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"One-trial tolerance" to the anxiolytic actions of benzodiazepines in the elevated plus-maze, or the development of a phobic state?

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Abstract. Diazepam (5 mg/kg) increased the number of shocks accepted by rats on two successive trials in the punished drinking test. Thus, the phenomenon of "one trial tolerance" to the anxiolytic effects of benzodiazepines in the elevated plus-maze does not generalise to this other animal test of anxiety. FG 7142 (20 mg/kg) and prior exposure to the odour of a cat had significant anxiogenic effects on two successive trials in the plus-maze. Thus the phenomenon of "one trial tolerance" does not generalise to these anxiogenic effects in the plus-maze. Furthermore, chlordiazepoxide retained its ability to counteract the anxiogenic effects in the plus-maze of prior exposure to cat odour, over successive trials. On the basis of these and previous experiments it is suggested that the state of anxiety generated on trial 2 in the plus-maze is close to a phobic state, against which benzodiazepines are relatively ineffective. Chlordiazepoxide (5 and 10 mg/kg) was also ineffective against the behavioural responses of rats during exposure to cat odour, another possible animal test of phobia. This contrasted with its efficacy against the anxiogenic effects of cat odour that subsequently generalised to and could be detected in the plus-maze.

Key words: Anxiety – Phobia – Plus-maze – Cat – Benzodiazepines

Mice and rats with previous experience of the elevated plus-maze have a reduced, or absent, anxiolytic response to benzodiazepines (Lister 1987; File 1990a; File et al. 1990). The present series of experiments was designed to determine the nature and generality of this phenomenon of "one trial tolerance". Experiment 1 was designed to determine whether the phenomenon could be demonstrated to the action of a benzodiazepine in another widely used animal test of anxiety, the punished drinking test. Tolerance does not develop to the anxiolytic effects of benzodiazepines in most tests before 14–21 days of drug treatment (see File 1990b for review), but most of the experiments have tested the animals on only one occasion, after the appropriate length of treatment. Experiment 1 therefore investigated whether there was a marked reduction in the effect of diazepam on the second occasion that the rats were tested in the punished drinking test.

Experiment 2 was designed to determine whether the phenomenon of "one trial tolerance" to drug actions in the elevated plus-maze extended to anxiogenic effects. The benzodiazepine inverse agonist, FG 7142, was chosen since this has anxiogenic actions in the plus-maze (Pellow and File 1986). In addition, we investigated the effects of a non-pharmacological means of generating an anxiogenic state, exposure to the odour of a predator, which generates an anxiogenic state, that can be detected in the plus-maze (Zangrossi and File 1992). Previous experiments have shown that the phenomenon of "one-trial tolerance" occurs whether trial 1 occurs undrugged, or after injection with a benzodiazepine or the benzodiazepine antagonist, flumazenil (File 1990a). Experiment 2 further explored whether the same phenomenon occurred if trial 1 took place after injection with an anxiogenic dose of FG 7142.

The results from experiments 1 and 2 indicated that there was little generality to the phenomenon of "one-trial tolerance". Experiment 3 therefore explored an alternative explanation for the lack of benzodiazepine efficacy on trial 2. It has been suggested that a single experience of the elevated plus-maze might change the nature of the anxiety state evoked by this test (Rodgers et al. 1992) and a factor analysis study confirmed that trials 1 and 2 were indeed measuring two independent factors (unpublished observations). We therefore propose that by trial 2 the nature of the anxiety state evoked by the plus-maze is close to that of a phobic state (e.g. a fear of heights or open spaces), against which the benzodiazepines are relatively ineffective (Marks 1987). If this hypothesis is correct, then the benzodiazepines should be equally ineffective in other animal tests of phobias. Animal tests of phobias are not well developed (see Lister 1991), but a good candidate is a rat's response to the odour of a predator (Blanchard et al. 1990; Zangrossi and File 1992). Experiment 3 therefore examined the effects of chlordiazepoxide on the responses of laboratory rats to the odour of a cat.

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Materials and methods

Animals

Male hooded Lister rats (Olac Ltd, Bicester), approximately 250 g, were housed in a room maintained at 22°C with lights on from 0700 to 1900 hours. Food and water were freely available unless otherwise specified. The rats were housed in groups of five, except for the experiments using exposure to cat odour, when they were singly housed so that they could be exposed to the odour while remaining in their home cages.

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 by an observer with no knowledge of the rat's treatment. The numbers of entries onto, and times spent on, open and enclosed arms were scored using a keyboard entry into an IBM PC. An entry was defined as both forepaws in the respective arm.

For the punished drinking test, the experimental chamber was a rectangular box 27 × 19.5 × 18 cm, with a metal grid floor, through which scrambled shocks were delivered (0.15 mA, 0.5 s). At one end of the box the rat had access to a stainless steel drinking spout. Licks were counted by automatically recording into a microcomputer the clicks made by a ball-bearing in the spout, using a directional and frequency-specific microphone.

Exposure to Cat odour

The cat odour was obtained by rubbing a damp cloth vigorously against the fur of a laboratory-housed domestic cat for 5 min. This procedure was carried out 1 h before the experimental session. The cat odour cloth was kept in a sealed plastic bag. Each cloth was used for four exposures only. Damp pieces from the same original cloth were used for the neutral odour.

Drugs

Chlordiazepoxide hydrochloride (Roche Products Ltd) was dissolved in distilled water; diazepam (Roche Products Ltd) and FG 7142 (Schering) were suspended by ultrasound in a distilled water/-Tween 20 solution. All drugs were given IP in an injection volume of 2 ml/kg, 30 min before testing. Control rats received water or water/-Tween injections, as appropriate.

Statistics

The data were analysed by multifactor analyses of variance, followed by Duncan's tests for comparisons of individual groups.

Experiment 1: punished drinking

Twenty rats were randomly allocated, ten to the vehicle control group and ten to diazepam (5 mg/kg). All rats were deprived of water for 22 h before a 15 min period of free drinking in the test apparatus; on this day all rats were undrugged. They were then returned to their home cages for a further 2 h of free drinking. The following day (22 h later) the rats were injected with vehicle or diazepam and given a 6 min test. For the first minute the licks were unpunished; for the next 5 min every 20th lick was punished with a foot-shock. The rats were then allowed 2 days of free drinking in their home cages, followed by a 22 h deprivation period. The following day the rats received trial 2 in the punished drinking test under the same conditions as trial 1.

Experiment 2

Effects of FG 7142. Table 1 shows the groups tested in this experiment and the number of rats in each group. Those allocated to injections only on trial 1 were injected with the appropriate drug, those in the "no injection" condition were picked up and weighed. Those allocated to a plus-maze trial were tested 30 min after injection. Each rat was placed in the central square of the plus-maze and allowed 5 min of free exploration. The rats were tested in an order randomised for drug treatment. The two trials were separated by 3 days and the test order remained the same on each day.

Effects of cat odour. Forty-seven rats were randomly allocated between control (distilled water) and chlordiazepoxide (CDP, 5 mg/kg) and then each drug treatment group was divided into neutral and cat odour exposure. Both drug and control groups received daily injections for 5 days, so as to minimise the sedative effects of CDP. Prior to any odour exposure or drug treatment all rats received a 5 min plus-maze exposure. One week later, the rats were injected with water or CDP, as appropriate and 30 min later given a 5 min odour exposure and 30 min afterwards given a 5 min plus-maze test, in a different room from the odour exposure. The same odour exposure was repeated the next day, 30 min after appropriate injections, and 30 min following the odour exposure a plus-maze test was given.

Experiment 3: exposure to cat odour

Seventy rats were randomly allocated between control (distilled water) and chlordiazepoxide (5 and 10 mg/kg for 5 days) groups and then to the neutral and cat odour conditions, such that there were 11–13 rats tested with each odour in each drug condition. All odour exposures took place in a separate small, dimly lit room and the neutral odour exposures always preceded the cat odour exposures in order to prevent any traces of cat odour. Before the first cat odour exposure an impregnated cloth was left in the test room for 10 min Each rat was carried to the exposure room in its home-cage which was placed next to an empty cage, with the odour cloth wedged between the cage tops, at the opposite end from the food and water containers. The odour exposure was 5 min and the rats were videotaped for later scoring. An identical procedure was followed for each rat on the following day. A cloth contact was defined as a direct contact or sniffing $\leq$ 5 cm from the cloth; sheltering was defined when the rat was underneath the food and water compartments.

Results

Experiment 1

Figure 1 shows that diazepam significantly increased the number of shocks accepted by the rats on both trials

Table 1. Groups tested in experiment 2 (n = number/group; INJ—injection; PLUS—plus-maze test; FG—FG 7142, 20 mg/kg; CON—control injection; DZ—diazepam, 6 mg/kg)

n	Day 1	Day 2
8	FG PLUS	DZ PLUS
8	CON PLUS	DZ PLUS
8	FG INJ	DZ PLUS
8	CON INJ	DZ PLUS
8	NO INJ	FG PLUS
8	NO INJ	CON PLUS
7	PLUS	FG PLUS
7	PLUS	CON PLUS

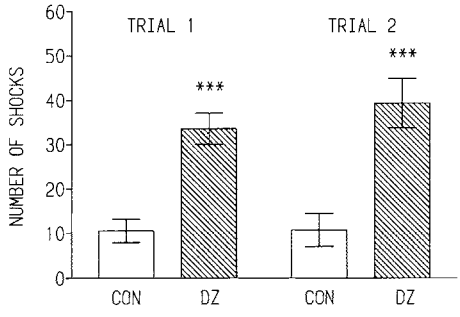

Fig. 1. Mean (± SEM) number of shocks received by rats on two trials in the punished drinking test 30 min after injections of distilled water (*CON*) or diazepam 5 mg/kg (*DZ*). *** $P < 0.001$ compared with control group

[$F(1,18) = 28.1$, $P < 0.0001$]. There was no evidence of a decreased effect on trial 2 [drug × trial interaction, $F(1,18) < 1.0$], and therefore no evidence of the phenomenon of "one-trial tolerance".

Experiment 2

Effects of FG 7142. Figure 2 shows the results for animals tested in the plus-maze after injection with diazepam, with and without previous plus-maze experience after control or FG 7142 injections. It can be seen from this figure that the response to diazepam is significantly higher in rats without previous plus-maze experience [$F(1,28) = 10.0$, $P < 0.005$ for % number; $F(1,28) = 11.4$, $P < 0.005$ for % time], but that the drug state during the previous experience (control or FG 7142) was unimportant [$F(1,28) = 1.4$ and < 1.0 for % number and % time, respectively]. This confirms the phenomenon of "one-trial tolerance" and provides further evidence as to the importance of previous experience of the plus-maze, rather than the previous drug state.

Figure 3 shows that the response to FG 7142 was significantly anxiogenic [$F(1,26) = 18.1$, $P < 0.0005$ for % number; $F(1,26) = 9.7$, $P < 0.005$ for % time] and that this was not modified by prior plus-maze experience [drug × experience interaction, $F(1,26) = 1.6$ and 1.0 for % number and % time, respectively].

Effects of cat odour. All the rats tested after odour exposure had received one previous 5-min exposure to the plus-maze. It can be seen from Fig. 4 that those exposed to the neutral odour and then tested in the plus-maze were unresponsive to chlordiazepoxide, i.e. displayed the phenomenon of " one-trial tolerance". The groups exposed to the cat odour showed significant reductions in the percentage of entries onto open arms and the percentage of time on the open arms, compared with the neutral odour groups [$F(1,22) = 4.9$ and 14.4, $P < 0.05$ and $P < 0.005$ for % number and % time, respectively]. In the cat odour group chlordiazepoxide had a significantly anxiolytic effect [$F(1,23) = 14.6$ and 12.8, $P < 0.001$ and $P < 0.005$ for % number and % time, respectively].

Figure 4 also shows that when the groups were retested the following day after a second exposure to the

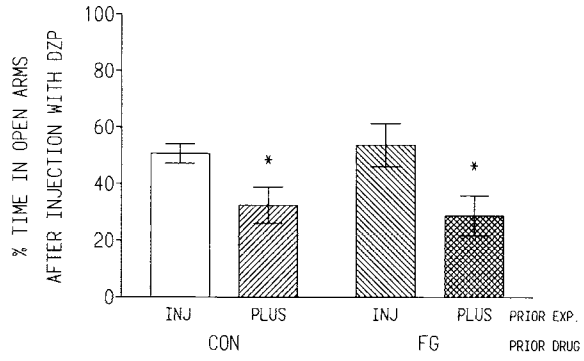

Fig. 2. Mean (± SEM) % of time (s) spent on the open arms of the elevated plus-maze by rats tested 30 min after injection with diazepam 6 mg/kg. The rats either had prior experience of injection alone (*INJ*) or also of the plus-maze (*PLUS*) after injection with water/Tween (*CON*) or FG 7142, 20 mg/kg (*FG*). * $P < 0.05$ compared with INJ group

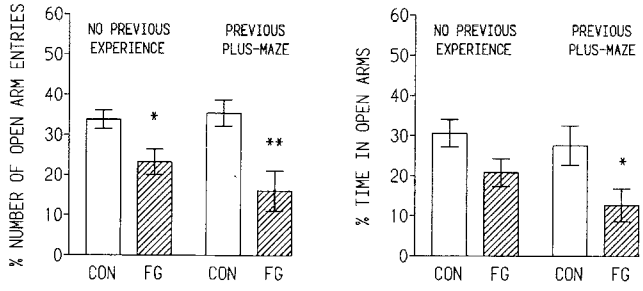

Fig. 3. Mean (± SEM) % number of entries onto open arms and % of time (s) spent on open arms by rats with no previous plus-maze experience or with one previous 5 min experience. All rats were tested 30 min after injection with water/Tween (*CON*) or FG 7142, 20 mg/kg (*FG*). * $P < 0.05$, ** $P < 0.01$ compared with relevant control group

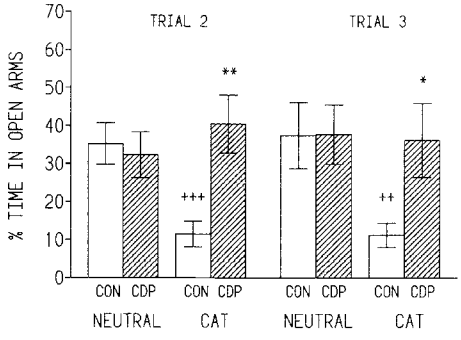

Fig. 4. Mean (± SEM) % of time (s) spent on the open arms of the elevated plus-maze on two trials by rats injected with distilled water (*CON*) or chlordiazepoxide (*CDP*) and previously exposed to a neutral or cat odour. All rats had one previous plus-maze experience: * $P < 0.05$, ** $P < 0.01$ compared with relevant control group. $^{++}P < 0.01$, $^{+++}P < 0.001$ compared with neutral odour group

odours, chlordiazepoxide again had a significant effect in antagonising the effects of cat odour, i.e. there was no tolerance to this effect [drug × trial interaction, $F(1,23) = < 1.0$ for both measures].

Table 2. Mean (± SEM) number of contacts and time (s) in contact with the odour cloth and time (s) spent sheltering under the hoppers for rats exposed to a neutral or cat odour, 30 min after injection with distilled water (CON) or chlordiazepoxide (CDP, 5 or 10 mg/kg)

	Trial 1			Trial 2		
	CON	CDP5	CDP10	CON	CDP5	CDP10
No. contacts						
Neutral	6.8 ± 0.6	4.4** ± 0.5	4.7* ± 0.6	8.1 ± 0.7	5.3* ± 0.9	4.0** ± 0.7
Cat	5.0 ± 0.9	4.2 ± 1.2	6.4 ± 0.9	2.5^{+++} ± 0.6	3.8 ± 0.9	4.5 ± 0.7
Time contact						
Neutral	75.4 ± 11.0	100.6 ± 17.6	72.8 ± 18.1	82.0 ± 12.3	113.1 ± 15.6	76.1 ± 21.4
Cat	39.9^{++} ± 8.1	53.6 ± 15.8	56.5 ± 9.0	26.6^{++} ± 10.0	51.2 ± 20.6	30.3 ± 5.9
Time sheltering						
Neutral	34.5 ± 12.8	39.2 ± 12.3	31.2 ± 12.3	25.2 ± 13.4	24.5 ± 9.7	77.0 ± 27.2
Cat	150.5^{++} ± 27.7	139.6 ± 31.5	105.6 ± 22.3	206.0^{+++} ± 28.4	167.9 ± 32.7	168.6 ± 21.0

* $P < 0.05$; ** $P < 0.01$ compared with respective control group $^{++}P < 0.01$; $^{+++}P < 0.001$ compared with neutral odour control group

Experiment 3

Table 2 shows that the undrugged rats exposed to cat odour made fewer contacts with the odour cloth, spent less time in contact with the cloth and spent longer sheltering under the hoppers than did the rats exposed to the neutral odour. The number of contacts with the neutral odour cloth was significantly reduced by chlordiazepoxide, but there were no significant drug effects on the responses to the cat odour [$F(2,33) = 1.4$, 0.6 and 0.7 for the number of contacts, time in contact and time sheltering, respectively, on trial 1; and 1.7, 0.9 and 0.6 respectively on trial 2].

Discussion

There was no evidence of "one-trial tolerance" in the punished drinking test, i.e. diazepam significantly increased the number of shocks accepted on both trial 1 and trial 2. Although the exact design of the "one-trial tolerance" procedure has not been used in other animal tests of anxiety, the lack of tolerance in the punished drinking test is in accord with other tests in which rats are repeatedly tested, e.g. the Geller-Seifter conflict test, in which benzodiazepines retain efficacy (Howard and Pollard 1991).

In contrast to the ineffectiveness of benzodiazepines in animals with prior plus-maze experience, there was no reduction in the sensitivity to the anxiogenic effects of either FG 7142 or exposure to cat odour. This suggests that both these treatments produced a state(s) of anxiety that was superimposed on that generated by the plus-maze itself and prevented the learning that changes the nature of the anxiety generated by the maze itself. Previously unhandled animals do not show the phenomenon of "one-trial tolerance" suggesting that the stress of handling also prevents the acquisition of a phobic anxiety state (File et al. 1992).

Rats that had been exposed to cat odour for 5 min showed a subsequent generalised anxiogenic response in the elevated plus-maze, i.e. a decreased percentage of open arm entries and a decreased percentage of time in the open arms. Interestingly, chlordiazepoxide retained its ability to counteract these anxiogenic effects over two trials. All the rats tested in this experiment had a plus-maze experience prior to odour exposure and the phenomenon of "one-trial tolerance" was shown by the absence of the effects of chlordiazepoxide in the neutral odour groups.

If, during the first 5 min of exposure to the plus-maze, the rats were acquiring a fear of heights or open spaces, it is interesting that their drug state during this exposure is of relatively little importance. Certainly, having an anxiolytic treatment does not prevent the acquisition of this fear (File 1990a), and although there was a trend for an enhanced effect when trial 1 was in the presence of an anxiogenic drug, this did not reach significance. So far, only an amnesic dose of chlordiazepoxide has prevented the phenomenon (File et al. 1990). It therefore seems likely that there is an innate propensity for rodents to rapidly acquire this fear/phobia. The fact that the control animals show very similar scores on both trials suggests that there is habituation of the state of anxiety generated on trial 1 and that the phobic anxiety is replacing rather than adding to the initial anxiety. If this suggestion is correct then it would be possible to have similar scores on both trials, a decrease on trial 2 or even an increase (e.g. Rodgers et al. 1992), depending on the relative extent of habituation of trial 1 anxiety and acquisition of trial 2 anxiety.

In contrast to the efficacy of chlordiazepoxide at counteracting the generalised anxiogenic effects detected in the plus-maze after exposure to cat odour, during the actual cat odour exposure period it was without significant effect. Blanchard et al. (1990) have also reported no effects, other than those probably attributable to sedation, of diazepam on the behaviour of rats exposed to cat odour. It is possible that the odour of a predator evokes

such intense anxiety that only very high doses of benzodiazepines will be effective. However, the lack of a benzodiazepine response raises the possibility that the responses *during* the actual odour exposure reflect a phobic state, whereas those subsequently expressed in the plus-maze or social interaction tests (Zangrossi and File 1992) may reflect a more generalised state of anxiety.

Further experiments are clearly needed to establish whether trial 2 in the elevated plus-maze and presentation of the odour of a predator can indeed be considered as animal tests of phobia. However, from the work of the Blanchards' group it is clear that the benzodiazepines have little effect on wild and laboratory rats' fear responses to the actual presence of a predator or on many of the responses to the odour of a predator (Blanchard et al. 1989, 1990). Mineka (1985) has distinguished two clear components of a phobic response: avoidance responses and behavioural disturbance. We consider the responses we measured in experiment 3 to be avoidance of the odour and the avoidance of the open arms in the plus-maze to be avoidance of the elevation and/or open spaces. The other behaviours measured in the Blanchard experiments may be more reflective of the behavioural disturbance caused by the predator odour and these were reduced by relatively high doses of diazepam. Thus these actions of diazepam could either reflect its sedative effects or could suggest that the benzodiazepines would be more effective against the behavioural disturbances caused by a phobic situation than they are against phobic avoidance.

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