Novel and simple behavioral paradigm for assessing anxiety in rats: effect of diazepam

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Abstract

OBJECTIVES: Anxiety is an emotional state experienced by people, and is not readily modeled in animals. In order to extend till now ethologically derived paradigms used in the evaluation of anxiety and fear in rodents, a modified open-field was designed.

METHODS: Spontaneous behavior of male rats was investigated in the elevated arena; the bottom was divided by inter-space of different width (2, 4, 6, 8 and 10 cm) into two identical parts. Anxiolytic effects of diazepam (DZP) at doses 0.5, 1.0, 2.5 and 5.0 mg/kg were investigated in the newly designed device and compared with the effects of similar doses in a large circular open-field arena.

RESULTS: In Experiment 1 the progressive extension of the inter-space prolonged the first passing, decreased the total number of passing, and increased the inter-space sniffing in intact animals. In Experiment 2 DZP at doses 1.0, 2.5 and 5.0 mg/kg significantly enhanced the readiness to cross, the frequency of passing the inter-space and decreased inter-swpace sniffing as compared to controls. In Experiment 3 we found that DZP at doses 0.1 and 0.3 mg/kg increased behavioral activity both along the perimeter and in the center of the arena, thus indicating lower level of anxiety.

CONCLUSION: The presented modified open-field test is a useful paradigm to investigate risk assessment behavior in rats, and may provide a sensitive novel model of anxiety and fear level.

INTRODUCTION

Animal behavioral models of anxiety have played an important role in the assessment of putative anxiolytics. There are currently many animal studies showing that several classes of anxiolytic drugs produce a dose-dependent effect with relative potency that is consistent with their clinical effectiveness in the treatment of human anxiety and fear. In rodents there are several traditional models of anxiety including paradigms based on exploratory-locomotor behavior (open-field, hole-board, elevated plus-maze, light-dark box, social interaction, mirror chamber) and conditioned or un-conditioned threat responses (for a review see Rodgers, 1997; File, 1985a; Blanchard et al., 1990a,b; Flint, 2003; Carobrez & Bertoglio, 2005; Lister, 1990; Ramos & Mormede, 1998; Treit, 1985). In the very popular elevated plus-maze paradigm two basic indices of decreased anxiety

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have been validated: the preference for the open arms and the increased time spent on the open arms, however, without altering the total number of arm entries (Pellow et al., 1985). In the light/dark paradigm drugs which increase the transitions from the dark to the light of the box at doses that do not increase locomotion activity are considered to be anxiolytic (Chaouloff et al., 1997; Crawley, 1981). Finally, in the open-field paradigm an increased entry and the time spent in the center of the arena (therefore, a decreased thigmotaxis) reflects decreased anxiety altered by an effective drug (File, 1985b; Robbins, 1977; Walsh & Cummins, 1976). Therefore, the drug effect on spontaneous locomotion activity itself should be carefully distinguished from an anxiolytic one. All these above-mentioned paradigms are considered as models of conflictive exploratory behavior by which unconditioned fear of animals is measured.

So far, there is no model making it possible to evaluate behavioral response of rodents following placing in the area in which spontaneous locomotion and exploration is affected and limited by an „inter-space" in the bottom of the sufficiently elevated testing arena. A differently wide inter-space can be a handicap for an animal to get on the opposite part of the arena; an animal cannot circumvent or to avoid the inter-space. Moreover, an animal is exposed to "anxiety and fear" induced by stimuli coming from the perceived deepness. We anticipate that under these conditions "anxiety and fear level" can be primarily indicated by the passing latency and by the total number of passing the inter-space. If it is true, then both mentioned behavioral parameters could be successfully modified by an anxiolytic drug. The use of such induced "anxiety and fear" in animals could be a model of the normal human anxiety everyone is faced when confronted with a stressful or threatening situation. We also assume this method could be a model to test behavioral effects of classical benzodiazepines and 5-HT_{1A} agonists.

In the present study, three experiments were designed. First, an ability to pass the inter-space of different width was examined in intact adult male rats. Based on the results, three "critical" inter-spaces were chosen. Second, to test the suitability of the designed method we employed a clinically effective benzodiazepin diazepam (DZP), the most used compound in studies investigating the anxiolytic-like action in animal studies. We anticipated that the DZP treated animals will be able to pass more readily "critical" inter-spaces. Third, the effect of DZP was investigated in the circular open-field in order to examine if there is a correlation between the findings in these two different tests. The open-field paradigm makes it possible to measure locomotion and time spent in the inner zone of the arena, another variable widely considered to reflect the anxiety and fear level. Moreover, it also serves as a convenient procedure to measure sedation or activation which enables to examine the influence of the used range of doses on behavior from this point of view. In our laboratory the circular open-field arena is used to measure behavioral output of stressed (immobilized) rats (Klenerová et al., 2008).

In summary, the purpose of the present study was: (a) to characterize risk assessment behavior of rats placed in the arena in which they are exposed to the danger of the fall into the deepness; (b) to extend till now ethologically derived paradigms used in the evaluation of anxiety and fear.

MATERIAL AND METHODS

Animals

At arrival, adult Wistar male rats (VELAZ, Prague, Czech Republic) aged 100 days (230–260 g) were housed in a temperature controlled room (20–22 °C) in standard plastic cages with three animals in each. All animals were maintained on a natural day-night period (March-April) for at least 2 weeks before the start of the experiment. Food and water were available ad libitum. The animals were daily handled by the same person.

Experimental design and procedure

Behavioral testing was conducted between 08.00 and 12.00 (a.m.) in the experimental room illuminated from the ceiling with a 25-W fluorescent tube (diffuse artificial light). An experienced observer was present in an adjacent room separated from the experimental one by a two-way window. In the first and the second experiment the testing arena (120 x 60 x 30 cm) made from transparent plastic was located 140 cm above the floor. It was divided into two identical parts by an inter-space of a different width: 2, 4, 6, 8 and 10 cm. In the first experiment intact animals were tested. Based on the results three "critical" inter-spaces were chosen (6, 8 and 10 cm), a critical value being defined as a significantly decreased ability of animals to pass the inter-space of a relevant width. In the second experiment, animals received intraperitoneally DZP (SPOFA, Prague, Czech Republic) in saline solution at doses 0.5, 1.0, 2.5 and 5.0 mg/kg of the body weight in a volume of 1 ml/kg, always 60 min before the testing. In both experiments, an animal was placed at the centre of one out of two parts of the arena and the testing lasted for 8 min. In the third experiment, the effect of DZP (0.1, 0.3 and 1.0 mg/kg) was measured in a circular arena with the diameter of 150 cm the wall being 50 cm high. Behavioral testing started 60 min after DZP administration and lasted 5 min. In all experiments the controls received saline. Also, before using another animal the arena was carefully washed and cleaned. Always, animals were randomly assigned to the control and DZP treated groups (always, n = 8 per group). Each animal was used only once; the total number of animals being as follows: Experiment 1, n = 40; Experiment 2, n = 120; Experiment 3, n = 32.

Table 1. Behavioral performance of intact male rats in the open-field arena divided by the inter-space of different width.

Behavioral variable	Inter-space width				
	2 cm	4 cm	6 cm	8 cm	10 cm
Locomotion latency (s)	20.4 ± 2,3	23.7 ± 4.6	25.0 ± 3.8	23.5 ± 2.6	22.7±4.4
1st passing latency (s)	31.5 ± 3.3	43.5 ± 5.3	63.3 ± 7.2	95.8 ± 10.8*°″	139.9 ± 21.9*°″
Total passing number	23.6 ± 4.0	18.8 ± 2.0	15.4 ± 2.4*	7.1±1.3*°″	4.0 ± 1.2*°″
Total locomotion time (s)	220.8 ± 8,9	215.0±11.5	206.8 ± 9.0	195.3±11.1	206.0±12.2
Total inter-space sniffing (s)	21.0 ± 2.6	30.3 ± 3.8	47.5 ± 5.6*″	77.9 ± 6.9*°″	100.7 ± 8.6*°″±
Total rearing number	11.3 ± 1.9	12.1±2.1	11.8 ± 1.7	13.5 ± 2.2	12.0 ± 1.9

For explanation of behavioral variables and inter-space width see Material and Methods.
Data are expressed as the mean+ S.E.M.; n = 8 for each group.
Statistical significance: Student-Newman-Keuls test, $p < 0.05$ * vs 2cm. ° vs 4 cm, " vs 6 cm, + vs 8 cm.

Behavioral measurement

In the first and the second experiment observed behavioral variables were recorded by typing keys on the keyboard of a computer. The following variables of spontaneous behavior were recorded: the latency time (s) of locomotion activity, the latency time (s) of the first inter-space passing, the total number of inter-space passing, the total time (s) spent in the locomotion, the total time (s) spent in sniffing the inter-space, the total number of rearing on the hind limbs. In the third experiment, behavior of rats was video-monitored by an automated activity monitoring system (AnyMaze, Stoelting, USA). Locomotor activities were recorded automatically; the experimenter measured rearing and grooming (face washing, body and genital grooming, body and paw licking, scratching).

Statistics

In the first experiment, one-way analysis of variance (ANOVA) with factor inter-space was used. In the second experiment, a two-way analysis of variance (ANOVA) with factors inter-space and treatment was used. In the case of a significant treatment x inter-space interaction, one way ANOVA for individual factors was done. In the third experiment, one-way ANOVA with factor dose was used. Always, ANOVA was followed with Student-Newman-Keuls method with the aim to compare a difference between two particular groups. Statistical significance was accepted when $p < 0.05$.

RESULTS

Experiment 1

The results are summarized in Table 1. Regarding the latency of the first inter-space passing, one-way ANOVA revealed a significant inter-space effect [$F(4,35) = 13.98$, $p < 0.0001$]. The first passing progressively prolonged with the extension of the inter-space being the longest in animals exposed to the inter-space wide 10 cm. As for the total number of passing, a significant inter-space effect was found [$F(4,35) = 11.54$, $p < 0.0001$]. The wider was the inter-space, the lower was the total number of passing. No significant difference in the total number of passing was revealed between those wide 2 and 4 cm. A significant effect was also found in the time spent in the inter-space sniffing [$F(4,35) = 31.84$, $p < 0.0001$]. This behavioral variable increased gradually with the extension of the inter-space being the lowest in inter-spaces 2 and 4 cm of width. No significant differences were found in the locomotion latency [$F(4,35) = 0.22$, $p = 0.93$], the total locomotion time [$F(4,35) = 0.84$, $p = 0.51$] and the total rearing number [$F(4,35) = 0.18$, $p = 0.95$].

Experiment 2

The results are presented in Fig.1. Regarding the locomotion latency, two-way ANOVA revealed a significant treatment effect [$F(4,105) = 5.57$, $p = 0.0004$], but no significant inter-space effect [$F(2,105) = 0.18$, $p = 0.84$] and treatment x inter-space effect [$F(8,105) = 0.31$, $p = 0.96$]. Animals given DZP at doses 1.0, 2.5 and 5.0 mg/kg had significantly shorter locomotion latency than the controls and those given 0.5 mg/kg dose. No difference was between the controls and those treated with the lowest DZP dose. As for the latency of the first inter-space passing, two-way ANOVA revealed a significant treatment effect [$F(4,105) = 52.64$, $p < 0.0001$], a significant inter-space effect [$F(2,105) = 53.46$, $p < 0.0001$] and a significant treatment x inter-space effect [$F(8,105) = 8.74$, $p < 0.0001$]. Subsequent one-way ANOVA showed that the passing latency shortened significantly with higher DZP doses [$F(4,115) = 21.48$, $p < 0.0001$], very short passing latency being in rats given 2.5 and 5.0 mg/kg dose. Regardless of the treatment, one-way ANOVA showed that the wider was the inter-space, the longer was the first passing latency [$F(2,117) = 16.23$, $p < 0.0001$]. The overall analysis (two-way ANOVA) of the total number of passing revealed a significant treatment effect [$F(4,105) = 49.12$, $p < 0.0001$], a significant inter-space effect [$F(2,105) = 48.66$, $p < 0.0001$] but no significant interaction effect [$F(8,105) = 1.26$, $p = 0.27$]. Compared with the control animals, DZP increased dose-dependently the total passing number under all three used inter-spaces. Nevertheless, the wider was

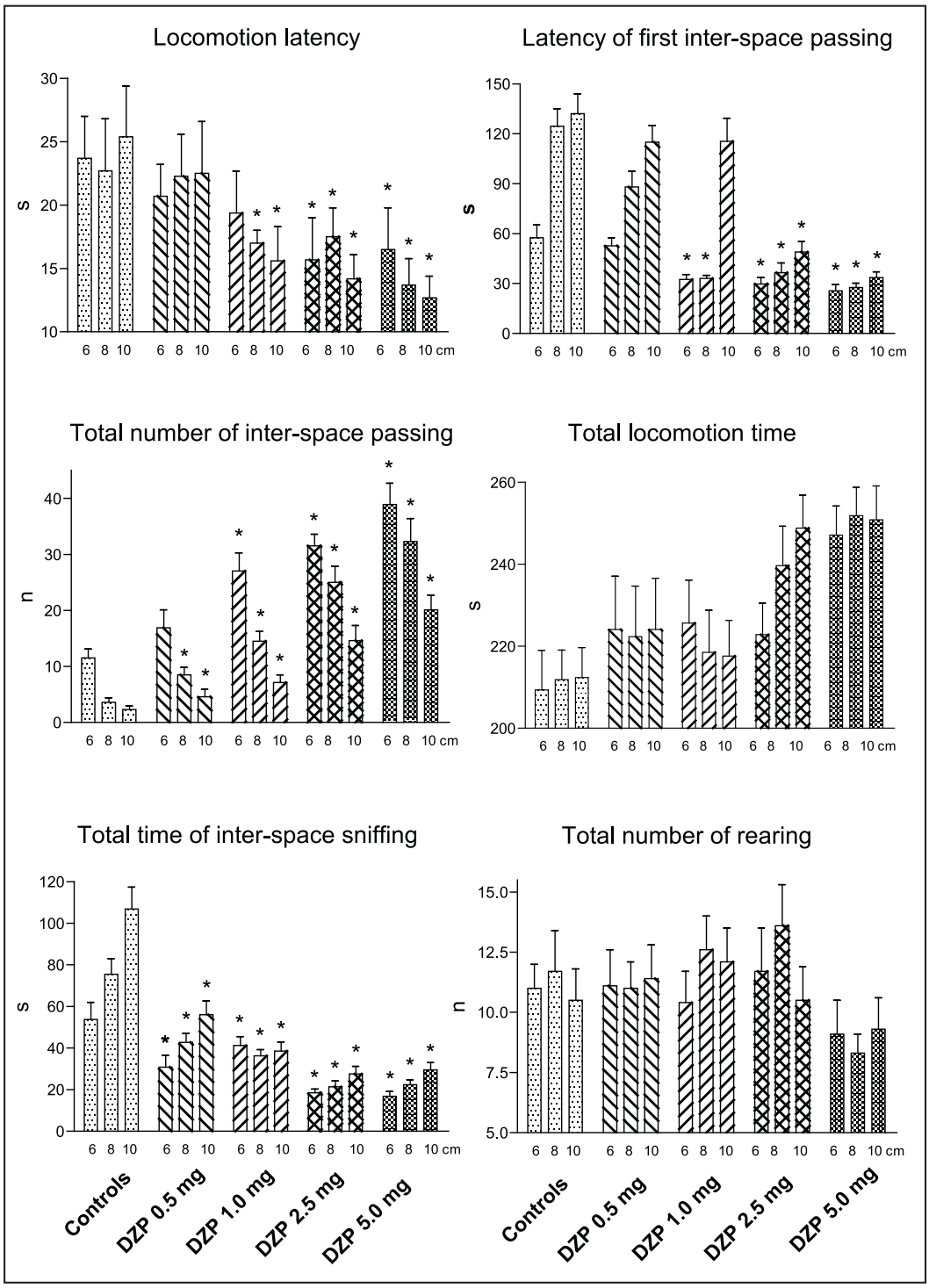

Fig. 1. Effect of different diazepam (DZP) doses on behavioral performance of male rats placed in the open-field arena divided by the inter-space of a different width (Experiment 2). Axis X: Inter-space width: 6, 8 and 10 cm. Doses of DZP for all parameters are given at the bottom of the third graphs on the page. Axis Y: Behavioral performance is expressed as the mean ± SEM. Statistical significance, $p < 0.05$: * For a clear view significant difference with a corresponding control group is indicated; for details see Results.

the inter-space, the lower was the total passing number. Regarding the total locomotion time, two-way ANOVA revealed a significant treatment effect [$F(4,105) = 7.83$, $p < 0.0001$] but no significant inter-space effect [$F(2,105) = 0.35$, $p = 0.71$], and no significant treatment x inter-space effect [$F(8,105) = 0.49$, $p = 0.86$]. Compared with the controls, 2.5 and 5.0 mg/kg DZP doses increased significantly locomotion time; the same holds true for animals given 5.0 mg/kg dose when compared with those given 0.5 and 1 mg/kg dose. As for total sniffing time, two-way ANOVA revealed a significant treatment effect [$F(4,105) = 55.77$, $p < 0.0001$], a significant inter-space effect [$F(2,105) = 16.96$, $p < 0.0001$] and a significant treatment x inter-space effect [$F(8,105) = 4.04$, $p =$

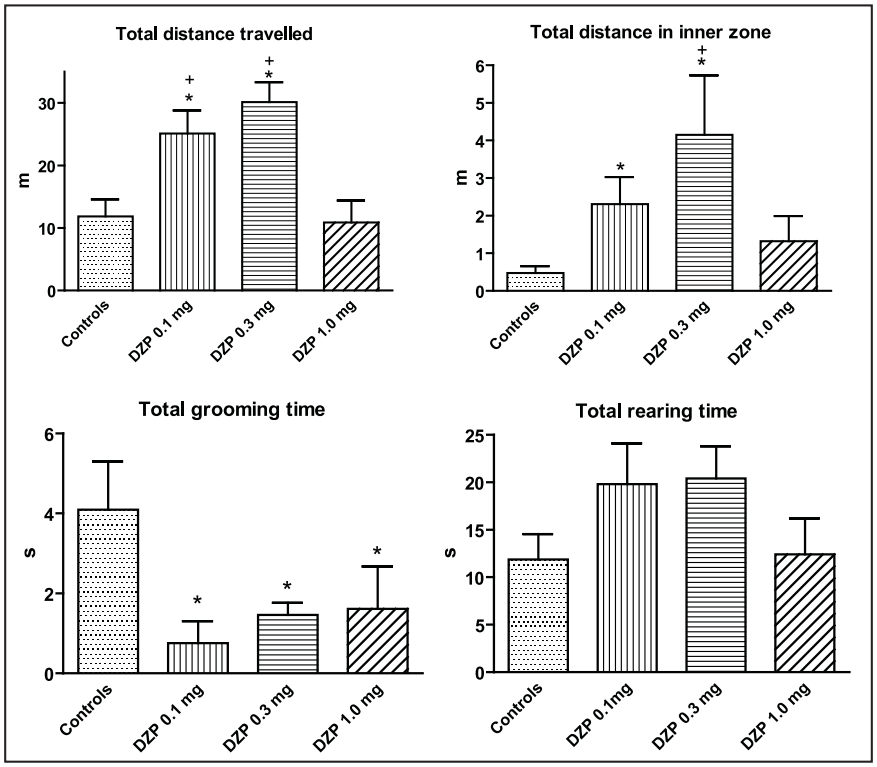

Fig. 2. Effect of different diazepam (DZP) doses on behavioral performance of male rats placed in a circular open-field arena (Experiment 3). Behavioral performance is expressed as mean ± SEM. Statistical significance, p < 0.05: * vs Control group, + vs DZP 1.0 mg group.

0.0003]. Subsequent one-way ANOVA showed that the sniffing time decreased significantly with higher DZP dose [F(4,115) = 37.45, p <0.0001] being very short in rats given 2.5 and 5.0 mg/kg DZP doses. Further, regardless of the treatment one-way ANOVA showed that the wider was the inter-space, the more time the animals devoted to the sniffing [F(2,117) = 5.51, p = 0.005]. Finally, with regard to the total number of rearing, two-way ANOVA revealed no significant effect of both treatment [F(4,105) = 2.11, p = 0.08] and inter-space effect [F(2,105) = 0.26, p = 0.78] and treatment x inter-space interaction [F(8,105) = 0.36, p = 0.94].

Experiment 3

The results are presented in Fig. 2. The one-way ANOVA revealed a significant effect of treatment on the total distance travelled [F(3,28) = 8.07, p = 0.001]. Compared with the controls as well as with animals given the highest DZP dose (1.0 mg/kg) the travelled distance was significantly increased in those given the lowest dose (0.1 mg/kg) and the medium dose (0.3 mg/kg) of DZP, no difference being between these DZP doses. Also, no difference was found between the controls and those given the highest DZP dose. The same holds true for the total distance travelled in the inner part of the arena: the overall analysis showed a significant dose-dependent difference [F(3,28) = 2.28, p = 0.03]. No significant dose-dependent effect was found in the total number of rearing [F(3,28) = 0.29, p = 0.83]. However, animals given all three DZP doses had significantly decreased total time spent in grooming [F(3,28) = 3.35, p = 0.03] which contrasted with the controls.

DISCUSSION

Adult male rats used in the present study (Experiment 1 and 2) were placed for the first time in an unfamiliar open-field arena divided in two identical parts by an inter-space of different width. To come on the opposite part of the arena an animal had to cross the inter-space without any opportunity to circumvent or to avoid this obstacle. Moreover, the bottom of the arena was at the height of 140 cm above the floor. We supposed that except for a fear of environment novelty, the inter-space in combination with the deepness can reinforce anxiety and fear level of animals.

In Experiment 1, intact rats safely passed the inter-space 2 and 4 cm of the width. Although there was no difference in the latency of the first passing of the inter-space wide 6 cm, the total number of passing decreased significantly. The passing frequency was very low and the first passing occurred after a long period of time in animals exposed to the inter-space wide 8 and 10 cm. The total sniffing time of the inter-space increased significantly in animals exposed to the inter-space 6, 8 and 10 cm of the width. We add that the majority of rats stopped on the edge of the inter-space 8 and 10 cm of width and sniffed it. Presumably, they hesitated shortly whether to go back or to proceed on the opposite side of the arena. The posture of rats was suggestive of the

stretched attend posture described as a risk assessment behavior (Blanchard et al., 1990a,b; Grewal et al., 1997; Mikics et al., 2005). No difference was found either in the locomotion latency or the total time spent in the locomotion and the total number of rearing. Based on these results we conclude that the probability of the passing decreased significantly with the extension of the inter-space. Therefore, the inter-space wide 6 cm and more was designated as a "critical" bar. The results confirmed that three behavioral parameters, i.e. the latency of the first passing, the total number of passing and the total inter-space sniffing, may provide a sensitive tool and a high predictive value for pharmacological characterization of tested substances.

In Experiment 2, DZP treated animals were more successful in coping with the inter-space of critical width than the controls. Above all, the latency of the first passing shortened with the administration of higher DZP doses, although it prolonged with a wider inter-space. Further, compared with the controls DZP increased the total number of passing irrespective of the inter-space width; nevertheless, the wider was the inter-space, the lower was the total number of passing. In every case, these results can be considered as a decrease or even a suppression of anxiety and fear level by DZP. These results are in agreement with the effectiveness of DZP using other behavioral paradigms (Cruz et al., 1994; Merlo-Pich & Samanin, 1989; Poschel, 1971; Rex et al., 1996; Rodgers & Cole, 1994; Rohmer et al., 1990; Yamamoto & Ueki, 1987). Our data also demonstrate that DZP decreased the inter-space sniffing. Although DZP increased dose-dependently both the total locomotion time and decreased the locomotion latency, a significant dose-dependency on the inter-space width was not revealed. This result excludes a possibility that the increase in the total number of passing and shortening the first passing latency are directly related to the stimulatory effect of the drug. Moreover, no stimulatory effect of DZP on the rearing was found.

The success of any behavioral paradigm is limited by the characterization of the phenomena that are being modelled. It is feasible to assume that our experimental paradigm represents situation, which rats may encounter in their natural habitat. In other words, we focused on the capacity of rats to cope with ecologically relevant problem. Even, the response to a novel situation, i.e. "a pit" that has to be vanquished can be considered as a novelty stress resulting in the increased anxiety and fear level. In the present study DZP evidently suppressed anxiousness and facilitated passing the inter-space that showed as a "critical" for intact animals.

In Experiment 3, the results obtained in the circular open-field arena principally agree with the outcome of most studies dealing with DZP, i.e. the increase of locomotor activity indicating anxiolytic effect (Prut & Belzung, 2003). A large, plain and illuminated arena is perceived as an aversive, frightening environment and the behavior of a subject being placed in it reflects the reaction of the subject to that stressful event. It has been widely accepted that stress induces anxiety-like behavior expressed in the open-field as an inhibition of locomotion; the increase in locomotion resulting from the effects of anxiolytic drugs then is considered to reflect lowering of the anxiety level (Prut & Belzung, 2003; Van Dijken et al., 1992).

In the present study the two lower doses of DZP increased the total distance traveled; the increase in rearing time did not reach significance. The 1.0 mg dose neither increased nor reduced the locomotion. Presumably, the sedative effect of that dose prevailed over the disinhibitory, anxiolytic one. Higher doses of DZP induce muscle-relaxation (Matsubara & Matsushita, 1982), however, the rearing score after the 1.0 mg dose, equal to the control values, does not indicate a stronger influence of the dose in this respect.

Another important factor determining rodent´s behavior in the open-field is their preference to walk close to the walls; in fact, traveling or staying close to the walls of the arena has been a prominent feature of behavior of laboratory rats, suggesting that the walls confer anxiety-relieving body contact (Eilam, 2003; Genaro & Schmidek, 2000). Locomotion and time spent in the inner zone of the arena is now widely considered another variable reflecting the anxiety level. In the present experiment the distance traveled by the control rats in the inner zone fell bellow 5% of the total traveled distance, the value being so low most probably due to the large size of the arena (Eilam, 2003). All three doses of DZP increased the inner zone ambulation, the enhancement after the two lower doses being significant.

Grooming behavior in the open field situation is considered a response to the low stress induced by introducing the animal in the novel environment (Spruijt et al., 1992; Gispen & Isaacson, 1981). Also, it has been accepted that grooming appears in connection with the lowering arousal following the stressful event. Yet, it is not quite clear if grooming appears as an indicator of de-arousal, or if it actively participates in the stress and arousal attenuation (Kalueff & Tuohimaa, 2004; Van Erp et al., 1994). DZP reduced grooming after all three doses and in our view, this effect indicates reduction of stress consequences and of the concomitant arousal.

These results show that in the used large, circular open-field arena, DZP clearly influenced the behavior in a way considered as indicating reduced anxiety level. Although the doses applied in the two experimental paradigms were not identical, it is conceivable to assume, that in the used dose range, DZP produced anxiolytic-like effects in both used models. These effects were produced with slightly lower doses in circular arena than in newly designed experimental model, which however offers the evaluation of some other behavioral parameters. The indication of sedative effect of 1.0 mg DZP in the plain open-field contrasted with the observed absence of depression after this and higher doses when the bottom of the arena was equipped with the inter-

space. The deepness stimuli may account for inducing higher level of emotions and consequently less responsiveness to the drug sedative action.

Although for animals the distinctions between fear and anxiety are vague, in humans fear and anxiety are alerting signals that warn the individual against impending danger and enable the individual to take defensive states. For example, behavioral responsiveness in humans is enhanced in anticipation of aversive stimuli coming before as well as during passing the gulf, climbing in the mountains or an air-plane flight. Experimental situation in the present study appears to posses a good predictive validity in that the passing an unknown inter-space is enhanced by an anxiolytic drug, such as DZP. In our view, the results show, that a new model fulfills requirements for face and construct validity.

In conclusion, the presented modified open-field test is a useful paradigm to investigate risk assessment behavior in rats, and may provide a sensitive novel model of anxiety and fear level.

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