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One-trial tolerance to midazolam is due to enhancement of fear and reduction of anxiolytic-sensitive behaviors in the elevated plus-maze retest in the rat

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Abstract

The anxiolytic-like effects of benzodiazepines (BZDs) in rats is reduced after a single exposure to the elevated plus-maze test (EPM). Several hypotheses have been formulated but no conclusive explanation exists for this phenomenon called "one-trial tolerance." In this study, we examined this phenomenon further by carrying out an ethopharmacological analysis of the behavior of rats submitted to the EPM in two trials. Rats injected with saline before both trials (control), treated with 1.0 mg/kg of midazolam before both trials (MM), or only before Trial 2 (SM), were exposed to the EPM. The SM group did not differ from the controls in the Trial 1 and Trial 2 conditions. The MM group showed a clear anxioselective profile in Trial 1 and no anxiolytic-like effects in Trial 2. Whereas midazolam injected before the first trial caused no significant change in immobility, there was a pronounced increase in immobility during Trial 2 for all three conditions. These data suggest that the anxiolytic-like action of midazolam in the first trial gives way to the fear-related insensitive behaviors (phobic/avoidance responses) responsible for the one-trial tolerance to BZDs in Trial 2. Furthermore, an additional experiment showed that midazolam does not seem to affect the acquisition of the learned avoidance response since it is present upon retesting even after midazolam administration in Trial 1 (MS group). Rather, the present data suggest an emotional shift from Trial 1 to Trial 2, which leads to change in the responsiveness of the animals to BZDs.

Keywords: Elevated plus-maze; Midazolam; One-trial tolerance; Ethological analysis

1. Introduction

The elevated plus-maze (EPM) has been extensively used as a reliable model for the investigation and measurement of anxiety in laboratory animals (Montgomery, 1955; for reviews, see Handley and McBlane, 1993; Rodgers and Cole, 1994; Griebel et al., 1993). Moreover, this test has shown good sensitivity to both anxiolytic and anxiogenic drugs (Pellow et al., 1985; File et al., 1993).

It is well known that the anxiolytic-like effects of the benzodiazepines (BZDs) are strongly reduced by a single previous undrugged experience in the EPM (Lister, 1987; File, 1990; Fernandes and File, 1996). This phenomenon, known as "one-trial tolerance," appears to be highly dependent on aversive learning from the first trial. Recent behavioral studies have proposed that the Trial 1–Trial 2 in the EPM results in a qualitative shift in emotional state (File and Zangrossi, 1993; File et al., 1993; Holmes and Rogers, 1998). The idea is that unconditioned fear in Trial 1 would shift to a learning avoidance in Trial 2. Alternatively, Trial 1 may represent the acquisition of a phobia-like response to the open arms, and the lack of anxiolytic-like effects of BZD in the second exposure to the EPM may be related to the well-known insensitivity of BZDs to phobic behaviors (Nutt, 1990; File et al., 1993; Bertoglio and Carobrez, 2000).

Blanchard et al. (1991) showed that immediate threat causes escape behavior, while potential threat generates a conflict between approach and risk assessment. In general, the organism is impelled to approach dangerous stimuli and the evaluation of such stimuli is critical for defining the direction of approach to safety cues and retreat form aversive

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cues. According to Gray and McNaughton (2000), this results in the inhibition of approach responses (i.e., inhibition of environmental involvement) as well as an increased arousal and attention to negative stimuli. These opposing situations would result in a conflict that could be responsible for the overall behavioral repertoire expressed by rats when they are submitted to the EPM (Gray and McNaughton, 2000). The EPM involves mixes of conditioned, innate, proximal and distal aversive mechanisms such that aversive cues detected at a distance could function as a negative incentive that activates a fear system, which guides the organism from danger present in the open arms of the maze (Graeff and Deakin, 1991). Accordingly, the nature of the threat present in Trial 1 and Trial 2 of the EPM, i.e., whether learned or innate and the nature of the appropriate response (emission or suppression of an action) has a bearing on drug responses (Handley et al., 1993).

There is a substantial amount of evidence to prove that ethological analysis has been a very important tool in the analysis of the functional relevance of behavioral responses to innate and acquired anxiety stimuli. Given that both kinds of stimuli appear to be present in the EPM, the present study attempted to evaluate the mechanisms involved in the one-trial tolerance to BZDs using ethological behavioral categories to assess exploratory behavior (as expressed by both traditional and nonstandard ethological measures). In addition, we have included immobility as an additional behavior during EPM evaluation as an indirect measure of fear. This seems to be extremely important since previous reports have described freezing, defecation, and increases in plasma corticosteroids as behavioral and physiological expressions of fear when the animals are restricted to the open arms (Pellow et al., 1985; Treit et al., 1993). Besides, it has been made clear that the degree of open arm aversion may be measured by the great avoidance drive elicited by their openness to the environment (Handley et al., 1993).

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 200–260 g and obtained from the animal house of the Campus of Ribeirão Preto of the University of São Paulo, were used. These animals were transported to a room adjacent to the test laboratory 72 h before the test. They were housed in groups of six per cage under a 12:12 dark/light cycle (lights on at 0700 h) at 23 ± 1 °C, and given free access to food and water. The animals were taken to the test laboratory at least 1 h prior testing.

2.2. Apparatus

The EPM was made of wood with two open arms (50 × 10 cm) and two enclosed arms of the same size, with 50 cm high walls. The level of illumination was 30 lx on the floor level of the closed arms of the maze. The maze was configured such that arms of the same type were opposite each other, and the whole maze was raised 50 cm from the floor. The walls of the closed arms of the standard maze were made of wood. A raised edge (0.5 cm) on the open arms provided additional grip for the rats.

All testing was conducted during the light phase of the LD cycle, between 0900 and 1300 h. Rats were placed individually in the center of the maze facing a closed arm and allowed 5 min of free exploration. The behavior of the animals was recorded by a video camera positioned above the maze allowing the discrimination of all behaviors, with the signal relayed to a monitor in another room via a closed-circuit TV camera. The maze was cleaned thoroughly after each test using damp and dry cloths.

An observer trained in measuring ethological plus-maze parameters subsequently scored the videotapes. The behavioral categories were scored using ethological analysis software (Observer) developed by Noldus (Netherlands). This software allowed measurement of the number of entries in both arms and the time spent in different parts of the maze, the ethological behaviors. Using separate location and behavior keys, this software allows the real-time scoring of videotapes (of any behavior test) by direct keyboard entry to a PC. This software only records the next behavior after a "stop" key is pressed, thus allowing for the recording of duration and frequency of prolonged behaviors as grooming, rearing, etc. Behaviors scored from videotape included traditional and nonstandard plus-maze parameters.

2.3. Ethological analysis

The performance of each animal in the maze was analyzed, taking the standard measurements recorded in each section of the maze into account (closed and open arms, central platform), comprising the frequency of open and closed arm entries (an arm entry or exit being defined as all four paws into or out an arm, respectively), total arm entries and the amount of time spent by the animals in each section of the maze. In addition, these data were used to calculate percentage of open arm entries, percentage of time spent in open arms, percentage of time spent in closed arms, and percentage of time spent on the central platform.

The items recorded were grooming, rearing, peeping out, stretched attend posture, flat back approach, scanning, head dipping, immobility and end-arm exploration. These categories were defined after studies in rats (Blanchard et al., 1991; Cruz et al., 1994; Anseloni and Brandão, 1997) and in mice (Rodgers and Johnson, 1995). Grooming: species-typical sequences beginning with snout, progressing to ears and ending with whole body groom, included scratching. Rearing: partial or total rising onto the hind limbs. Scanning: scrutinizing in any direction, including sniffing (olfactory exploration of maze floor and walls). Head dipping: exploratory movement of head/

shoulders over sides of the maze and down towards the floor. End-arm exploration: number of times the rat reached the end of an open arm. Peeping out: stretching of the head/shoulders from the closed arms to the central platform. Stretched attend posture (SAP): when the animal stretches to its full length and turns back to the anterior position without any forward motion of the hind legs. Flat back approach (FBA): locomotion when the animal stretches to its full length and cautiously moves forward. Immobility: arrest of movement for a time period greater than 10 s at any arm of the maze.

2.4. Procedure and statistics

Midazolam (Roche Products, Brazil) (1 mg/kg) was dissolved in saline solution (0.9%) shortly before use. Selection of midazolam dose and the time for testing were based on previous studies (Motta and Brandão, 1993; Motta et al., 1995; Anseloni and Brandão, 1997; Anseloni et al., 1995). All rats were tested twice, with an interval of 24 h. For analysis of the effects of midazolam on standard and ethological variables of the EPM test, the rats were randomly allocated to three groups of 16 subjects each: (a) control group (C), injected with isotonic saline solution before the first and the second trials; (b) SM group, injected with saline before the first trial and with midazolam before the second trial; and (c) MM group, administered with midazolam before Trials 1 and 2. The injections were administered intraperitoneally 15 min before trials. The data obtained are expressed as mean ± S.E.M. and were analyzed by an analysis of variance (ANOVA) with drug treatment as the independent factor and trials as the repeated measure. Tukey post hoc comparisons were carried out if significant overall F values were obtained.

3. Results

The effects of midazolam 1 mg/kg on the behavior of rats submitted to Trials 1 and 2 are summarized in Table 1. Statistically significant effects for group, trial, and the interaction (Group × Trial) were observed on number of open arm entries [$F(2,45)=15.01$, $P<.001$; $F(1,45)=96.28$, $P<.001$; $F(2,45)=14.31$, $P<.001$]; percentage of time in closed arms [$F(2,45)=18.09$, $P<.001$; $F(1,45)=48.29$, $P<.001$; $F(2,45)=5.37$, $P<.01$]; and percentage of time spent in open arms [$F(2,45)=8.05$, $P<.01$; $F(1,45)=39.83$ $P<.001$; $F(2,45)=7.41$, $P<.005$]. Significant effects were detected for group and trial for the percent open arm entries [$F(2,45)=18.54$, $P<.001$; $F(1,45)=45.85$, $P<.001$] and total arm entries [$F(2,45)=5.96$, $P<.01$; $F(1,45)=60.21$, $P<.001$]. Post hoc analysis showed that the group differences were due to the group treated with midazolam before Trial 1 (MS) compared to the control group (SS) and the group injected with midazolam before the second trial (MM) (Fig. 1).

For the number of entries in the closed arms [$F(2,45)=0.43$, $P>.05$] and the percentage of time spent in the center of the maze [$F(1.85)=2.9$, $P>.05$], no significant interactions were observed.

In the analysis of the ethological behavioral items, significant effects for group, trial and the interaction Group × Trial were observed for end-arm exploration [$F(2,45)=7.5$, $P<.05$; $F(1,45)=65.6$, $P<.001$; $F(2,45)=9.27$, $P<.001$], head dipping [$F(2,45)=19.82$, $P<.001$; $F(1,45)=93.23$, $P<.001$; $F(2,45)=10.97$, $P<.001$], stretched attended postures [$F(2,45)=5.07$, $P<.05$; $F(1,45)=6.34$, $P<.05$; $F(2,45)=7.10$, $P<.01$], scanning [$F(2,45)=12.76$, $P<.001$; $F(1,45)=41.71$, $P<.001$; $F(2,45)=7.60$, $P<.01$] and duration of immobility [$F(2,45)=4.00$, $P<.05$; $F(1,45)=22.27$, $P<.001$; $F(2,45)=4.49$, $P<.05$]. In all of these cases,

Table 1
Traditional and ethological measures of rats in standard plus-maze: effects of the administration of midazolam (1 mg/kg) before Trial 1 and the effects on Trial 2

	C		MM		SM	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
No. open arm entries*,#,a	4.8 ± 0.7	1.8 ± 0.7	12.1 ± 1.3	4.13 ± .7	5.0 ± 0.5	2.3 ± 0.4
No. closed arm entries	8.4 ± 0.7	6.3 ± 0.9	8.5 ± 0.8	7.4 ± 1.2	8.6 ± 0.7	6.0 ± 0.9
% Open arm entries*,#	35.0 ± 3.7	13.0 ± 4.2	57.3 ± 3.7	36.5 ± 2.7	35.9 ± 2.1	22.5 ± 4.7
% Time open arms*,#,a	20.3 ± 3.5	11.9 ± 6.3	49.2 ± 6.2	11.8 ± 2.2	18.9 ± 2.0	8.0 ± 2.3
% Time closed arms*,#,a	80.1 ± 3.6	93.8 ± 2.4	44.0 ± 6.0	75.9 ± 6.1	79.0 ± 2.5	90.8 ± 3.0
Total entries*,#	13.2 ± 1.2	8.1 ± 1.4	20.8 ± 1.5	11.5 ± 1.9	13.6 ± 1.2	8.3 ± 1.3
% Time in center	5.0 ± 0.6	4.6 ± 1.7	8.9 ± 2.3	13.5 ± 5.3	10.1 ± 2.4	5.0 ± 1.6
End-arm exploration*,#,a	3.6 ± 0.7	1.5 ± 0.6	9.3 ± 1.4	2.6 ± 0.7	3.8 ± 0.7	1.2 ± 0.2
Head dipping*,#,a	13.9 ± 1.9	4.8 ± 1.1	36.8 ± 4.6	10.7 ± 1.7	15.1 ± 1.4	3.8 ± 0.7
SAP*,#,a	10.6 ± 0.9	8.7 ± 1.0	4.8 ± 1.1	6.4 ± 1.3	10.8 ± 0.9	5.8 ± 1.1
Grooming*	7.6 ± 0.9	8.8 ± 0.8	4.0 ± 0.6	3.8 ± 0.4	5.8 ± 0.6	3.9 ± 0.8
Scanning*,#,a	26.6 ± 1.9	23.0 ± 1.6	47.6 ± 4.6	25.8 ± 2.8	28.3 ± 1.7	16.8 ± 2.1
Flat back approach#,a	3.6 ± 0.5	2.4 ± 0.6	1.8 ± 0.6	3.1 ± 0.8	4.6 ± 0.7	1.8 ± 0.4
Rearing#	12.8 ± 1.0	10.0 ± 1.0	12.0 ± 1.2	8.8 ± 1.5	12.5 ± 0.7	5.7 ± 1.4
Peeping out*	4.7 ± 0.7	4.8 ± 0.6	0.7 ± 0.3	2.0 ± 0.3	4.1 ± 0.5	3.2 ± 0.8
Immobility*,#,a	3.0 ± 1.3	14.8 ± 6.2	4.3 ± 2.7	38.7 ± 12.4	1.2 ± 0.4	73.8 ± 20.5

Scores are means ± S.E.M.; $n=16$ for each group. * Indicates significant effects on group, # on trial and a Group × Trial interaction (see text). C = control; MM = midazolam–midazolam; SM = saline–midazolam.

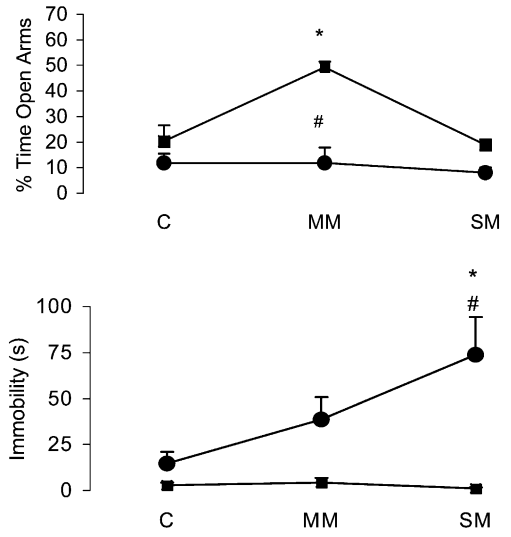

Fig. 1. Effects of midazolam (1.0 mg/kg ip) on the percentage of time in open arms (above) and duration of immobility (below) of rats submitted to Trial 1 (■) and Trial 2 (●) in the EPM. Each animal was injected before each trial with saline (C), midazolam (MM) or saline–midazolam (SM). * Different from the C group; # different from the respective Trial 1 ($P<.05$, Tukey test). $n=16$ in each group.

differences were detected between the C and MM groups, except for immobility, which significantly increased in MM and SM groups in relation to the control on the second trial and also in relation to the same groups on the first trial. For flat back, significant effects were obtained for trial [$F(1,45)=5.15$, $P<.05$] and the interaction Group × Trial [$F(2,45)=8.69$, $P<.001$]. Only effects per group were obtained for peeping out [$F(2,45)=12.48$, $P<.001$] and grooming [$F(2,45)=17.10$, $P<.001$]. For rearing, only significant effects for trial were observed [$F(1,45)=22.73$, $P<.001$].

Taking into account that the lack of anxiolytic-like effects of midazolam 1 mg/kg on the second day of test could be due to its sedative action we tested the possibility that a lower and less sedative dose (0.5 mg/kg) could have an anxiolytic-like activity on retesting. In this additional experiment the rats were randomly allocated to four groups of eight subjects each: (a) control group (C), injected with isotonic saline solution before the first and the second trials; (b) SM group, injected with saline before the first trial and with midazolam before the second trial; (c) MM group, administered with midazolam before Trials 1 and 2; (d) MS group, administered with midazolam before Trial 1 and saline before Trial 2. The procedure was similar to that described above for the experiment with midazolam 1 mg/kg.

As expected the effects obtained with 0.5 mg/kg were less pronounced but qualitatively similar to the dose of 1.0 mg/kg. Statistically significant effects for trial and for the interaction Group × Trial were observed on number of open arm entries [$F(1,28)=46.27$, $P<.001$; $F(3,28)=3.68$, $P<.05$], percent of entries on open arms [$F(1,28)=33.17$, $P<.001$; $F(3,28)=3.43$, $P<.05$], time on the open arms [$F(1,28)=68.98$, $P<.001$; $F(3,28)=4.86$, $P<.05$], percent of time on open arms [$F(1,28)=69.16$, $P<.001$; $F(3,28)=4.85$, $P<.05$] and percent of time on closed arms [$F(1,28)=48.19$, $P<.001$; $F(3,28)=3.03$, $P<.05$]. Post hoc analysis showed that the group differences were due to the groups treated with midazolam before Trial 1 compared to the control group. Whereas significant effects were detected on trial for total arm entries [$F(1,28)=25,77$, $P<.01$] no significant effects were observed for the number of entries in the closed arms [$F(3,28)=1.44$, $P>.05$].

In the analysis of the ethological behavioral items, significant effects for group and trial were observed only for head dipping [$F(3,28)=4.19$, $P<.05$; $F(1,28)=86.41$, $P<.001$; $F(2,45)=10.97$, $P<.001$] and stretched attended posture [$F(3,28)=4.06$, $P<.05$; $F(1,28)=7.40$, $P<.05$]. In both cases, differences were detected between the MM and MS groups in relation to the controls in Trial 1. Only effects per group were obtained for grooming [$F(3,28)=5.09$, $P<.01$]. Effects on trial only could be detected for end-arm exploration [$F(1,28)=34.23$, $P<.001$], peeping out [$F(1,28)=6.69$, $P<.05$]. A significant increase in immobility could be detected in Trial 2 in relation to Trial 1 [$F(1,28)=7.73$, $P<.01$], independent of the groups tested.

4. Discussion

The EPM has been one of the most useful tests for detecting anxiolytic and anxiogenic drug effects and for disclosing their mechanisms of action (File, 1992; Handley and MacBlane, 1993; Trullas et al., 1991; Motta and Brandão, 1993; Cruz et al., 1994; Anseloni et al., 1995). Standard anxiolytic drugs, such as diazepam, increase the percentage of entries and the time spent in the open arms of the maze. Our results are consistent with these reports, in that the injection of midazolam before the first trial raised the percentage of open arm entries and caused a selective increase in the percentage of time spent in the open arms, and on the total number of entries, whereas no significant effect could be detected in the closed arm entries. Regarding the ethological measures, midazolam reduced stretched attend postures, flat back approach and peeping out (effects that may due to reduced fear of leaving safe areas of the maze) with increased total head dips and end-arm exploration indicating an enhanced tendency to actively explore the potentially dangerous areas (Cole and Rodgers, 1993). At the same time, midazolam injected before the first trial caused no change in immobility. These results are consistent with the notion that indices of fear and anxiety in the EPM test may be dissociated pharmacologically. Accordingly, drug responses depend on the nature of the threat present in Trial 1 and Trial 2 of the EPM, i.e., whether learned or innate and the nature of the appropriate response (emission or suppression of an action) (Handley et al., 1993).

Overall, the animals are impelled to approach dangerous stimuli for the evaluation of such stimuli. In other words, an animal faced with a danger in the vicinity must be prepared both to remain still and assess whether it has been detected, and to flee and have a natural tendency to concomitantly approach and avoid dangerous situations leading to conflict (Gray and McNaughton, 2000). In other words, both anxiety and fear may be concurrent in the EPM test. Thus, the use of the EPM as an animal model of anxiety is based on the measures of all ethological categories that reflect the conflict resulting from the natural tendency of the animals to approach and avoid dangerous situations. Immobility used here as a measure of fear implies a phobic reaction to the openness and height of the open arms of the EPM. Thus, midazolam promoted a clear anxiolytic-like effect in this study, reducing avoidance of the open arms without changing the activity of the animals into the closed arms. These effects were not observed when this compound was injected before the second trial, confirming several reports in the literature that show the inefficacy of BZDs under these conditions (Lister, 1987; File, 1990; File and Zangrossi, 1993; File et al., 1993; Holmes and Rodgers, 1998). Indeed, in Trial 2 a completely different pattern of effects was observed in the present study; midazolam 1 mg/kg did not change the anxiety-related measures and caused a significant increase in immobility. This assumption is further strengthened by the data obtained with the use of a lower dose of midazolam. Indeed, although 0.5 mg/kg of this BZD did not produce an overall anxiolytic-like effect as the dose of 1.0 mg/kg it still increased the number of entries in the open arm entries and the head dipping on the first trial whereas no significant effect whatsoever on the second trial could be observed. Thus, the lack of anxiolytic-like effects of midazolam on Trial 2 could not be attributed to a shift from an anxiolytic-like action on the test to a sedative effect in Trial 2 probably due to a reduction of the level of arousal when the animal could be familiar to the apparatus.

We assume that fear and anxiety as described above are represented by the overall behavioral repertoire expressed by rats tested on the EPM. BZDs appear to attenuate the conflict resulting from these opposing situations. The present results allow us to go one step further in the proposal that the Trial 1–Trial 2 in the EPM results in a qualitative shift in emotional state, so that unconditioned fear in Trial 1 would shift to a learning avoidance in Trial 2 (File and Zangrossi, 1993; File et al., 1993; Holmes et al., 1998). We believe that the lack of anxiolytic-like effects of BZDs in Trial 2 is due to its inefficacy on indices of fear, which predominate in Trial 2. Indeed, Rodgers and Shepherd (1993) suggested that the loss of diazepam efficacy in Trial 2 might reflect a relative absence of an approach/avoid conflict. In other words, prior knowledge of the maze (e.g., escape is not possible via open arms) would reduce the tendency to explore these natural aversive areas, thereby reducing conflict and eliminating a possible response to diazepam. Indeed, introduction of conflict-generating elements in Trial 2 also reinstates the efficacy of BZDs on the Trial 2 (Pereira et al., 1999).

The data obtained in this study with the dose of 1.0 mg/kg left open the question whether midazolam was inactive on anxiety indices upon retesting because a sedative action predominates on Trial 2, which could probably result from a reduction of the level of arousal when the animal could be familiar to the apparatus. This prediction was not confirmed by the data obtained in the experiments with the use of 0.5 mg/kg of midazolam. Indeed, although this dose still caused anxiolytic-like effects it did not produce any effect at all on the entries into or time spent on the closed arms. Thus, the immobility evident during Trial 2 could not be due to an eventual sedative action of the drug. On the contrary, in agreement with several reports this may represent avoidance or phobic responses (File and Zangrossi, 1993; File et al., 1993; Holmes et al., 1998). Midazolam does not seem to affect the acquisition of learned avoidance/phobic responses since they are also present upon the retesting even after midazolam administration in Trial 1 (MS group). Rather, the present data suggest an emotional shift from Trial 1 to Trial 2, which leads to a change in the responsiveness of the animals to BZDs.

From a neurological point of view, it is recognized that there could be two different types of anxiety: anxiolytic-sensitive (presumed to reflect overactivity in the septo-hippocampal system) and counteracted by the action of anxiolytics; and anxiolytic-insensitive (presumed to reflect overactivity in the amygdala, dorsomedial hypothalamus and dorsal periaqueductal gray). These structures will receive information about concurrently activated, conflicting goals and this will result in inhibition of aversive motivation, increases in arousal mediated by the amygdala (Gray and McNaughton, 2000). The precise balance of activity between the areas involved in the processing of these stimuli will be determined by their interconnections and by the modulating influences of GABA, neuropeptides, dopamine and serotonin, as well as pituitary–adrenal hormones (see Brandão et al., 1999 for a review).

In the case of the present study, our analysis of the phenomenon called one-trial tolerance suggests that it is the result of the predominance of anxiolytic-insensitive fear behaviors in Trial 2 in animals that had previously experienced and solved the conflict approach avoidance in the first trial. Finally, the present analysis is not at variance with the current hypothesis to be found in the literature. We only describe further the kind of qualitative shift in emotional state that is present in EPM Trial 1–Trial 2.

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