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Absence of repeated-trial tolerance to the anxiolytic-like effects of chlordiazepoxide in the rat triple test

Thaize D. Wehrmeister, Geison S. Izídio, Elayne Pereira, Gustavo Izídio, André Ramos *

Laboratório de Genética do Comportamento, Departamento de Biologia Celular, Embriologia e Genética, Universidade Federal de Santa Catarina, 88.040-900, Florianópolis, SC, Brazil

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ABSTRACT

The triple test, recently developed to assess anxiety-related behaviors in rodents, combines three widely used behavioral tests: the open field (OF), elevated plus maze (EPM) and light/dark box (LDB). The EPM and LDB, individually, are normally sensitive to the anxiolytic effects of benzodiazepines only in the first trial, due to the phenomenon of one-trial tolerance, which limits their use in longitudinal studies. The main objective of the present investigation was to verify whether the anxiolytic-like effects of chlordiazepoxide (CDZ), previously observed in naive animals submitted to the triple test, would persist after repeated testing. To this end, three experiments were carried out where male Wistar rats received CDZ (10 mg/kg) 30 min before the triple test for 2, 3 or 20 consecutive days. Except for the first day of drug treatment following a previous test experience in an undrugged state, CDZ had enduring anxiolytic-like effects under all schedules, promoting an increase in the exploration of the EPM open arms (and in some cases of the white compartment of the LDB), without affecting the number of closed-arm entries. The finding that rats did not develop tolerance to CDZ even with chronic treatment and repeated exposures to the triple test suggests that this new device is a promising tool to be used in longitudinal studies involving pharmacological manipulations of anxiety-related behaviors.

1. Introduction

The triple test is a new experimental tool that has been proposed as an alternative to the use of behavioral test batteries in the preclinical study of anxiety (Ramos, 2008). It physically integrates three of the most widely used tests of anxiety, the open field (OF), the elevated plus maze (EPM) and the light/dark box (LDB), all interconnected, thus allowing the measurement of anxiety- and locomotion-related behaviors from each of the three devices within one single trial. Preliminary pharmacological studies showed that some of these behaviors (i.e. avoidance of the EPM open arms) responded as expected to classical anxiolytic and anxiogenic substances (i.e. chlordiazepoxide, midazolam and pentylenetetrazole in rats; diazepam and alprazolam in mice) (Fraser et al., 2010; Ramos et al., 2008). In addition, the new test was shown to be sensitive to genotypic differences in anxiety- and locomotion-related behaviors, as inbred strains of rats (Lewis and Spontaneously Hypertensive Rats) and mice (BALB/cJ, DBA/2J and C57BL/6J) displayed behavioral differences in the triple test that were comparable to those found in the classical individual tests (Fraser et al., 2010; Ramos et al., 2008).

Taken together, the aforementioned evidence suggested that the triple test was advantageous in relation to a sequence of the three individual tests not because of 'what' it measures (that is, both combined and individual conditions seemed to assess similar phenomena), but because of 'how' it measures (i.e. simultaneously rather than successively) a certain number of anxiety-related behaviors. Thus, for providing quasi-simultaneous measures from three different devices, which are thought to reflect distinct facets of emotionality, the triple test seems to circumvent two drawbacks of the increasingly popular test batteries: the undesirable effects of previous test experience (Ballaz et al., 2007; Cryan and Holmes, 2005; Paylor et al., 2006; Voikar et al., 2004) and of uncontrolled, intraindividual fluctuations in behavior that may occur between successive tests (Izídio et al., 2005a; McClearn, 2006; Ramos, 2008).

Yet, one of the reasons that led to the widespread use of the OF, EPM and LDB is their simplicity. From the experimenter's point of view, these tests are inexpensive, quick (typically 5 min), can be fully automatized and are not very large (usually <1 m^2). From the animal's point of view, each of these tests offers a simple choice between two contrasting and contiguous environments. However, one of the putative consequences of such a simplicity is that the approach/avoidance conflict that characterizes these tests (and makes them sensitive to anxiolytic treatments) can be resolved during a single test session. That is, the preference for the protected areas seems to be so well established at the end of the first trial that the animals are no longer motivated to approach the aversive areas in subsequent trials (Carobrez and Bertoglio, 2005). Therefore, without a motivational

* Corresponding author. Laboratório de Genética do Comportamento, Departamento de Biologia Celular, Embriologia e Genética, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88.040-900, Florianópolis, SC, Brazil. Tel.: +55 48 3721 5153; fax: +55 48 3721 5148.
E-mail address: andre@ccb.ufsc.br (A. Ramos).

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conflict, the effects of anxiolytic benzodiazepines are no longer present, a phenomenon that affects the EPM and LDB and is known as one-trial tolerance (File, 1990; File and Zangrossi, 1993; Holmes et al., 2001; Rodgers and Shepherd, 1993). Thus, an additional question that remained unanswered about the new triple test, in comparison with its individual components, is: can its higher spatial complexity, where the animals can choose among several different compartments, change the nature of each test and contribute to maintain the approach/avoidance conflict (and hence the anxiolytic effects of drugs) across trials? If so, for how long?

In order to address the aforementioned questions, three experiments were carried out to investigate the effects of repeated testing on the pharmacological sensitivity of the triple test. The first experiment used a classical two-day protocol, where rats were tested twice after receiving either chlordiazepoxide (CDZ) or its vehicle in both trials. In the second experiment, all rats were tested once without receiving any pharmacological treatment and then were tested for three more days with or without CDZ treatment. Finally, in the third experiment, rats received either CDZ or its vehicle before being submitted to the triple test for 20 consecutive days.

2. Materials and methods

2.1. Subjects

A total of 84 male Wistar rats, born and raised in our own laboratory, were used in a series of three experiments. All animals were weaned and separated by sex at 3–4 weeks of age and thereafter were kept in collective plastic cages (5–6 rats per cage, which was 17-cm high, 41-cm long and 34-cm wide) with food and water available ad libitum, under a 12-h light/dark schedule (lights on at 7:00 am), at 22 ± 2 °C. All animals were tested between 9 and 11 weeks of age with all tests being carried out between 1:00 and 6:00 pm. All procedures were carried out according to the guidelines of the local Committee for Animal Care in Research (CEUA/UFSC, protocol number PP00191).

2.2. Apparatus

The triple-test apparatus, as previously described (Ramos et al., 2008), was an integration of three well known tests of anxiety/emotionality, namely the OF, EPM and LDB. Briefly, the OF was made of wood covered with white formica, had a square floor of 100 × 100 cm (divided by black lines into 25 squares of 20 × 20 cm) and white walls, 40-cm high. The EPM was made of wood covered with black formica and had four elevated arms (52 cm from the floor) 50-cm long and 10-cm wide. The arms were arranged in a cross-like disposition, with two opposite arms being enclosed (by 40-cm high walls) and two being open, having at their intersection a central platform (10 × 13.5 cm). The open arms were surrounded by a raised ledge (1-mm thick and 5-mm high). Finally, the LDB was made of wood covered with formica and presented two compartments. One larger (27 × 27 × 27 cm) and white, with the floor divided by black lines in nine squares, being strongly illuminated by a 40-W white bulb. The other smaller (27 × 18 × 27 cm high) and black, with the floor divided by white lines in six squares, being illuminated by a 40-W red bulb. The bulbs were located 30 cm above the apparatus' floor. The two compartments were connected by a small square opening of 7 × 7 cm. The OF, EPM and LDB were physically connected by placing them side by side at the same height (52 cm from the floor) and by adding rectangular openings at the interface between them (i.e. a 10 × 40-cm opening between the periphery of the OF and one enclosed arm of the EPM and a 10 × 7-cm opening between the other enclosed arm of the EPM and the dark compartment of the LDB). The only illumination in the test room was provided by the lights of the LDB, which produced 10 lux (lx) inside the OF, 15 lx inside the EPM, 20 lx inside the dark area of the LDB and 750 lx inside the light area of the LDB.

2.3. Drug

Chlordiazepoxide (CDZ) (CENTAUR, India) was suspended in 0.5% methylcellulose (MC) as previously described (Ramos et al., 2008) and administered intraperitoneally (2 ml/kg) 30 min prior to the test at 10 mg/kg. The control groups received a similar volume of 0.5% MC 30 min before being tested. Injection volume was chosen based on previous rat studies (Chaouloff et al., 1997; Pellow et al., 1985; Ramos et al., 2008), whereas CDZ dose and injection timing were chosen, also based on previous studies (Chaouloff et al., 1997; Ramos et al., 2008), in order to maximize the chances of having an anxiolytic effect in the triple test, to allow daily repeated testing and to minimize the number of animals used.

2.4. Procedures

2.4.1. Experiment 1: effects of CDZ on trials 1 and 2

This was a preliminary study carried out to investigate the existence of one-trial tolerance (File, 1990; File and Zangrossi, 1993; Holmes et al., 2001; Rodgers and Shepherd, 1993) in rats tested for 2 consecutive days in the triple test. To this end, a simple test/retest protocol was designed where rats, 30 min before being introduced in the triple test for 2 consecutive days (with 24 h between trials), received either CDZ or MC. All rats were assigned to the same pharmacological treatment (CDZ or MC) in both testing days, waited to be tested for 30 min in their home-cages and were then introduced in the central area of the OF compartment, as previously described (Ramos et al., 2008). Through the use of three video cameras mounted above the apparatus (one for each main area) and connected to a computer located in an adjacent room, the following behaviors were registered for 15 min: OF — number of squares crossed (referred to as locomotion) in either the inner (away from the walls) or outer (adjacent to the walls) areas; EPM — number of entries and time spent in either the closed or open arms (% entries and % time spent in the open arms were calculated in relation to the total entries and total time spent in both types of arms); LDB — number of squares crossed (referred to as locomotion) in either the light or dark compartments and number of entries into the light compartment (referred to as transitions). Number of entries, number of crossings and time spent were considered when all four paws entered a compartment, crossed one line of a square or were located inside a compartment, respectively. Number of entries in the closed arms of the EPM included entries from the central platform, like in the classical EPM test, and also entries (or transitions) from the OF and LDB. At the end of each test session, the apparatus was cleaned with a 10% alcohol solution and dried with a paper towel.

2.4.2. Experiment 2: effects of CDZ administered for 3 consecutive days in test-experienced rats

This study was designed to investigate the effects of repeated treatment with CDZ for 3 consecutive days in rats that had been previously exposed to the triple test without having received any pharmacological treatment. To this end, in the first day of experiment, all rats were exposed to the triple apparatus for 15 min after receiving only an injection of the vehicle MC. On days 2, 3 and 4, always 24 h after the previous test session, the rats were re-submitted to the triple test after receiving an injection of either MC or CDZ, with the same treatment being repeatedly applied to the same rats. Testing protocol, recording methods and cleaning procedures were carried out as in experiment 1.

2.4.3. Experiment 3: effects of chronic CDZ treatment

This experiment was carried out to investigate the effects of chronic CDZ treatment in rats that were tested daily in the triple test.

To this end, for 20 consecutive days, rats received a daily injection of either CDZ or its vehicle, 30 min before being submitted to the triple test. All rats received the same treatment throughout the entire experiment. Testing protocol, recording methods and cleaning procedures were carried out as in experiments 1 and 2.

2.5. Statistics

In all experiments, data were analyzed through an ANOVA for repeated measures (daily testing) having pharmacological treatment (CDZ or MC) as the independent variable. Duncan test was used whenever a significant effect of day (or day × treatment interaction) was revealed by the ANOVA. In experiment 2, because all rats received the same treatment (MC) on day 1, the statistical analysis included only days 2, 3 and 4. All analyses were performed using the Statistica® 8.0 software package.

3. Results

3.1. Experiment 1

The results of experiment 1 are shown in Figs. 1 and 2. The ANOVA for repeated measures (days 1 and 2) revealed no significant ($p>0.05$) interactions between day and treatment for any of the variables analyzed. In the OF, the ANOVA showed a main day effect ($F_{(1,28)}=25.9$, $p<0.001$) for the locomotion in the outer area, with a drop being observed on day 2 (Fig. 2). No main treatment effects ($p>0.05$) were observed for locomotion scores in either the inner or outer areas of the OF (Figs. 1 and 2). In the EPM, a significant day effect was found for the % entries ($F_{(1,28)}=27.9$, $p<0.001$) and % time ($F_{(1,28)}=18.9$, $p<0.001$) spent in the open arms, with an overall drop being observed for both variables on day 2. A significant treatment effect was also observed for the % entries ($F_{(1,28)}=12.3$, $p<0.01$) and % time ($F_{(1,28)}=9.8$, $p<0.01$) spent in the open arms, with an overall increase being produced by CDZ treatment for both variables regardless of the day of test (Fig. 1). No significant ($p>0.05$) effects were seen for the number of closed-arm entries as well as for any measure from the LDB (Figs. 1 and 2). Data on the total number of entries and total time spent in both types of arms of the EPM, for this and the two subsequent experiments, are presented in Table S1 (Supplementary data).

3.2. Experiment 2

The results of experiment 2 are presented in Figs. 3 and 4. The ANOVA for repeated measures (days 2–4) revealed no significant

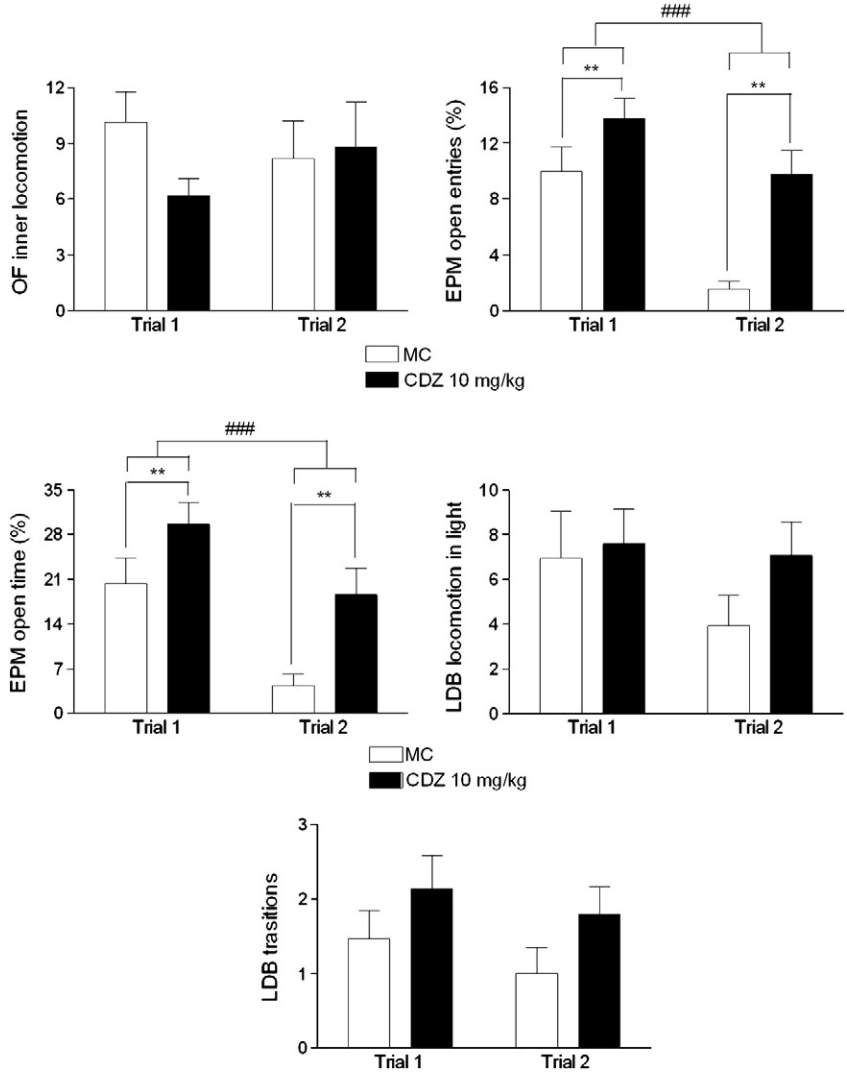

Fig. 1. Anxiety-related behaviors of male Wistar rats treated with either methylcellulose 0.5% (MC) or chlordiazepoxide (CDZ) at 10 mg/kg before being submitted for 2 consecutive days (with a 24-h interval between trials) to the triple test. Each trial lasted 15 min and the following variables were analyzed: inner locomotion in the OF, % entries and % time spent in the open arms of the EPM, locomotion in the light compartment of the LDB, and number of transitions in the LDB. Bars and vertical lines represent the means and S.E.M. of animals grouped by pharmacological treatment ($n=15$). ** represents a significant ($p<0.01$) effect of CDZ treatment, over both trials. ### represents a significant ($p<0.001$) effect of the trial, over both treatments (ANOVA for repeated measures).

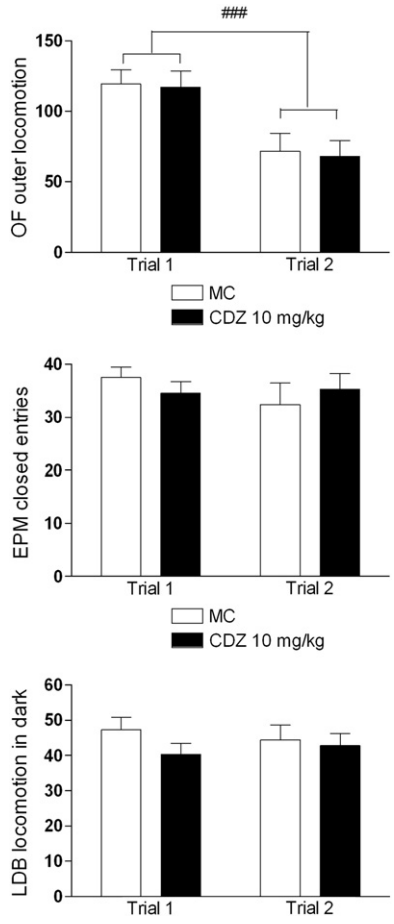

Fig. 2. Locomotion-related behaviors of male Wistar rats treated with either methylcellulose 0.5% (MC) or chlordiazepoxide (CDZ) at 10 mg/kg before being submitted for 2 consecutive days (with a 24-h interval between trials) to the triple test. Each trial lasted 15 min and the following variables were analyzed: outer locomotion in the OF, number of entries in the closed arms of the EPM, locomotion in the dark compartment of the LDB. Bars and vertical lines represent the means and S.E.M. of animals grouped by pharmacological treatment ($n=15$). ### represents a significant ($p<0.001$) effect of the trial, over both treatments (ANOVA for repeated measures).

($p>0.05$) day or day × treatment effects in the OF. However, a significant effect of the treatment was found for both inner ($F_{(1,28)}=8.8$, $p<0.01$) and outer ($F_{(1,28)}=28.6$, $p<0.001$) locomotion, with both variables being overall decreased by CDZ treatment (Figs. 3 and 4). When transformed into percentage of the total OF locomotion (data not shown), the locomotion performed in the inner area was not affected ($p>0.05$) by CDZ. In the EPM, there was a significant day × treatment interaction for the % entries ($F_{(2,56)}=4.9$, $p<0.05$) and % time ($F_{(2,56)}=3.3$, $p<0.05$) spent in the open arms. Post hoc tests revealed that animals that received CDZ displayed higher levels of approach towards the open arms when compared to their controls on days 3 ($p<0.05$) and 4 ($p<0.001$). No treatment differences were observed on day 2 (Fig. 3). The ANOVA revealed no significant ($p>0.05$) effects for the number of closed-arm entries (Fig. 4). In the LDB, no significant ($p>0.05$) day or day × treatment effects were found. However, a significant overall treatment effect was found for the locomotion scores in the light ($F_{(1,28)}=12.1$, $p<0.01$) and dark ($F_{(1,28)}=9.8$, $p<0.01$) areas and for the number of transitions between dark and light areas ($F_{(1,28)}=9.4$, $p<0.01$). For all these measures, rats treated with CDZ displayed higher scores than their controls, regardless of the day of test (Figs. 3 and 4). An additional t-test analysis for dependent samples was performed within the MC (vehicle-treated) group to compare the baseline behavior in days 1 and 2. No significant ($p>0.05$) changes were found for any behavioral variable between the first 2 days of test.

3.3. Experiment 3

The results of experiment 3 are presented in Figs. 5 and 6. In the OF, the ANOVA for repeated measures (days 1–20) revealed a significant ($F_{(19,342)}=1.7$, $p<0.05$) day × treatment interaction for inner locomotion, with rats that received CDZ showing significantly ($p<0.05$) lower scores than their vehicle-treated controls only in the 7th day of test. There was also a significant overall day effect ($F_{(19,342)}=2.8$, $p<0.001$) for this variable. Post hoc tests comparing the overall daily mean values revealed that between days 4 and 20, the rats showed a higher ($p<0.05$) locomotion in the center of the OF than they did in the first day of test (Fig. 5). An overall day effect ($F_{(19,342)}=7.3$, $p<0.05$) was also found for the outer locomotion in the OF, however, in this case, post hoc tests showed that between days 2 and 20, the rats significantly ($p<0.05$) decreased this behavior in comparison with day 1 (Fig. 6). No treatment or day × treatment effects were found for this variable. In the EPM, the ANOVA revealed a significant overall treatment effect for the % entries ($F_{(1,18)}=7.7$, $p<0.05$) and % time ($F_{(1,18)}=7.6$, $p<0.05$) spent in the open arms. In both cases, rats treated with CDZ displayed higher scores than controls regardless of the day of test. There was also a significant overall day effect (with no day × treatment interaction) for both variables ($F_{(19,342)}=5.8$, $p<0.001$ and $F_{(19,342)}=3.6$, $p<0.001$, respectively), with rats showing a decrease on day 2 and an increase on days 13 and 15–20 in relation to day 1 for the % open entries; and a decrease on day 2 and an increase on day 18 in relation to day 1 for the % open time (Fig. 5). There was also a significant day effect ($F_{(19,342)}=2.3$, $p<0.01$) for the number of closed-arm entries, with a drop being observed in all days (excepting day 6) in relation to day 1 (Fig. 6). In the LDB, no significant ($p>0.05$) treatment or day × treatment effects were observed. Overall day effects were found for the locomotion in the light area ($F_{(19,342)}=2.1$, $p<0.01$), locomotion in the dark area ($F_{(19,342)}=4.5$, $p<0.001$) and number of transitions ($F_{(19,342)}=3.0$, $p<0.001$). In relation to the scores from the first day of test, there was an overall drop: in the light-area locomotion on days 10, 11, 17, 18 and 20; in transitions on days 5, 7–12 and 15–20; and in the dark-area locomotion on days 7–10, 12, 15–17 and 19–20 (Figs. 5 and 6).

4. Discussion

In a previous recent study, rats that were submitted to the triple test for the first time were shown to respond to acute benzodiazepine treatment by increasing their approach towards the aversive open arms of the EPM, which can be seen as a sign of anxiolysis (Ramos et al., 2008). In the present study, such anxiolytic-like effects were not only confirmed but also found to persist for at least 20 consecutive days of daily injection and testing. These promising findings, together with recent results showing that strain differences in the triple test are not changed with repeated trials (Fraser et al., 2010), suggest that this new device, differently from the standard EPM and LDB tests (File, 1990; File and Zangrossi, 1993; Holmes et al., 2001; Rodgers and Shepherd, 1993), may be adequate for longitudinal studies of anxiety-related behaviors involving chronic pharmacological and/or genetic treatments.

A few years after the publication of the seminal paper by Pellow et al. (1985), who have thoroughly validated the EPM as a pre-clinical test of anxiety, File (1990) elegantly demonstrated that a single exposure to this test, regardless of the pharmacological state of the animal during that exposure, abolished the effects of CDZ in rats retested 24 h later in the same apparatus. Such a phenomenon, known as one-trial tolerance, was later confirmed by numerous studies using different classes of drugs in both rats and mice (for a review see Carobrez and Bertoglio, 2005). The same kind of effect was later observed in other anxiety-related tests, such as the four-plate (Hascoet et al., 1997), the cat odor test (McGregor and Dielenberg, 1999) and the LDB (Holmes et al., 2001). Moreover, one-trial

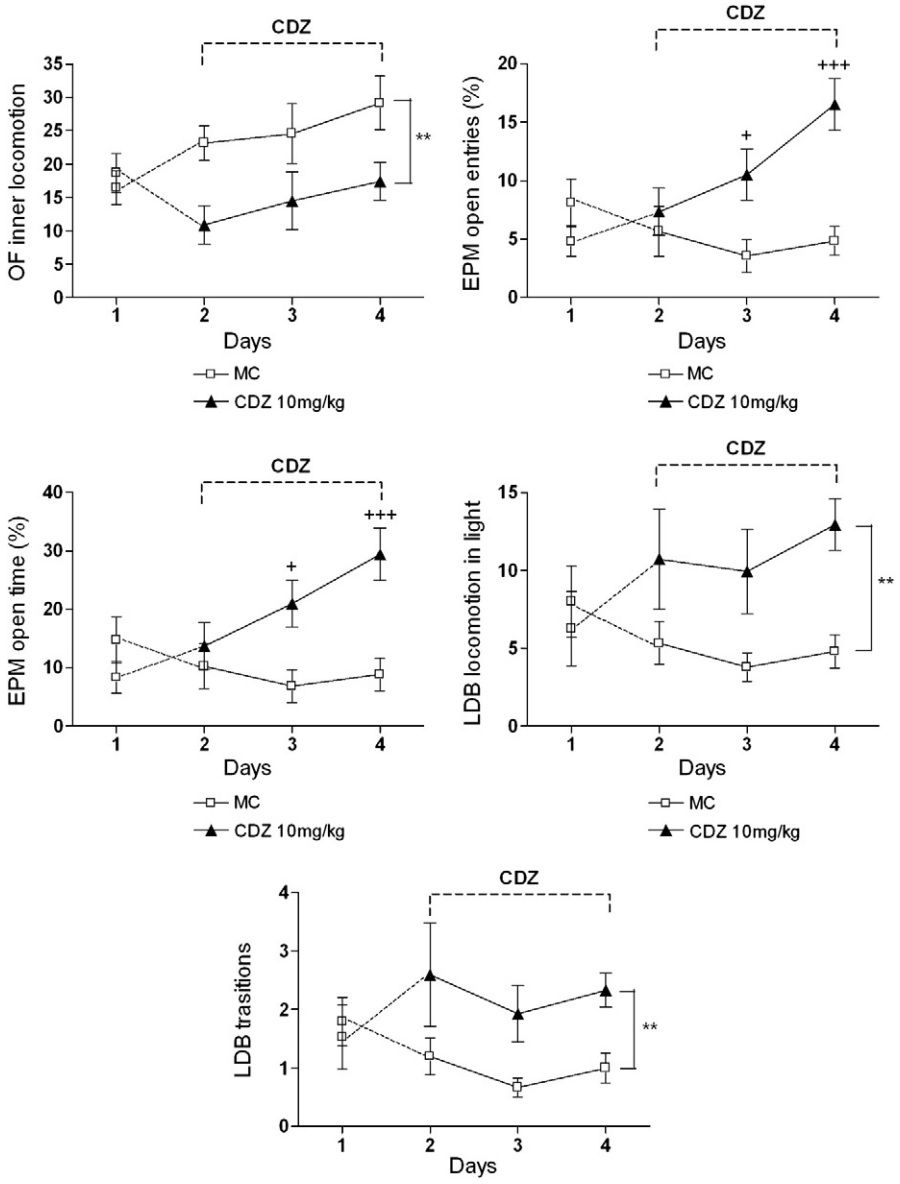

Fig. 3. Anxiety-related behaviors of male Wistar rats treated with methylcellulose 0.5% (MC) on day 1 and with either MC or chlordiazepoxide (CDZ) at 10 mg/kg on days 2, 3 and 4 (with a 24-h interval between trials) before being submitted to the triple test. Each trial lasted 15 min and the following variables were analyzed: inner locomotion in the OF, % entries and % time spent in the open arms of the EPM, locomotion in the light compartment of the LDB, and number of transitions in the LDB. Symbols and vertical lines represent the means and S.E.M. of animals grouped by pharmacological treatment ($n=15$). The dotted connecting lines represents the period in which the animals had never received CDZ. ** represents a significant ($p<0.01$) effect of CDZ treatment over all trials. + and +++ represent a significant ($p<0.05$ and $p<0.001$, respectively) effect of CDZ treatment within a specific testing day (ANOVA for repeated measures followed by Duncan test whenever a significant day × treatment interaction was found; only days 2–4 were included in the analysis).

tolerance has been shown to occur even across tests, that is, a single experience in the EPM was sufficient to abolish the effects of diazepam in the LDB applied 8 days later (Rodgers and Shepherd, 1993).

The exact causes and the clinical relevance of this phenomenon have not been completely clarified but, having been a matter of investigation during the past 20 years, it is generally agreed that the behaviors assessed in trial 2 of the EPM reflect an emotional state that is qualitatively different from those measured in trial 1. According to this view, such a behavioral shift would result from a learning process taking place during the first few minutes of the first EPM trial; it would persist over time and across subsequent trials; and it would produce a new emotional state (controlled by specific neurobiological mechanisms) that could not be reversed by classical anxiolytic drugs (Carobrez and Bertoglio, 2005; File et al., 1999; Holmes and Rodgers, 1998). It is interesting to notice, however, that strain differences seen in rats submitted to the EPM for the first time neither diminished nor disappeared in a second trial performed 24 h later (Izídio et al., 2005b). Somewhat more controversial is the inter-trial stability of baseline behaviors measured in the EPM. Whereas most studies report a clearcut increase in the avoidance towards the open arms by undrugged rats and mice following the first trial (see Carobrez and Bertoglio, 2005; Holmes and Rodgers, 1998), a few studies have shown steady levels of avoidance behavior between trials (File, 1990; Holmes and Rodgers, 1999; Pellow et al., 1985). The stress of injection prior to EPM testing, present in some protocols but not in others, has been pointed out as an experimental factor potentially responsible for such an inconsistency (Holmes and Rodgers, 1999).

In the present study, when rats were injected with either CDZ or its vehicle 30 min before being introduced in the triple test for 2 consecutive days, there was a significant and overall increase in anxiety-related behaviors (i.e. a decrease in % entries and % time spent

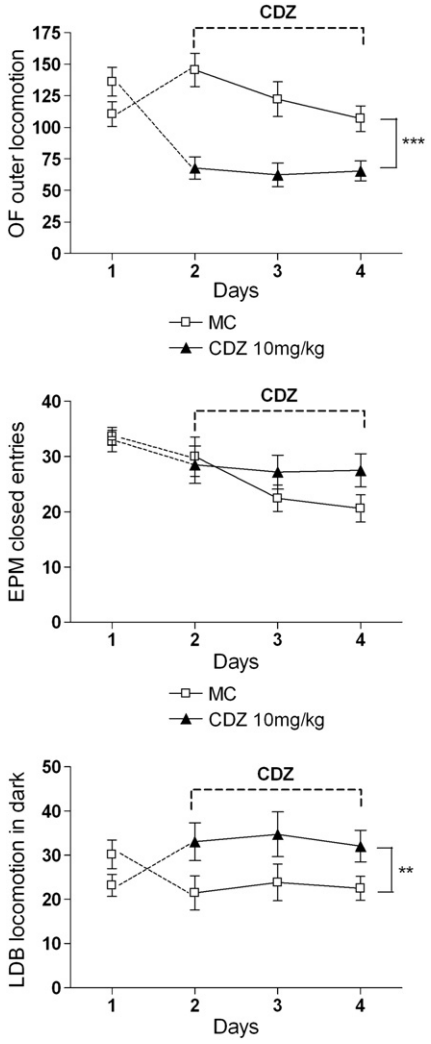

Fig. 4. Locomotion-related behaviors of male Wistar rats treated with methylcellulose 0.5% (MC) on day 1 and with either MC or chlordiazepoxide (CDZ) at 10 mg/kg on days 2, 3 and 4 (with a 24-h interval between trials) before being submitted to the triple test. Each trial lasted 15 min and the following variables were analyzed: outer locomotion in the OF, number of entries in the closed arms of the EPM, locomotion in the dark compartment of the LDB. Symbols and vertical lines represent the means and S.E.M. of animals grouped by pharmacological treatment ($n = 15$). The dotted connecting lines represents the period in which the animals had never received CDZ. ** and *** represent a significant ($p<0.01$ and $p<0.001$, respectively) effect of CDZ treatment over all trials (ANOVA for repeated measures; only days 2–4 were included in the analysis).

in the EPM open arms) between trials 1 and 2, thus being consistent with the majority of the studies on the EPM alone, as mentioned earlier (Carobrez and Bertoglio, 2005). Moreover, CDZ produced a decrease in open-arm avoidance, as expected, but the one-trial tolerance phenomenon was not observed in this first experiment. On the contrary, the anxiolytic-like effect of CDZ seemed to be even stronger in the second day of test, although the interaction between day and treatment did not reach significance levels. Interestingly, CDZ had no effects on measures of locomotion in the protected areas of the triple test, thus suggesting an anxiety-related selectivity of the drug effect. In the LDB area, only non-significant trends could be seen and these were consistent with the day and drug effects on anxiety-related behaviors measured in the EPM area. No drug effect was found in the OF area, but a drop in the outer locomotion was seen between trials 1 and 2, which is indicative of habituation. The absence of one-trial tolerance in the triple test suggests that a single exposure to this integrated apparatus, differently from what occurs with the individual tests, does not cause a shift in the behavioral/emotional state of the animals. In the standard EPM, rodents are thought to learn about the spatial layout during the first trial and to use this spatial knowledge in subsequent trials (Holmes and Rodgers, 1999), in which the approach/avoidance conflict would be no longer present (Carobrez and Bertoglio, 2005). In the triple test, possibly due to its higher spatial complexity, animals may take longer to learn about all possible safe and unsafe locations, thus keeping the approach/avoidance conflict for a longer period of time, which would then lead to a persistent response to anxiolytic drugs. These enduring drug effects, together with the increased anxious-like behavior shown in trial 2, suggest that there is a quantitative rather than a qualitative change in emotionality between the first two repeated trials in the triple test.

The lack of significant anxiolytic-like effects in the OF and LDB areas, observed in experiment 1, in spite of being seemingly contradictory, agreed with previous reports on the triple test for both rats (Ramos et al., 2008) and mice (Fraser et al., 2010). Pilot experiments carried out in our laboratory (unpublished), showed that the standard LDB and OF tests, even when applied individually, were not sensitive to different doses of CDZ (0.5, 5 and 10 mg/kg) given to rats of the same strain as the one used herein. Taken together, these findings suggest that the LDB and the OF, at least in our hands, are not nearly as sensitive to benzodiazepine treatment as the EPM. This hypothesis is further supported by Chaouloff et al. (1997), who showed that rats tested in the LDB did not respond to doses of diazepam and CDZ that were effective in the EPM, and by Prut and Belzung (2003), who found that between 44 and 66% of all studies looking at the effects of benzodiazepines administered to rats or mice before the OF test either had no effect or showed unexpected anxiogenic-like effects. Further studies are necessary to investigate the potential sensitivity of the behaviors measured in the OF and LDB areas of the triple test to other classes of psychoactive drugs.

In experiment 2, where rats received only vehicle before the first triple-test trial and were then retested for three more days after receiving either CDZ or vehicle, no significant changes in behavior were observed between trials 1 and 2 in the vehicle-treated (MC) group (in this experiment, because CDZ was not used in trail 1, MC was the only group to provide valid information on the effect of a single test experience). However, a non-significant trend towards increased anxiety was seen in the second trial for all anxiety-related measures from the EPM and LDB. Such a trend is consistent with the significant effects seen in the EPM and the non-significant trends observed in the LDB data from experiment 1. Also consistently with experiment 1, CDZ produced an increase in the levels of exploration of the EPM open arms. However, such an effect was only observed on days 3 and 4, being larger and more significant in the latter than in the former. On the one hand, as far as days 3 and 4 are concerned, the present results totally corroborate the hypothesis that the triple test is not susceptible to repeated-trial tolerance. On the contrary, the response to CDZ increased with repeated testing. On the other hand, the lack of drug effect on day 2 (first day of CDZ treatment) suggests the existence of one-trial tolerance in the triple test when rats have been previously exposed to it in an undrugged state, as it has been widely demonstrated in the classical EPM (see Carobrez and Bertoglio, 2005). This intriguing observation could give rise to several tentative explanatory hypotheses. For example, the persistent effects of CDZ might only be observed in rats that had been previously exposed to the triple test under the effect of drugs (in this case, CDZ) that could cause some kind of learning impairment. However, such a hypothesis, as well as the conclusion that one-trial tolerance was indeed observed in the present study, should be considered with caution, as the baseline mean values of the two groups (CDZ and MC) were not the same on day 1, probably due to random variations between groups, and because tolerance did not appear in days 3 and 4. Further studies on that specific issue are warranted.

As aforementioned, the effect of one-trial tolerance has been previously observed also in mice retested in the classical LDB (Holmes

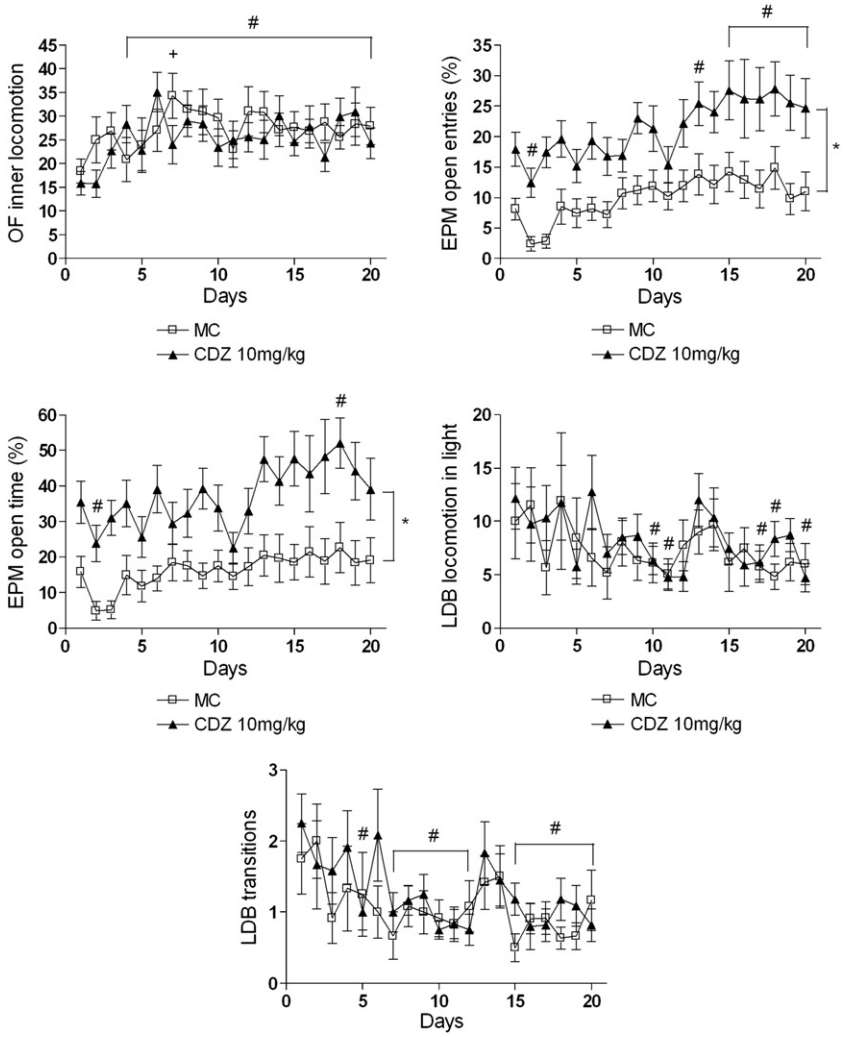

Fig. 5. Anxiety-related behaviors of male Wistar rats treated with either methylcellulose 0.5% (MC) or chlordiazepoxide (CDZ) at 10 mg/kg before being submitted for twenty consecutive days (with a 24-h interval between trials) to the triple test. Each trial lasted 15 min and the following variables were analyzed: inner locomotion in the OF, % entries and % time spent in the open arms of the EPM, locomotion in the light compartment of the LDB, and number of transitions in the LDB. Symbols and vertical lines represent the means and S.E.M. of animals grouped by pharmacological treatment ($n = 12$). * represents a significant ($p < 0.05$) effect of CDZ treatment over all trials. + represents a significant ($p < 0.05$) effect of CDZ treatment within a specific testing day. # represents a significant ($p < 0.05$) difference in relation to day 1 over both treatments (ANOVA for repeated measures followed by Duncan test).

et al., 2001). Surprisingly, in experiment 2, CDZ produced anxiolytic-like effects in the LDB, by increasing the number of squares crossed and the number of entries into the light compartment, regardless of the day of test (2, 3 or 4). These findings, when compared with those of experiment 1 (which showed only a trend towards anxiolysis on trial 2) and with two previous studies on rats and mice (which showed no anxiolytic effect on trial 1) (Fraser et al., 2010; Ramos et al., 2008), could suggest that the effects of benzodiazepines in the LDB area only occur with repeated testing. However, as discussed later, this hypothesis is not supported by the data from experiment 3. Other than considering these findings as a false positive result, an alternative explanation would be that anxiolytic effects can only be seen in the LDB if rats are first exposed to it undrugged. Although unlikely, since it contradicts the results obtained in the EPM, this hypothesis needs to be systematically tested in future studies.

In spite of not affecting the number of entries in the closed arms of the EPM, considered as a measure of general locomotion (Fraser et al., 2010; Ramos et al., 2008), CDZ produced a decrease in both inner and outer locomotion in the OF, regardless of the day of test. This general drop in the exploration of the OF did not affect the relative preference for its central area, thus probably reflecting only an increased preference of CDZ-treated rats for the other two devices of the triple test (EPM and LDB vs OF). The fact that CDZ increased all measures from the LDB area, including the locomotion in the dark compartment, supports this idea.

The results from experiment 3, where rats received a daily injection of either CDZ or its vehicle before being exposed to the triple test for 20 consecutive days, confirm that rats, on the second day of test, show increased indices of anxiety in the EPM in comparison with day 1. Such an effect, which was significant in experiment 1 but was only a trend in experiment 2, indicates that a single experience in the triple test increases anxiety-related behaviors in a subsequent trial, as it has been often shown in the classical EPM test (Carobrez and Bertoglio, 2005; Holmes and Rodgers, 1998). Yet, this increased anxiety is temporary, as it tends to go back to the original levels as early as at the third trial and then gradually decreases, to the point of being totally reversed in later trials. In spite of these overall temporal changes, CDZ had anxiolytic-like effects in the EPM throughout the whole 20-day period, thus confirming the absence of repeated-trial tolerance in the rat triple test.

One can hypothesize, as aforementioned, that the higher spatial complexity of the triple test, compared to its individual components where the animals can only choose between two types of environment, prevents (or at least slows down) the resolution of the conflict

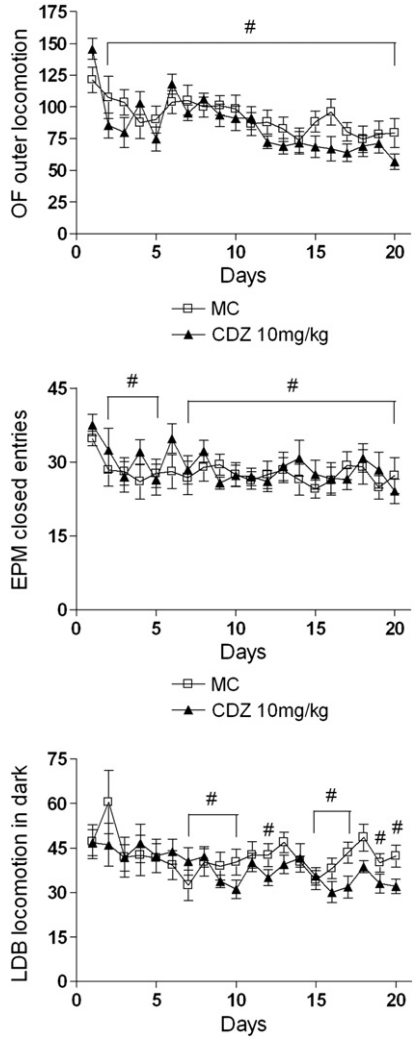

Fig. 6. Locomotion-related behaviors of male Wistar rats treated with either methylcellulose 0.5% (MC) or chlordiazepoxide (CDZ) at 10 mg/kg before being submitted for twenty consecutive days (with a 24-h interval between trials) to the triple test. Each trial lasted 15 min and the following variables were analyzed: outer locomotion in the OF, number of entries in the closed arms of the EPM, locomotion in the dark compartment of the LDB. Symbols and vertical lines represent the means and S.E.M. of animals grouped by pharmacological treatment ($n=12$). # represents a significant ($p<0.05$) difference in relation to day 1 over both treatments (ANOVA for repeated measures followed by Duncan test).

between exploring and avoiding potentially dangerous areas such as the open arms. According to this hypothesis, this lasting conflict would be responsible for the enduring anxiolytic effects of a classical benzodiazepine drug such as CDZ. Alternatively, it could be suggested that the absence of tolerance observed in the triple test could be explained solely by its trial duration. File et al. (1993) have shown in rats that the development of tolerance to diazepam in the test/retest protocol of the EPM could be prevented by increasing the duration of trials 1 and 2 from 5 to 10 min, possibly due to the rapid extinction of the secondary emotional state generated during the first few minutes of the test. Interestingly, very similar results were obtained in mice treated with CDZ and retested in the EPM (Holmes and Rodgers, 1999). Thus, because each trial in the triple test lasts 15 min, one could hypothesize that 15-min long trials in the EPM would also result in persistent anxiolytic drug effects, even with chronic administration and testing. To our knowledge, however, the effect of 10-min trials preventing the loss of drug effects in the EPM has never been reported for periods longer than two testing days. Moreover, three aspects cast doubt upon the idea that the effectiveness of the triple test in chronic protocols would be simply the result of longer trials. Firstly, unpublished data from our laboratory reveals that rats and mice submitted to the triple test spend, in average, only 332 and 324 s, respectively, inside the EPM area, which is much less than the reported 600 s necessary for the maintenance of the anxiolytic drug effects in the individual EPM (File et al., 1993; Holmes and Rodgers, 1999). Secondly, the present study showed that one-trial tolerance may have appeared in the triple test, even with 15-min long sessions, when rats were submitted undrugged to the first trial. Finally, it has been shown that the introduction of extra physical stimulation in the traditional EPM (e.g. a strong light or wind in the closed arms during trial 1 or a novel object in the open arms during trial 2) can prevent the development of one-trial tolerance, probably due to the preservation of the conflict across trials (Andreatini et al., 2003; Pereira et al., 1999). These findings provide further support to the idea that an enriched (or more complex) testing environment would be more adequate to test the predictive validity of chronic treatments and/or to model psychological traits and disorders that are typically complex and enduring (Lesch, 2001).

In the present study, the prolonged experience in the triple test led to a decrease in the outer locomotion and an increase in the inner locomotion in the OF, thus revealing an increasing preference for the central zone of the OF throughout time, which is consistent with the notion that the avoidance of the OF center, being fear-related, should diminish with repeated exposure (Archer, 1973). At the same time, the fact that inner locomotion did not respond to the anxiolytic-like effects of chronic CDZ, being thus partially consistent with experiment 1 (no effect) and experiment 2 (anxiogenic-like effect), suggests that this behavioral index may be sensitive to classes of drugs other than benzodiazepines, such as serotonin 5-HT1A receptor agonists, for example (Prut and Belzung, 2003). As far as the LDB is concerned, the results from the 20-day experiment did not confirm the hypothesis that the anxiolytic-like effect of CDZ could be perceived within this device under repeated testing. Excepting the explanatory hypotheses already proposed earlier (see discussion of experiment 2 in the previous page), the inconsistent effects of CDZ in the LDB area of the triple test remain difficult to explain.

In summary, the present study provides further support to the notion that the triple test, for allowing quasi-simultaneous measurements from the OF, EPM and LDB, is an interesting alternative to the use of the three individual tests performed as a battery (Ramos, 2008). Furthermore, it indicates that the triple apparatus, besides representing "a new way of using old tests" (i.e. simultaneously rather than successively), as originally proposed (Fraser et al., 2010), also adds behavioral information that cannot be gathered using the standard, separate apparatuses. Such a new feature, possibly generated by its higher spatial complexity, renders the triple test sensitive to the effects of CDZ for at least 20 consecutive days, therefore placing it as a promising tool to the longitudinal study of experimental anxiety.

Supplementary materials related to this article can be found online at doi:10.1016/j.pbb.2010.08.014.

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