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Trial 2 in the elevated plus-maze: a different form of fear?

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Abstract. A factor analysis of the scores from rats given two trials in the elevated plus-maze showed that four independent factors emerged. Measures of anxiolytic activity on trial 1 (number of open arm entries and time spent on open arms) loaded on factor 1, measures of anxiolytic activity on trial 2 loaded on factor 2, the measure of general activity (number of closed arm entries) on both trials loaded on factor 3, and a measure of decision time (time spent in central square) for both trials loaded on factor 4. The independence of trials 1 and 2 anxiety measures raises the possibility that the state of anxiety/fear on the second trial in the plus-maze is qualitatively different from that on trial 1. This difference is reflected in the loss of anxiolytic action of diazepam (2 mg/kg) on trial 2. However, this occurs only when the trials are short (5 min); when they are longer (10 min) diazepam retains anxiolytic efficacy. It is concluded that during a brief (5 min) trial in the plus-maze rats acquire a specific phobic anxiety, which is relatively resistant to benzodiazepines. With a longer exposure to the plus-maze this form of fear extinguishes.

Key words: Anxiety – Fear – Benzodiazepines – Tolerance

It has been reported that mice and rats with previous experience of the elevated plus-maze have a reduced, or absent, anxiolytic response to benzodiazepines (Lister 1987; File 1990; Rodgers et al. 1992). This phenomenon of "one-trial tolerance" is not dependent on the drug treatment on trial 1, but is controlled by crucial learning on trial 1 that is associated with experience of the open arms (File et al. 1990).

Previous experience of the plus-maze not only modifies the behavioural response to benzodiazepines in the plus-maze, but also changes the effects of chlordiazepoxide on GABA release from cortical slices (File et al. 1992) and the effects of diazepam in blocking the antinociceptive effects of plus-maze experience (Rodgers et al. 1992). On the basis of the inability of diazepam to block the anxiogenic effects they observed on trial 2, Rodgers et al. (1992) suggested that a single experience of the plus-maze might change the nature of the anxiety reaction provoked by this test. If this explanation is correct, the reason for the lack of efficacy of benzodiazepines on trial 2 could be that they are relatively ineffective against this type of anxiety.

A recent factor analysis study has shown that measures derived from three different animal tests of anxiety load on independent factors (File 1991), suggesting that each test is measuring a different type of anxiety. In order to determine whether the measures in the plus-maze on trials 1 and 2 were reflecting a different type of anxiety, in experiment 1 a factor analysis was conducted on the scores from two trials.

In contrast with the reports of "one-trial tolerance", studies in which rats were repeatedly tested in the plus-maze, receiving several doses in a randomised order, found no evidence for a diminished efficacy of diazepam with plus-maze experience (Critchley and Handley 1987; Almeida et al. 1991). However, one difference between these studies and those in which "one-trial tolerance" was found was the use of 10-min trials in the former, as opposed to 5-min trials in the latter, cases. We therefore conducted a pilot study using two 10-min trials and confirmed the lack of tolerance. It is unlikely that the inter-trial interval is a crucial factor since the phenomenon of "one-trial tolerance" has been found with intervals from 24 h to 2 weeks (File et al. 1990). Experiment 2 therefore investigated the importance of trial duration to the phenomenon of "one-trial tolerance", by directly comparing groups tested in 5- and 10-min trials. Experiment 3 then explored the relative importance of the duration of trial 1 versus trial 2.

Materials and methods

Animals

Male hooded Lister rats (Olac Ltd, Bicester), approximately 220–240 g in weight, were housed in groups of five in a room with

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lights on from 0600 to 1800 hours; food and water were freely available.

Apparatus

The elevated plus-maze was made of wood, with two opposite open arms, 50 × 10 cm and two opposite enclosed arms of the same size, but with walls 40 cm high. The arms were connected by a central square and thus the maze formed a plus-sign. It was elevated 50 cm above the floor. The rats were observed on a TV monitor in an adjacent room and the number of entries onto, and time spent on, open and enclosed arms were scored. An entry was defined as both forepaws in the respective arm. The time spent in the central square was also recorded.

Drugs

Diazepam (Roche Products Ltd) was suspended in distilled water with Tween 20 (1 drop/20 ml) and sonicated in an ultrasonic water bath for 30 min prior to injection; control rats received water/Tween solution. All injections were IP in a volume of 2 ml/kg, 30 min before testing.

Statistics

The data from experiment 1 were analysed by factor analysis using a principal component solution with an orthogonal rotation of the factor matrix, with the Orthotran/Varimax transformation method. Using the method default extraction rule, four factors emerged and a maximum likelihood factor analysis (of the non-singular 8 × 8 matrix of raw measures) confirmed that the residual matrix only became non-significant when four factors were extracted.

The data from experiment 2 were analysed with split-plot analyses of variance, with drug treatment as the independent factor and trials as the repeated measure. The trial 2 data for experiment 3 were analysed by one-way analyses of variance.

Procedure

Experiment 1. Each rat was placed in the centre of the plus-maze and allowed 5 min free exploration; the maze was cleaned after each trial. Each rat received a second 5-min trial 24 h later. One hundred rats received both trials 30 min after injection with distilled water. This placebo injection was included to produce the same experimental conditions to the later drug study.

Experiments 2 and *3.* Each rat was given two trials in the plus-maze, separated by 24 h, with the same control or diazepam treatment on each trial. Rats were randomly assigned, $n = 7$ or 8 in each of the groups shown in Table 1. The groups assigned to no previous experience of the plus-maze (0 min) were handled, injected and returned to their home cages.

Table 1. Duration of exposure (0, 5 or 10 min) to the plus-maze on trials 1 and 2 for rats injected with vehicle (CON) or with diazepam (DZ, 2 mg/kg) for experiments 2 and 3

	Trial 1	Trial 2
Experiment 2		
	CON	5	5
	DZ	5	5
	CON	10	10
	DZ	10	10
Experiment 3		
	DZ	0	5
	DZ	0	10
	DZ	5	5
	DZ	10	10
	DZ	5	10
	DZ	10	5
	CON	5	5
	CON	10	10
	CON	5	10
	CON	10	5

Table 2. Orthogonal factor loadings from trials 1 and 2 in the plus-maze, for rats tested undrugged

	Factor 1	Factor 2	Factor 3	Factor 4
Tr1\ no. open	0.81	—	—	—
Tr1\ time open	0.92	—	—	—
Tr1\ no. closed	—	—	0.76	—
Tr1\ time closed	− 0.97	—	—	—
Tr1\ centre time	—	—	—	0.94
Tr2\ no. open	—	0.83	—	—
Tr2\ time open	—	0.84	—	—
Tr2\ no. closed	—	—	0.76	—
Tr2\ time closed	—	− 0.90	—	—
Tr2\ centre time	—	− 0.67	—	0.58

Results

Experiment 1

Table 2 shows the loadings of each variable in the plus-maze on the independent factors that were found from the factor analysis. The factor loading for each variable provides an estimate of how well that variable reflects a particular factor. A value of 1.0 would be a perfect reflection, whereas loadings of less than 0.4 suggest a particular variable is a poor reflection of the factor. Only loadings > 0.4 are shown in Table 2.

It can be seen from Table 2 that the two variables which reflect anxiety in the plus-maze (number of open arm entries, time in open arms) loaded highly on factor 1 if they were trial 1 scores and on factor 2 if they were trial 2 scores. Since by definition the factors extracted by this analysis are independent, the results confirm that the variables are reflecting different underlying processes. The measures loading highest on factor 3 were the numbers of closed arm entries on trials 1 and 2. The measures loading highest on factor 4 were the times spent in the central square on trials 1 and 2.

The correlations between the various variables confirms the results of the factor analysis (see Table 3). Thus, whilst the number of open arm entries correlated highly with the time on the open arms on each trial, the numbers of open arm entries for trials 1 and 2 had a much lower correlation, as did the times spent on the open arms in trials 1 and 2.

Table 3. Correlation matrix of trial 1 and trial 2 scores in the plus-maze (only correlations ≥ 0.40 are shown. # = number of entries; t = time (s); op = open arms; cl = closed arms; sq = central square; 1 = trial 1; 2 = trial 2; all measures are for 5-min trials

	# op 1	t op1	# cl1	t cl1	t sq1	# op2	t op2	# cl2	t cl2	t sq2
# op 1	1.0									
t op 1	0.87	1.0								
# cl 1	0.45	—	1.0							
t cl 1	− 0.59	− 0.68	—	1.0						
t sq 1	—	—	—	− 0.47	1.0					
# op 2	0.54	0.55	—	− 0.46	—	1.0				
t op 2	0.46	0.48	—	− 0.40	—	0.89	1.0			
# cl 2	—	—	—	—	—	—	—	1.0		
t cl 2	—	—	—	0.41	—	− 0.71	− 0.71	− 0.49	1.0	
t sq 2	—	—	—	—	0.47	—	—	0.54	− 0.88	1.0

Experiment 2

It can be seen from Fig. 1 that when the rats received 5-min trials in the plus-maze there was no longer an anxiolytic response to diazepam on trial 2 [drug × trial interaction, $F(1,13) = 5.5$, $P < 0.05$ for % number of entries onto open arms; $F(1,13) = 7.5$, $P < 0.05$ for % time spent on open arms]. In contrast, for the rats given 10-min trials there was a significant diazepam effect on both trials [drug effect, $F(1,13) = 15.2$, $P < 0.005$ for % number and $F(1,13) = 10.6$, $P < 0.01$ for % time].

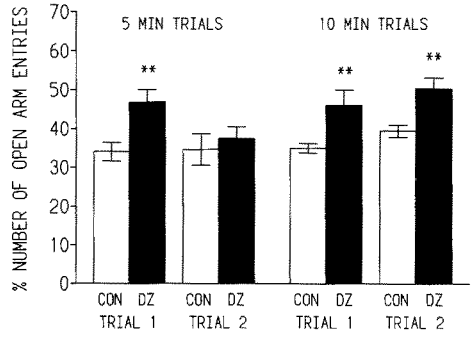

Fig. 1. Mean (± SEM)% of entries onto open arms made by rats injected with vehicle (*CON*) or diazepam (*DZ*, 2 mg/kg). $^{**}P < 0.01$, compared with relevant control group

Experiment 3

Figure 2 shows the relative importance of a 10-min trial on trial 1 versus trial 2. In all cases the scores are shown for trial 2; the previous experience was either no previous plus-maze experience (0 min) or 5 or 10 min. It can be seen that when the rats were naive to the plus-maze, diazepam had a significant effect, whether the trial was 5 or 10 min. When the rats had a previous 5-min exposure to the maze, diazepam no longer had a significant anxiolytic effect. When both trials were 10 min in duration, diazepam had a significant anxiolytic effect. However, when trial 1 was 10 min and trial 2 was 5 min, the effect did not reach significance [$F(1,12)$ 3.7, $P = 0.07$]. Because of this marginal result we also analysed the scores for the first 5 min of trial 2 for the groups given 10 min on trial 1 in experiment 2. This gave scores for the percentage number of open arm entries of 36.8 ± 1.5 for the control group and 45.0 ± 3.8, for the diazepam group [$F(1,13) = 4.5$, $P = 0.054$].

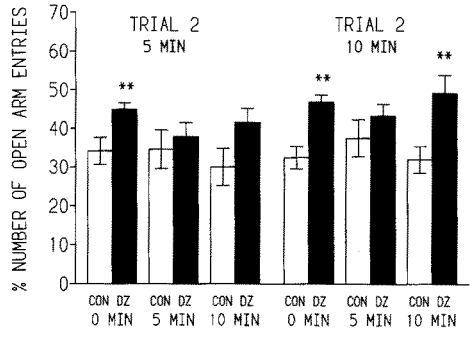

Fig. 2. Mean (± SEM)% of entries onto open arms made by rats injected with vehicle (*CON*) or diazepam (*DZ*, 2 mg/kg), with no previous experience of the plus-maze (0 min) or with a 5- or 10-min previous experience. $^{**}P < 0.01$, compared with relevant control group

Discussion

A previous factor analysis (File 1991) showed that the number of open arm entries and time spent on the open arms are as good measures of anxiolytic activity in the plus-maze as are the percentage scores. The number of closed arms was previously found to be the best measure of general activity in the plus-maze and these measures from both trials loaded on the same factor.

In contrast, the measures of anxiolytic activity loaded on two factors, depending on whether they came from trial 1 or 2. This suggests that there is a different underlying anxiety on trial 1 from that on trial 2. This situation is quite different from that found in the social interaction test of anxiety, where the scores from both trials 1 and 2 had high loadings (> 0.80) on the same factor (File 1991). Thus, the plus-maze may be unique in providing a test situation where the nature, rather than the extent, of the anxiogenic state changes with experience of the maze. There is no evidence from our measures that trial 2 anxiety is greater than trial 1 anxiety, but if the trial 1 state

is primarily triggered by unfamiliarity and the openness of the arms, and on trial 2 the state is primarily a fear of heights, the relative strength of the two is unimportant. We would expect any anxiety state generated by novelty to habituate over trials and thus the phobic anxiety state is replacing, rather than adding to, the anxiety generated by novelty.

Previous experiments (File et al. 1990) showed the importance of learning and experience of the open arms to the phenomenon of "one-trial tolerance", i.e. to the disappearance of an anxiolytic action of a benzodiazepine. The present results suggest that during the initial 5-min exposure to the maze the animals are acquiring a second form of fear. It is this state that is relatively unresponsive to benzodiazepine treatment. Since benzodiazepines are relatively ineffective against phobias, this raises the possibility that trial 2 behaviour reflects a phobic anxiety state, perhaps a fear of heights. The rapid acquisition of this phobia suggests that there may be a genetic predisposition for such a fear. We have shown that the phenomenon of "one-trial tolerance" is much reduced in rats that had not been handled prior to elevated plus-maze exposure (File et al. 1992). It is therefore possible that the stress of handling, in previously unhandled rats, prevented the acquisition of this second form of fear.

The results from experiments 2 and 3 show that this second form of fear is not expressed if the exposure to the plus-maze is longer (i.e. 10 min on each trial). This raises the possibility that the second form of fear rapidly extinguishes with further exposure to the maze. A total of 2 $\times$ 10 min is sufficient for extinction, whereas the groups that received only 15 min (10–5 and 5–10) showed incomplete extinction. This rapid extinction is consistent with the possibility that trial 2 behaviour in the plus-maze represents a phobic state, since phobias do diminish as a result of exposure to the phobic situation (Marks 1987).

If the hypothesis is correct that there is a different state of "anxiety" or "fear" on trials 1 and 2 in the plus-maze, then the pharmacological investigation of trial 2 behaviour in the maze should prove fruitful. Variations in the extent to which the rats are handled and/or whether they are naive or experienced with the plus-maze, might give rise to different sensitivity to pharmacological agents. For example a different response to drugs acting on the serotonergic system might arise, since the animals' prior handling history not only interacts with the phenomenon of "one-trial tolerance" in the plus-maze it also interacted with the effects of plus-maze experience on release of 5-HT from hippocampal slices (File et al. 1992).

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