kg) 30 min before the test. Control rats received saline plus propylene glycol (40%, v/v, in saline). Data are reported as means ± SEM for 8-10 animals in each group. Dose zero corresponds to control (ANOVA, $P>0.05$).

	Dose (µg/kg)			
	0	100	250	500
Open-field				
Freezing (sec)	3.00 ±1.60	7.29 ±2.62	3.00 ±1.38	8.42 ±2.73
Grooming (sec)	19.50 ±5.79	12.25 ±3.21	10.14 ±1.16	13.57 ±2.78
Ambulation (count)	72.50 ±5.18	78.38 ±5.09	77.86 ±4.89	74.71 ±5.79
Rearing (count)	22.38 ±1.49	22.14 ±4.16	26.29 ±2.55	16.14 ±6.91
Hole-board				
Head-dip (count)	10.88 ±1.56	10.13 ±1.87	9.14 ±1.18	9.13 ±1.52
Head-dipping (sec)	16.75 ±2.11	15.13 ±3.53	18.86 ±2.91	15.75 ±3.39

On the basis of these considerations, we conclude that CTX increases the emotionality of rats.

CTX treatment reduced both ambulation and rearing, in agreement with the literature which reports them as positively correlated behaviors (17). Since ambulation is an inverse measure of emotionality (15), the reduction of ambulation and rearing may be a consequence of the CTX-increased emotional state.

Furthermore, since the association of ambulation with rearing can be regarded as an exploratory behavior (15), another approach was to investigate the ability of the CTX-treated animals to explore the hole-board, a validated test for exploration. In agreement with the results obtained with animals submitted to the open-field, a decreased exploratory behavior was observed in the hole-board, which shows that the ex-

ploratory ability of rats is negatively influenced by CTX. Since it has been reported that high emotionality inhibits exploration (15), the diminished exploratory behavior can also be a consequence of the CTX-increased emotional state.

Other relevant aspects that may be cited are that anxiogenic drugs like ß-carbolines decrease open-field exploratory behavior (18) as does CTX treatment, and that the anxiogenic effect of these carbolines can be reversed by diazepam (19). Thus the influence of diazepam on the CTX-induced behavioral alterations was investigated. Diazepam treatment reversed these alterations confirming that they are the expression of an animal's increased emotional state and suggesting that CTX may be a benzodiazepine inverse agonist like ß-carboline. Nevertheless, since diazepam by itself increases ambulation and decreases grooming (20), one cannot rule out a physiological antagonism between CTX and benzodiazepines. Further studies are needed to determine if CTX plays a role in the gabaergic-benzodiazepine receptor complex.

One might conclude that the reduction of open-field activity as well as exploratory behavior may be attributed, at least in part, to neuromuscular junction blockade by CTX. However, in addition to reducing these activities, CTX increased grooming, which is inconsistent with a blockade of the neuromuscular junction by this toxin.

In conclusion, these results, which show that CTX reduces open-field activity and the exploratory behavior as well, suggest that these effects are the result of an increased emotional state induced by this toxin.

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