

Temporal analysis of the rat's behavior in the plus-maze: effect of midazolam

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Abstract

The aim of the present study was to carry out a temporal analysis of the midazolam (MDZ)-induced anxiolysis in rats submitted to the elevated plus-maze (EPM) test. Male Wistar rats received either MDZ (0.5, 1.0 and 1.5 mg kg⁻¹) or saline (0.9%) and were submitted to the EPM test. Temporal analysis revealed that the group receiving MDZ (1.5 mg kg⁻¹), as well as the group treated with saline, displayed low %Open arm entries, which suggests increased anxiety over the test period. Motor activity, evaluated by the enclosed arm entries, was also decreased in both experimental groups, thus suggesting locomotor habituation. The treatment with MDZ (1.5 mg kg⁻¹) induced a clear anxiolysis during the first 3 min, but not at the end of the test, since only the %Open arm time remained increased. The data are discussed with reference to the lack of the test's sensitivity to alterations in the level of anxiety over time and with respect to a qualitative shift in the experimental anxiety at the end of the session. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

The elevated plus-maze (EPM) test [13] has been widely used to study experimental anxiety in both rats [18] and mice [16], as well as to screen new anxiolytic drugs [4,12,14,19]. The maze consists of two elevated open arms arranged at a right angle with two other arms enclosed by lateral walls. It has been proposed that animals exhibit lower exploration of the open relative to the enclosed arms due to the animal's inability to engage thigmotaxic behavior in open spaces [22]. The percentage of either entries into or time spent in the open arms (%Open arm entries and %Open arms time, respectively) represents behavioral variables which negatively correlate with the level of anxiety of the animals, since drugs with recognised anxiolytic activity increase the %Open arm entries and the %Open arm time, while drugs with anxiogenic activity decrease both of these variables [18].

Although most studies have expressed the animal behavior as an average of the total time of the session, a more

detailed analysis has been developed, by dividing the session into 1-min blocks [15,20,21]. This kind of analysis represents a useful tool to assess the temporal distribution of each experimental variable through the session. In the case of mice, it has been shown that the behavioral profile by the end of the session is different to that seen at the beginning, with evident open arm avoidance from the second minute of the session [15,20].

However, the literature lacks an equivalent analysis for the exploratory behavior of rats during the EPM experience. Moreover, a temporal analysis of the effect of anxiolytic drugs is also needed. The aim of the present study was to carry out a more detailed analysis ("min by min") of the exploratory behavior of rats treated with midazolam (MDZ) in the EPM test.

2. Method

2.1. Animals

Male Wistar rats (supplied by the Central Animal House of the Federal University of Santa Catarina) weighing approximately 250 g were housed in groups of five in

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polypropylene cages (49 × 34 × 16 cm) and underwent a period of adaptation for 7 days with free access to food and water, under a light/dark cycle of 12 h (lights on at 06:00 h). The animals were handled for weighing, drug administration and cleaning of the cages only. All the experimental procedures were conducted in compliance with recommendations of the “Principles of Animal Care” (NIH, 1985) and of the “Ethical Principles of Animal Experimentation” of the Brazilian College of Animal Experimentation (COBEA, 1991).

2.2. Apparatus

The EPM was made of wood and consisted of two opposed open arms (50 × 10 cm) and two other opposed arms of the same size, enclosed by opaque walls 40 cm high, except for the entrance. In order to avoid falls, the open arms were surrounded only by a short (1 cm) Plexiglas edge. The four arms were arranged in such a way as to form a cross. The arms extended from a central platform (10 × 10 cm) and the whole was raised 50 cm above the floor [18]. Four 15 W fluorescent lights arranged as a cross at 100 cm above the maze were used as the single source of illumination and provided a level of illumination of 120 and 60 lux in the open and enclosed arms, respectively. Each experimental session was recorded by a video camera and the videotapes were analysed for the whole session and for each 1-min block.

2.3. Drug

MDZ (Roche, Brazil) was dissolved in saline solution (0.9% w/v) and administered by i.p. route in a volume of 0.15 ml/100 g of body weight.

2.4. Procedures

The animals were submitted to an acute treatment with MDZ (0.5 mg·kg⁻¹, *n* = 15; 1.0 mg·kg⁻¹, *n* = 15 or 1.5 mg·kg⁻¹, *n* = 23) and were tested in the EPM 30 min later. The control group (*n* = 27) received an equivalent volume of saline solution. Each rat was placed at the centre of the maze facing an enclosed arm and allowed to explore the maze for 5 min. The standard spatio-temporal measurements, such as the number of entries into either the open or enclosed arms, the total number of arm entries and the time spent in the open arms were recorded. The exploratory behavior in the open arms was expressed as the percentage of entries into (%Open arm entries) and the time spent (%Open arm time). Arm entry and arm exit were defined as all four paws into and out of an arm, respectively. Between animals, the maze was thoroughly cleaned with alcohol 20% v/v and dried. Any animal which fell off the maze was excluded from the experiment. All the experiments were carried out between 13:00 and 17:00 h.

2.5. Data analysis

The statistical analysis of the full session was done by using one-way analysis of variance (ANOVA), while the data relative to the temporal analysis were analysed by two-way ANOVA, with the variable *Drug* as one factor and the variable *Time* (1 min blocks) as the second. Both one- and two-way ANOVA were followed by Duncan's test for multiple comparisons. Probability values less than 5% were considered significant.

3. Results

Fig. 1 shows the results of one-way ANOVA, by taking into account the full session pattern, of the data from the groups treated with different doses of MDZ. ANOVA revealed a significant difference between groups in the %Open arm entries ($F(3,91) = 9.1992$, $p < 0.0001$) and %Open arm time ($F(3,91) = 12.3032$, $p < 0.0001$). Duncan's test revealed that rats receiving the highest dose of MDZ (1.5 mg·kg⁻¹) displayed a significant increase in both %Open arm entries ($p < 0.0001$) and %Open arm time ($p < 0.0001$), relative to the group treated with saline. Rats receiving MDZ at the dose of 1.0 mg kg⁻¹ showed a significant increase in the %Open arm entries ($p < 0.05$), but not %Open arm time ($p = 0.0968$). There was no significant difference between the group treated with saline and the group treated with MDZ (0.5 mg·kg⁻¹), neither in the %Open arm entries nor in the %Open arm time. With regard to the number of entries into the open arms (Fig. 2), ANOVA revealed a significant difference between groups ($F(3,93) = 6.7116$, $p < 0.001$), with an increase in the open arm entries in the group treated with MDZ (1.5 mg·kg⁻¹) only ($p < 0.001$). There were no significant differences either in the enclosed

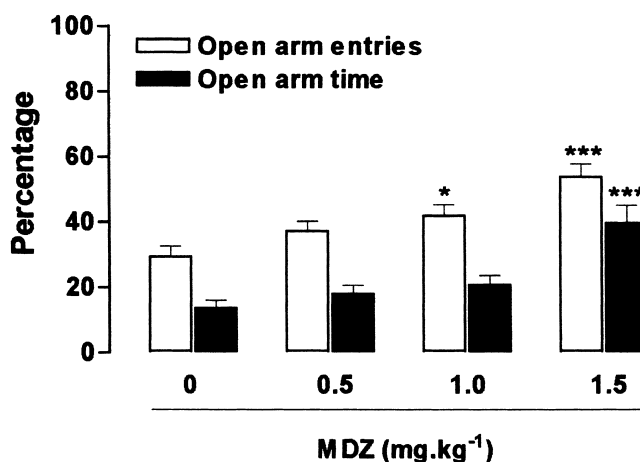


Fig. 1. Percentage of entries into and time spent in the open arms in rats treated acutely with either MDZ (0.5, 1.0 and 1.5 mg·kg⁻¹) or saline, 30 min prior to the EPM test. Each column represents the mean ± S.E.M. * $p < 0.05$ and *** $p < 0.0001$ relative to the control group (one-way ANOVA followed by Duncan's test for multiple comparisons).

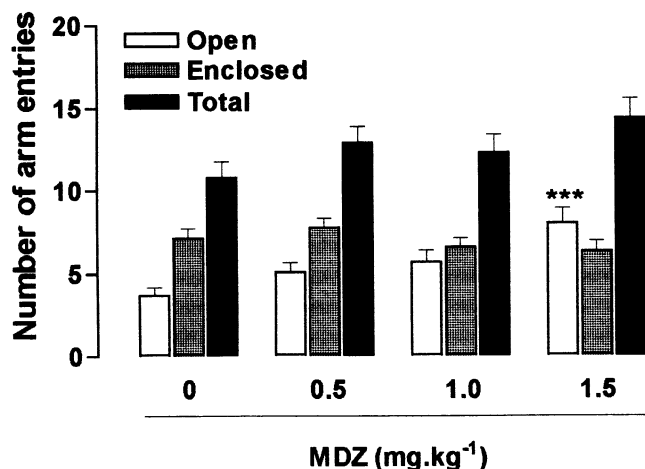


Fig. 2. Number of entries into the open and closed arms together with the total arms entries in rats treated acutely with either MDZ (0.5, 1.0 and 1.5 mg.kg⁻¹) or saline, 30 min prior to the EPM test. Each column represents the mean \pm S.E.M. *** p < 0.0001 relative to the control group (one-way ANOVA followed by Duncan's test for multiple comparisons).

arm entries ($F(3,91) = 1.0549$, $p = 0.3723$) or in the total arm entries ($F(3,91) = 1.8479$, $p = 0.1441$).

Data illustrated in Fig. 3 represent the temporal distribution of both %Open arm entries and %Open arm time over the duration of the EPM test. Two-way ANOVA revealed that rats receiving either saline or MDZ (1.5 mg.kg⁻¹) exhibited decreased %Open arm entries over the test ($F(4,240) = 9.3765$, $p < 0.0001$); in the group treated with MDZ (1.5 mg.kg⁻¹), the %Open arm entries was decreased in the fourth and fifth minutes (Duncan's test, $p < 0.05$), while in the group treated with saline, the same variable was decreased in the fourth minute only (Duncan's test, $p < 0.05$), relative to the respective first minute of the test (Fig. 3a). ANOVA failed to detect a significant difference within groups in the %Open arm time over the test in both groups (Fig. 3b). Previous treatment with MDZ (1.5 mg.kg⁻¹) induced an increase in the %Open arm entries ($F(1,240) = 31.7500$, $p < 0.0001$) in the first ($p < 0.05$), second ($p < 0.01$) and third ($p < 0.01$), but not in the fourth ($p = 0.0863$) and fifth ($p = 0.0621$) minutes of the test, relative to the group treated with saline (Fig. 3a). The %Open arm time was increased ($F(1,240) = 53.1446$, $p < 0.0001$) in the first ($p < 0.001$), second ($p < 0.001$), third ($p < 0.001$), fourth ($p < 0.001$) and fifth ($p < 0.05$) minutes of the session, relative to the group treated with saline (Fig. 3b).

Two-way ANOVA also revealed a significant difference in the open ($F(4,240) = 10.9965$, $p < 0.0001$), enclosed ($F(4,240) = 30.5055$, $p < 0.0001$) and total arm entries ($F(4,240) = 38.3624$, $p < 0.0001$) during the successive 1-min blocks (Fig. 4). In the group treated with saline, the open arm entries were decreased in the third ($p < 0.001$), fourth ($p < 0.001$) and fifth ($p < 0.001$) minutes, while in the group treated with MDZ (1.5 mg.kg⁻¹), the open arm entries were decreased in the fourth ($p < 0.001$) and fifth ($p < 0.001$) minutes, relative to the first minute of the

session (Fig. 4a). Previous treatment with MDZ (1.5 mg.kg⁻¹) induced increased open arm entries in the second ($p < 0.001$) and third ($p < 0.001$) minutes of the test, relative to the group treated with saline (Fig. 4a).

In the group treated with saline, the enclosed arm entries was significantly decreased in the second ($p < 0.0001$), third ($p < 0.0001$), fourth ($p < 0.0001$) and fifth ($p < 0.0001$) minutes of the session, while in the group treated with MDZ (1.5 mg.kg⁻¹) the reduction occurred in the second ($p < 0.001$), third ($p < 0.0001$), fourth ($p < 0.0001$) and fifth ($p < 0.0001$) minutes of the session, relative to the respective first minute (Fig. 4b). There were no significant differences between the groups in the enclosed arm entries over the test period ($F(1,240) = 1.3143$, $p = 0.2527$).

Duncan's test indicated that in the group treated with saline, total arm entries was decreased in the second ($p < 0.0001$), third ($p < 0.0001$), fourth ($p < 0.0001$) and fifth ($p < 0.0001$) minutes of the session, while in the group treated with MDZ, the total arm entries was decreased in the

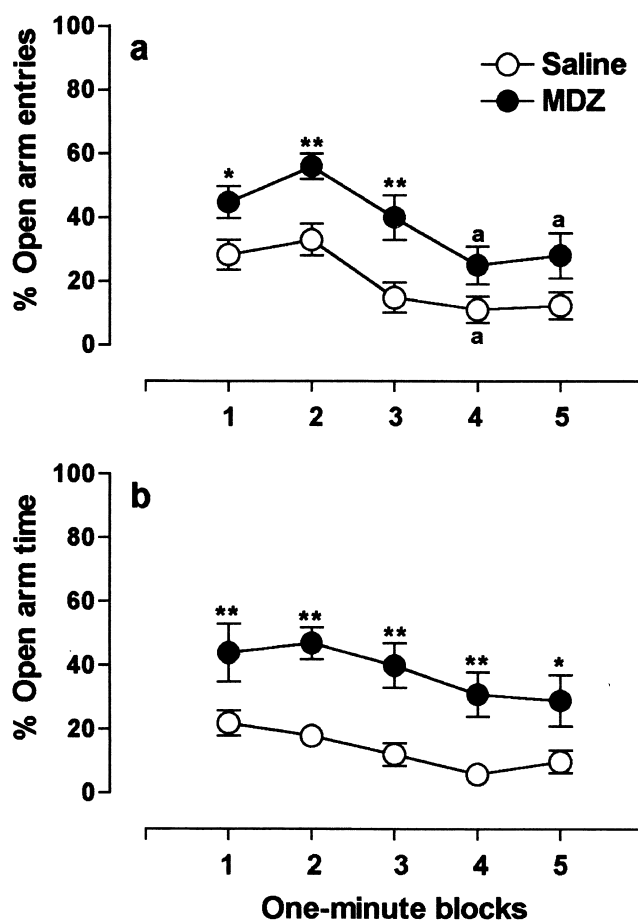


Fig. 3. Minute-by-minute distribution of the percentage of entries into (a) and time spent in (b) the open arms in rats treated acutely with either MDZ (1.5 mg.kg⁻¹) or saline, 30 min prior to the EPM test. Data are presented as the mean \pm S.E.M. by 1-min blocks. * p < 0.05 and ** p < 0.001 relative to the group treated with saline; ^a p < 0.05 relative to respective first minute of the test (two-way ANOVA followed by Duncan's test).

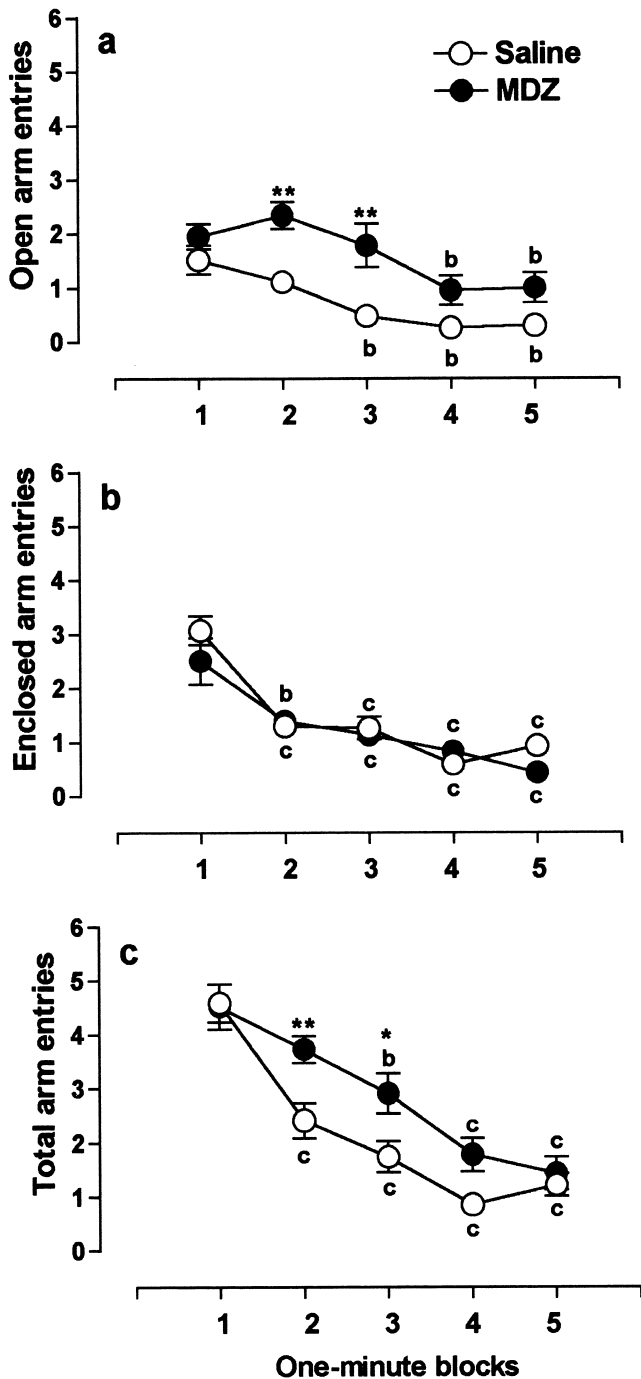


Fig. 4. Minute-by-minute distribution of the number of entries into the open (a) and enclosed (b) arms, together with the total number of arm entries (c) in rats treated acutely with either MDZ ($1.5 \text{ mg}\cdot\text{kg}^{-1}$) or saline, 30 min prior to the EPM test. Data are presented as the mean \pm S.E.M. by 1-min blocks. * $p < 0.05$ and ** $p < 0.001$ relative to the group treated with saline; ^b $p < 0.001$ and ^c $p < 0.0001$ relative to respective first minute of the test (two-way ANOVA followed by Duncan's test).

third ($p < 0.001$), fourth ($p < 0.0001$) and fifth ($p < 0.0001$), relative to the first minute of the session. Between-groups comparison revealed that the group receiving MDZ displayed a higher total arm entries in the second ($p < 0.001$), third ($p < 0.05$), but not in the fourth

($p = 0.0570$) and fifth ($p = 0.6274$) minutes, relative to the group treated with saline (Fig. 4c). There were no drug \times time interactions, neither in the %Open arm entries ($F(4,240) = 0.4454$, $p = 0.7756$), %Open arm time ($F(4,240) = 0.3244$, $p = 0.8614$), Open arm entries ($F(4,240) = 1.2666$, $p = 0.2837$), enclosed arm entries ($F(4,240) = 1.2543$, $p = 0.2886$) nor in the total arms entries ($F(4,240) = 1.9625$, $p = 0.1009$).

4. Discussion

Underlying the behavior of rats in the EPM test is a conflict between the animal's motivation to explore the maze and avoid the open arms, motivated by the fear [17] elicited by the rat's inability to engage thigmotaxic behavior in open spaces [22]. Thus, the animal exhibits a reluctance to explore the open arms of the maze and displays a clear closed arms preference, characterized by a higher number of entries into and time spent in this kind of arm, which is protected by lateral walls [18].

Factor analysis studies of the rat's behavior in the EPM test have demonstrated that the %Open arm entries and %Open arm time represent variables which have a high loading with anxiety factor, thus they are used as anxiety indices, while the variable enclosed arm entries loads with an independent factor, related to motor activity [8]. Thus, it is possible to determine whether a given drug's effect (i.e. anxiolytic or anxiogenic) is being disguised by alterations induced by the drug in the motor activity of the animals. On the other hand, the total arm entries reflect a variable which load simultaneously with anxiety and motor activity factors and therefore, in contrast with the enclosed arm entries, do not represent a reliable approach to evaluate neither anxiety nor motor activity [8].

It has been shown that benzodiazepine-like drugs induce increased exploration of the open arms, by increasing both %Open arm entries and %Open arm time, with no change in overall motor activity; typically, these studies have evaluated the anxiolytic effect of the drug by evaluating the animal's behavior as an average of the total time of the test [2,7,9,18,22]. Similarly, the present study showed that previous treatment with MDZ ($1.5 \text{ mg}\cdot\text{kg}^{-1}$) induced an evident anxiolytic effect, since both %Open arm entries and %Open arm time were increased, with no change in the enclosed arm entries. Thus, the effect of the MDZ cannot be ascribed to non-specific action of the drug.

The data relative to temporal analysis revealed a significant reduction in the %Open arm entries in the group treated with MDZ at the end of the test, specifically at the fourth and fifth minute, even the animals treated with saline displayed the same arm preference at the end and at the beginning of the test. In contrast, the %Open arm time remained unchanged through the test in both experimental groups. We have no satisfactory explanation for this result, since an evident avoidance of the open arm over the

duration of the test has been previously demonstrated in mice [15,20]. However, it is possible that the increased anxiety during the EPM test may not be so evident in the case of rats. On the other hand, it is also possible that the absence of increased anxiety over the test, when evaluated by the %Open arm time, may be reflecting a lack of the sensitivity of the EPM test to detect alterations in the level of anxiety over time [6]. This question could be addressed by using either a plus-maze without ledges or an ethological approach for the temporal analysis, thus increasing the test's sensitivity to changes in the level of anxiety.

The temporal analysis also revealed a reduction in exploratory behavior in both kinds of arms over the test period. In both groups, the enclosed arm entries decreased from the second minute of the session, thus indicating reduced motor activity over the test, which may be associated with locomotor habituation to the maze. In contrast to the present study, an evident increase in enclosed arm entries from the second minute of the test has been shown in mice [15,20]. It is possible that these contradictory results may be explained by organismic or procedural factors, especially the criteria used to define arm entry/exit previously used (i.e. arm entry defined as all four paws into an arm and arm exit defined as two paws onto the central platform; [15]). This difference in arm entry/exit scoring can represent a significant source of variation and it is important therefore, that the same criteria are used for both arm entry and exit, in order to avoid variations both within and between laboratories [19].

The present study also showed that the reduction in motor activity precedes and is followed by increased anxiety in the last 2 min of the test, since in both groups there was a reduction in the enclosed arm entries in the second minute of the test, when the animals still exhibited the same level of anxiety as in the first minute.

When the BDZ-induced anxiolysis was divided into 1-min blocks, it became clear that an evident anxiolytic effect was occurring at the beginning of the session, specifically during the first 3 min, since both %Open arm entries and %Open arm time were increased. In the fourth and fifth minutes, MDZ was no longer able to increase the ratio of open: total arm entry. The anxiolysis induced by MDZ still remained at the end of the test, when evaluated by the %Open arm time. Whether the impaired MDZ-induced anxiolysis at the end of the test is due to a lack of sensitivity in the test requires further investigation [6].

On the other hand, it has been reported in the literature that a second exposure to the EPM induces increased anxiety in rodents [1,5,20,22]. Moreover, a previous exposure to EPM also counteracts the anxiolytic effect of benzodiazepines [7,10,11], the explanation for which has varied between “one trial tolerance” [7], locomotor habituation [3] and a shift in the experimental anxiety at the second trial [10,11], against which the benzodiazepines are clinically ineffective; the latter is particularly noteworthy, since anxiety is a heterogeneous psychiatric disorder and the

benzodiazepines are not able to suppress all of its different clinical manifestations. Consequently, if rats display a different kind of anxiety during the second trial on the EPM, it is possible that such an emotional state may be manifested even during the first exposure. Although we did not carry out a second exposure to the EPM, the present results, showing the impaired MDZ-induced anxiolysis at the end of the session, lend support to this presumption.

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