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Research report

Prior test experience compromises the anxiolytic efficacy of chlordiazepoxide in the mouse light/dark exploration test

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Abstract

It is now well established that prior test experience can alter behavioural baselines and attenuate/abolish the anxiolytic efficacy of benzodiazepines in the elevated plus-maze paradigm. In view of evidence that different models of anxiety measure qualitatively distinct forms of anxiety-like behaviour, it is important to establish whether the effects of prior experience extend to other widely-used tests. The present study assessed the behavioural and pharmacological sequelae of a single undrugged prior exposure to the light/dark exploration (L/D) test in mice, using ethological scoring methods. One group of adult male Swiss-Webster mice was given a single undrugged exposure to the L/D test 24 h prior to drug testing, while another group was completely naïve to the apparatus. On test day, half the animals in each experiential condition were treated with saline and half with an anxiolytic dose (10 mg/kg) of chlordiazepoxide (CDP). When administered to test-naïve animals, CDP induced a clear reduction in anxiety-like behaviour as evidenced by significant increases in exploration of the light compartment (line crossings, % line crossings, and % time) as well as reductions in stretched attend postures (SAPs) and the proportion of SAPs displayed toward the light compartment. The behavioural specificity of these effects was confirmed by the absence of a drug effect on line crossings in the dark compartment, total rearing and grooming. In complete contrast, with the sole exception of a decrease in total SAPs, CDP was without significant behavioural effect in test-experienced mice. As prior test experience did not significantly alter behavioural baselines in the L/D test, a second experiment was designed to investigate the possibility that handling/intraperitoneal injection may have precluded detection of experientially-induced changes in baseline behaviour. Results showed that handling/injection had no effect upon L/D behavioural profiles in either test-naïve or test-experienced subjects, and confirmed that prior experience itself did not modify the primary indices of anxiety in this test. Present data indicate that prior test experience seriously compromises the anxiolytic efficacy of CDP (10 mg/kg) in the mouse L/D test and, together with recent findings in the four-plate test, appear to confirm that an experientially-induced reduction in sensitivity to the anxiolytic effects of benzodiazepines is by no means unique to the elevated plus-maze.

Keywords: Light/dark exploration test; Animal model of anxiety; Test experience; Retesting; Chlordiazepoxide; Injection effects; Mice

1. Introduction

Traditional animal models of anxiolytic activity (e.g. Geller–Seifter conflict) often employ repeated measures designs in which benzodiazepines retain their efficacy in test-experienced subjects [24,41]. In contrast, there is mounting evidence that prior test experience radically alters behavioural and pharmacological responses in a widely-used exploratory model of anxiolytic activity, the elevated plus-maze. Although early findings suggested good test–retest stability for this test [26,45,50], a substantive literature now indicates that a single prior undrugged exposure to the maze usually results in increased open arm avoidance on subsequent trials [19–21,29,31,39,44,55–58,62,66]. In addition to these observations, prior test experience also appears to fundamentally alter the nature of future emotional re-

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sponses to the plus-maze. More specifically, the primary indices of anxiety from trials 1 and 2 load on independent factors [21,24,27–29,39], while the anxiolytic efficacy of benzodiazepines is either markedly reduced or completely abolished by prior undrugged test experience [23,39,45,57,58]. Such findings have led to the proposal that 'the plus-maze may be unique in providing a test situation in which prior experience in some way makes the animals unresponsive to benzodiazepines' [24]; p. 200.

Although consistent with the retention of benzodiazepine efficacy following repeat testing in other animal models of anxiolytic activity (e.g. Geller–Seifter conflict, Vogel conflict, social interaction test; [27,41]), this view of the plus-maze is seemingly challenged by recent findings in the mouse four-plate test of punished exploration. Thus, Hascoet and colleagues [34] have reported that a single prior undrugged experience of this test also results in a significant retest reduction in punished responding as well as a marked attenuation in the anxiolytic efficacy of benzodiazepines. The obvious question arises as to the further generality of these findings, an issue that is particularly important given recent evidence that ostensibly similar rodent tests measure qualitatively different forms of anxiety-like behaviour [5–7,25,46,52].

The light/dark exploration (L/D) test is one of the most commonly-used murine models of anxiety [38]. Devised by Crawley and colleagues 20 years ago [16,18], this model permits mice to freely explore two inter-connected compartments that vary in size (2:1), colour (white:black) and illumination (bright:dim). Results showed that intact mice tend to avoid the brightly-lit chamber and that conventional anxiolytics (benzodiazepines, barbiturates, meprobamate), but not drugs of other classes, increase the number of inter-compartmental transitions. Since then, the L/D test has been widely adopted as an anxiolytic screening test in mice [15,42,49,59,67] and extended for use with rats [51,60,64]. In parallel with these developments, additional indices of anxiolytic activity have been championed, e.g. relative behavioural activity/time spent in each compartment [15,35,59,67]. To date, however, rather limited attention has been paid to the behavioural and pharmacological sequelae of prior experience in the L/D test. As such, the primary aim of the present study was to assess the influence of this experiential variable on responsivity to the prototypical benzodiazepine anxiolytic, chlordiazepoxide. In addition, we have assessed the influence of prior test experience on behavioural baselines in the L/D test, as well as the potential influence of pre-trial handling/intraperitoneal injection stress on behaviour patterns in test-naïve and test-experienced subjects. Ethological scoring methods were used to generate comprehensive behavioural profiles that included both conventional test indices (e.g. transitions and relative time spent in the light area) and measures related to rodent defence (e.g. stretched attend postures and thigmotaxis).

2. Materials and methods

2.1. Animals

Subjects were adult male Swiss-Webster mice (Bantin and Kingman, Hull, UK), 11–12 weeks old at the time of testing. They were housed in groups of 10 (cage size: $45 \times 28 \times 13$ cm), under a 12 h reversed light cycle (lights off: 07:00 h), in a temperature (21 ± 1°C) and humidity (50 ± 5%) controlled environment. Food and drinking water were freely available except during brief test periods. All animals were experimentally naïve at the start of the study, and had been handled only for the purpose of routine husbandry.

2.2. Drugs

Chlordiazepoxide hydrochloride (CDP; Sigma, UK) was dissolved in physiological saline which, alone, served as vehicle control. Solutions were freshly prepared and administered intraperitoneally (i.p.) (10 ml/kg) 30 min prior to testing. The dose of CDP (10 mg/kg) was selected on the basis of earlier findings in the L/D test [18] and its reliable anxiolytic profile in Swiss-Webster mice tested in the plus-maze under local conditions [11,12].

2.3. Apparatus

The L/D test apparatus comprised an open-topped arena ($45 \times 27 \times 27$cm), one third painted black and two-thirds white. The two compartments were separated by a wooden partition (height 27 cm) which had a small opening (7.5×7.5 cm) cut into its centre at floor level. The outer walls of the apparatus were constructed from metal and the floor (wood) was marked (in both compartments) into 9 cm squares. The white compartment was illuminated by bright, direct white light and the dark compartment by dim, indirect red light (both provided by 2×60 W anglepoise lamps). In accord with the procedure adopted for plus-maze research in our laboratory, all testing was conducted during the early-mid dark phase of the LD cycle, i.e. 1000–1500 h [39,54].

2.4. General procedure

On test days, animals were transported to the dimly illuminated laboratory and left undisturbed for at least 1 h prior to testing. Test sessions began when mice were individually placed in the centre of the white compart-

ment (facing the dark compartment). A test duration of 5 min was employed and, between subjects, the apparatus was thoroughly cleaned with wet and dry cloths. All sessions were videorecorded by a camera (positioned directly above the apparatus) linked to a monitor and VCR in an adjacent laboratory and, to avoid unnecessary distractions, the experimenter retreated to this location during testing.

2.4.1. Experiment 1

Forty-eight mice were used, of which 24 were test-naïve while 24 had been pre-exposed undrugged to the L/D test 24 h earlier (trial 1 = 5 min). Within each main experimental group, animals were randomly allocated to treatment conditions ($n = 11–12$), injected i.p. with saline or 10 mg/kg CDP, and placed in individual holding cages ($17 \times 7 \times 6$cm) until testing. Thirty minutes later, mice were exposed to the L/D test in an order counterbalanced for test experience and drug treatment. To assess a possible influence of 'injection stress' on the response of naïve mice to the L/D test, the scores from a random sample of 13 animals were taken from the pre-exposure day itself and these constituted a no injection/test-naïve control condition.

2.4.2. Experiment 2

Forty mice were used, of which 20 were test-naïve while 20 had been pre-exposed undrugged to the L/D test 24 h earlier (trial 1 = 5 min). Within each main experimental group, animals were randomly allocated to treatment conditions ($n = 10$), and either given a single pre-trial i.p. saline injection prior to placement in individual holding cages ($17 \times 7 \times 6$ cm) or placed directly (without injection) in individual holding cages. Thirty minutes later, mice were exposed to the L/D test in an order counterbalanced for test experience and pre-trial injection.

2.5. Behavioural measures

Videotapes were scored blind by a highly trained observer (intra-rater reliability ≥ 0.9) using ethological analysis software ('Hindsight'; [68]). Behavioural parameters comprised conventional indices [15,18] as well as ethological measures derived from recent plus-maze research [39,53]. Conventional indices were: the initial latency (seconds) to move from the light to the dark compartment; the frequencies of (whole body) inter-compartmental transitions and (whole body) line crossings in each compartment; total rearing; and the percentages of both line crossings and time spent in the light [both measures = (score for light/total score) × 100]. Ethological measures comprised the frequency of stretched-attend postures (SAPs: exploratory posture in which the body is stretched forward then retracted to the original position without any forward locomotion) and the total duration (seconds) of grooming (licking, scratching and washing of the head and body). In view of the importance of thigmotactic cues to rodent exploration [3,33,66], the duration (seconds) of thigmotactic ambulation was also scored: in accord with Treit and Fundytus [65], this behaviour was defined as the duration of ambulation occurring in contact with, or within 1 cm of, any of the vertical surfaces of the apparatus. Furthermore, in parallel to recent plus-maze methodology [39], thigmotaxis and SAP were differentiated as a function of their spatial distribution within the apparatus, i.e. in view of the relative security afforded by the dark compartment, 'percent dark' scores were additionally calculated for these specific defensive behaviours (e.g. [dark SAP/total SAP] × 100).

2.6. Statistics

Data from Experiment 1 were subjected to two-factor independent (test-experience × drug treatment) analyses of variance (ANOVA), with further comparisons performed using Newman–Keuls post-hoc tests. In addition, a one-factor independent ANOVA was used to compare scores from the no injection/test-naïve and pre-trial injection/test-naïve groups. Data from Experiment 2 were subjected to two-factor independent (test-experience × pre-trial injection) ANOVA.

2.7. Ethics

The research described in this paper was licensed by the Home Office under the Animals (Scientific Procedures) Act 1986.

3. Results

3.1. Experiment 1

3.1.1. Control profiles

Uninjected test-naive mice initially entered the dark compartment with an average latency of 45 s and, over the 5 min session, displayed high overall levels of line-crossings and rearing together with moderate overall levels of SAPs and thigmotaxis (Table 1). Given the relative sizes of the two compartments, the scores for % line crossings and % time spent in the light compartment (both around 50% relative to an expected 67%) indicated a strong basal preference for the dark compartment. The greater aversiveness of the light compartment was further suggested by the finding that thigmotactic ambulation was almost exclusively (i.e. ~92%) displayed in the light compartment, and also by the high percentage of SAPs displayed from the dark compartment towards the light compartment (67%). Results also showed that handling/i.p. saline injection

Table 1
Effects of chlordiazepoxide (10 mg/kg) on L/D test behaviour in test-naïve and test-experienced male Swiss-Webster mice ($n = 11$–12)

	Test-naïve			Test-experienced	
	No injection	Saline	CDP	Saline	CDP
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Total L/D transitions	9.67 ± 1.74	7.38 ± 1.32	11.09 ± 1.68	8.64 ± 1.32	8.77 ± 2.04
Line crossings in light	44.00 ± 7.76	28.54 ± 6.09	[a]68.73 ± 10.48	27.64 ± 5.26	[d]32.38 ± 7.76
Line crossings in dark	43.58 ± 5.19	37.85 ± 2.82	37.09 ± 5.55	36.64 ± 4.19	39.69 ± 4.79
% line crossings in light	50.24 ± 4.74	42.99 ± 6.53	[a]64.95 ± 5.44	43.01 ± 4.93	[f]44.92 ± 6.42
% time in light	49.74 ± 6.16	34.43 ± 5.59	[b]56.73 ± 6.51	29.87 ± 4.33	[e]28.71 ± 6.18
Latency to enter dark (s)	45.03 ± 6.91	34.97 ± 7.92	48.96 ± 10.28	14.84 ± 3.36	[f]19.67 ± 5.21
Supported rears	34.33 ± 5.22	33.77 ± 4.26	29.73 ± 5.05	32.73 ± 3.98	30.77 ± 5.88
Thigmotaxis (s)	15.03 ± 2.61	13.00 ± 3.25	7.08 ± 2.07	13.24 ± 3.86	13.29 ± 4.97
% thigmotaxis in dark	8.39 ± 5.75	*30.87 ± 8.21	8.99 ± 5.75	27.90 ± 10.42	35.66 ± 12.49
SAPs	11.08 ± 2.12	*18.54 ± 1.66	[b]10.55 ± 2.33	16.00 ± 1.90	[a]6.46 ± 1.29
% SAPs in dark	66.76 ± 6.47	75.55 ± 5.27	[c]52.96 ± 8.93	86.17 ± 4.75	[f]80.99 ± 7.84
Grooming (s)	2.38 ± 0.77	*7.86 ± 2.45	6.05 ± 2.47	10.33 ± 2.60	15.04 ± 3.16

[a] $P < 0.001$;
[b] $P < 0.01$;
[c] $P < 0.05$ vs. saline.
[d] $P < 0.001$;
[e] $P < 0.01$;
[f] $P < 0.05$ vs. CDP/test-naïve.
* For the effects of pre-trial injection per se on test-naïve profiles; $P < 0.05$ vs. no injection.

30 min prior to testing had a mild effect on the behaviour of mice naive mice to the test. Consistent with a weak anxiogenic-like action, significantly higher levels of total SAPs, grooming, and % thigmotaxis in the dark [all $F(1, 23) \geq 4.26$, $P < 0.05$] were found in saline-injected naïve mice, compared to non-injected naïve subjects.

3.1.2. Effects of prior test experience on behavioural baselines and response to CDP

The effects of CDP on L/D behaviour in test-naïve and test-experienced mice are summarised in Table 1. ANOVA yielded significant test-experience × CDP interactions for total line crossings in the light, % line crossings in the light and % time spent in the light [all $F(1, 44) \geq 4.10$, $P < 0.05$]. Newman–Keuls analysis showed that CDP significantly increased all three measures in test-naïve mice, but completely failed to alter them in mice that had been pre-exposed (undrugged) to the apparatus 24 h previously (see also Fig. 1). The contrasting effect of CDP in test-naïve and test-experienced mice was further emphasised by the significantly lower levels of line crossings in light, % line crossings in light and% time in light in drug-treated test-experienced subjects compared to similarly treated test-naïve animals. ANOVA also indicated significant main effects of CDP on total SAPs and % SAPs in the dark [both $F(1, 44) \geqslant 4.02$, $P < 0.05$]. Newman–Keuls comparisons confirmed that CDP significantly reduced the total number of SAPs regardless of test-experience, whereas the % SAPs displayed in the dark was significantly reduced by CDP in test-naïve subjects only. The influence of prior test experience on the latter measure was further highlighted by a significant main effect for this factor [$F(1, 44) = 7.78$, $P < 0.01$], as well as the significantly higher level of % SAPs (dark) in drug-treated test-experienced subjects relative to their test-naïve counterparts. A significant main effect for test-experience was also found for latency to enter the dark area [$F(1, 44) = 11.90$, $P < 0.001$]; post-hoc analysis revealed that, although this measure was reduced by test-experience in both treatment conditions, the change was statistically reliable only for CDP-treated subjects. Grooming was generally increased by prior test experience [$F(1, 44) = 4.41$, $P < 0.05$].

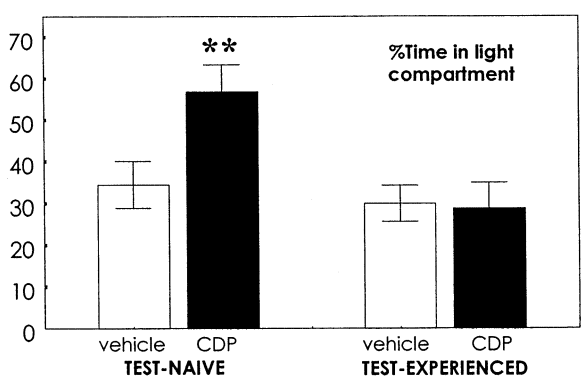

Fig. 1. Effect of chlordiazepoxide (10 mg/kg) on % time spent in the light compartment in L/D test-naïve and L/D test-experienced male Swiss-Webster mice ($n = 11$–12). $^{**}P < 0.01$ vs. saline.

Table 2
Influence of pre-trial saline injection on the effects of repeated L/D testing in male Swiss-Webster mice ($n = 10$)

	No injection		Pre-trial injection	
	Test-naive	Test-experienced	Test-naïve	Test-experienced
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Total L/D transitions	8.00 ± 1.31	7.90 ± 1.45	9.70 ± 1.13	8.80 ± 1.37
Line crossings in light	31.00 ± 6.14	36.20 ± 6.98	38.70 ± 4.12	37.30 ± 6.40
Line crossings in dark	52.10 ± 5.35	49.40 ± 4.87	44.70 ± 3.89	55.90 ± 5.76
% line crossings in light	34.16 ± 6.13	40.98 ± 3.40	46.05 ± 3.37	38.11 ± 4.57
% time in light	30.30 ± 5.87	30.35 ± 3.96	37.62 ± 4.01	27.03 ± 4.62
Latency to enter dark (s)	19.79 ± 3.17	19.01 ± 7.18	26.05 ± 5.61	13.78 ± 3.67
Supported rears	16.40 ± 1.91	20.30 ± 4.14	16.80 ± 2.35	26.40 ± 4.19
Thigmotaxis (s)	11.27 ± 1.68	12.62 ± 1.23	12.66 ± 1.49	10.52 ± 1.76
% thigmotaxis in dark	28.93 ± 11.95	24.73 ± 4.25	22.28 ± 5.68	28.70 ± 5.79
SAPs	17.60 ± 3.28	10.10 ± 1.93	14.40 ± 2.13	8.80 ± 1.50
% SAPs in dark	79.89 ± 5.44	82.48 ± 4.53	78.56 ± 6.45	80.58 ± 8.04
Grooming (s)	4.32 ± 1.54	13.90 ± 3.27	9.74 ± 2.93	12.41 ± 4.66

3.2. Experiment 2

3.2.1. Control profiles

As observed for experiment 1, uninjected test-naive mice showed a distinct aversion to the light compartment, as evidenced by the low scores for activity (% line crosses) and time spent in this part of the test arena (Table 2). If anything, profile comparisons with the corresponding treatment condition in the first experiment would suggest a somewhat higher baseline level of anxiety in experiment 2: e.g. lower scores for % line crosses and time spent in the light compartment, and higher levels of total SAPs and % SAPs displayed from the dark compartment.

3.2.2. Effects of handling/i.p. saline injection on changes with test-experience

The effects of handling/i.p. saline injection 30 min prior to testing on L/D behaviour in test-naïve and test-experienced mice are summarised in Table 2. ANOVA failed to reveal any significant test-experience × pre-trial injection interactions [all$F(1, 36) = 2.70$, $P > 0.05$], or any significant main effects for pre-trial injection [all$F(1, 36) = 1.00$, $P > 0.05$]. However, significant main effects of test experience were found for supported rears [$F(1, 36) = 4.15$, $P < 0.05$] and total SAPs [$F(1, 36) = 8.08$, $P < 0.01$], with injection-independent increases in the former and decreases in the latter. There were no other significant effects of test-experience [$F(1, 36) = 3.47$, $P > 0.07$].

4. Discussion

Present results show that test-naïve Swiss-Webster mice exhibit high overall levels of activity (i.e. line crosses/rearing) in the light/dark exploration (L/D) test and, as for many mouse strains [10,15–18,34,49,67], display a distinct aversion to the light compartment. Thus, in experiment 1, vehicle-treated test-naïve animals spent approximately 35% of the test session in the light compartment and exhibited around 43% of their locomotor activity in this area, both values being substantially lower than that predicted on the basis of random activity in unequal-sized compartments ($\sim$ 67%). Of the ethological measures profiled in the present study, stretched-attend postures (SAPs) and thigmotactic ambulation were clearly evident while levels of grooming were low. SAPs are believed to reflect risk assessment in potentially dangerous environments [9,39,47,61], a role consistent with their predominant display (76%) from the dark compartment towards the light area in the present study. Thigmotaxis (or 'wall-hugging') is another integral component of the rodent defensive repertoire [65,66], and one which is thought to reduce an animal's physical exposure in open and/or well-lit spaces. Consistent with this functional perspective, the spatial distribution of thigmotaxis in the L/D test indicated an almost exclusive ($> 90\%$) association with the more aversive light compartment.

In replication of previous reports for mice [2,15,16,18,22,34,42,49,59,67], rats [13,14,51,64], and even wild voles [36], present data confirm that CDP (10 mg/kg) blocked the aversion shown by test-naïve animals towards the brightly-lit compartment. Thus, drug treatment not only increased % time and % line crossings in the light area but did so to levels approximating unbiased behaviour. The observation that these changes were not paralleled by increases in either rearing, or the number of line crossings made in the dark area, suggests that CDP specifically reduced the natural aversion of mice towards the light area, rather than producing

general locomotor stimulation. An anxioselective interpretation of these effects is further supported by the significant CDP-induced reductions in total SAPs, a behavioural measure that (in a variety of threatening contexts) has been found to be highly sensitive to benzodiazepine and non-benzodiazepine anxiolytics [8,30,32,47,55,62]. Indeed, in a further parallel with benzodiazepine effects in the plus-maze, the proportion of SAPs displayed from the dark ('safe') area towards the light ('potentially dangerous') area was also significantly reduced by CDP. Although CDP did not significantly alter either the total duration of thigmotaxis, or the proportion of this behaviour displayed in the dark area, both measures were clearly reduced by the drug [48,65]. In view of concerns about the reliability of light/dark transitions and light/dark latencies as behavioural measures sensitive to anxiolytic activity [2,15,35,42,49,67], it is pertinent to note that neither measure was significantly altered by CDP at doses that, in the present study, clearly reduced avoidance of the light compartment.

In contrast to results obtained in test-naïve mice, our findings clearly show that the anxiolytic response to 10 mg/kg CDP was almost completely absent in Swiss-Webster mice that had been exposed to the maze, undrugged, 24 h earlier. In test-experienced animals, and irrespective of whether statistical comparisons involved saline-treated test-experienced controls or drug-treated test-naïve animals, CDP completely failed to increase % time in light, line crossings in light or % line crossings in light. Importantly, this finding cannot be explained by experientially-induced changes in baseline responses to the L/D test since, other than a notable (though non-significant) reduction in latency to enter the dark area, there were no effects of prior test-experience on baseline scores in saline-treated controls. However, it is interesting to note that one specific behavioural effect of CDP was not affected by prior test experience, i.e. total SAPs were reduced to the same extent as in test-naïve subjects. Although consistent with evidence that certain benzodiazepine effects in the plus-maze can remain at least partially intact in test-experienced mice [40,58], it should be emphasised that prior experience of the L/D test did eliminate the inhibitory effect of CDP on the proportion of SAPs displayed towards the relatively more aversive light compartment. Overall, present results are fully in accord with the experientially-induced loss of benzodiazepine anxiolysis in the mouse elevated plus-maze [40,57,58] and four-plate [34] paradigms, and with the effects of prior plus-maze exposure on subsequent response to diazepam in the L/D test [58]. This finding represents an important extension to the previous literature given the growing consensus that different tests for anxiety in rodents are measuring different forms of anxiety-related behaviour [5–7,25,46,52]. Thus, while there may well be qualitative differences in behavioural responses to the elevated plus-maze and the L/D tests, the consequences of repeated testing for the detection of benzodiazepine anxiolysis are remarkably similar in both procedures.

Despite this strong evidence for cross-test generality in the effects of prior experience on the anxiolytic efficacy of benzodiazepines, our data are at variance with two previous reports involving repeated exposure to the L/D test. Blumstein and Crawley [10] found that diazepam retained its ability to increase light/dark transitions when mice were either given up to three trials with constant inter-trial-interval (ITI) (2–3 days) or two trials with variable ITI (1–7 days). Unfortunately, any direct comparison between present data and those of Blumstein and Crawley [10] is compromised by the use of different mouse strains (C57BL/6J vs. Swiss-Webster), the lack of CDP effect on light/dark transitions in our test-naïve mice, and the fact that their protocol involved diazepam on every trial whereas our subjects received drug treatment only on the retest trial. However, in a test variant that employed a 24 h undrugged pre-exposure (as a procedural means of reducing the variability of behavioural responses), Artaiz et al. [2] found that diazepam produced a significant increase in % time spent in the light compartment in test-experienced subjects. It is difficult to account for the discrepancy between this finding and the results of the present study since both experiments used closely related mouse strains (Swiss and Swiss-Webster), undrugged pre-exposure to the L/D test, and a 24 h interval between pre-exposure and drug testing. Despite these major similarities, however, Artaiz and colleagues [2] reported a substantial ($\sim$50%) between-trials reduction in exploration of the light area. From data cited (though not detailed) in their paper, it would appear that undrugged test-naïve mice spent around 30% of their time in the light area and showed approximately 35 line crosses in this part of the apparatus. Comparison with corresponding values reported in Table 1 (test-naïve, no-injection) shows that our intact mice were not only more active in the light area ($\sim$44 crossings) but also spent significantly more time there ($\sim$50%). While this difference in basal anxiety may be relevant, logic would suggest that our test conditions (i.e. relatively low anxiety on trial 1) would have been more, not less, likely to reveal an experientially-induced increase in anxiety. Moreover, a comparison between scores for test-naive and test-experienced saline-treated mice in experiment 2 revealed no difference in behaviour, even though basal anxiety scores for test-naive mice in that study were similar to those reported for test-naive mice by Artaiz et al. [2]. In this context, it is pertinent to note that, while others have also observed apparent retest increases in anxiety in the L/D test, such changes have usually been evident only after mul-

tiple retest trials [4,49]. Nevertheless, in view of the results of Artaiz et al. [2], as well as the enhanced anxiety levels that are normally seen following a single undrugged exposure to the mouse plus-maze [20,39,44,55–58] and four-plate [34,63] tests, the question of why test–retest baseline changes were not observed in the present study assumes some importance.

Of possible relevance to this issue is the profile comparison between the two control groups used in experiment 1, i.e. uninjected test-naïve vs. saline-injected test-naïve animals. From Table 1, it is evident that there were few major effects of pre-trial injection on L/D test behaviour, i.e. despite increases in total SAPs, grooming and % dark thigmotaxis in saline-treated test-naïve subjects, no differences were apparent in the primary indices of anxiety. These findings render it unlikely that pre-trial injection per se could have increased anxiety in (saline treated) test-naïve mice [1,43], thereby preventing detection of a further shift in anxiety when comparisons were made with (saline treated) test-experienced mice. Nevertheless, to more directly address this possibility, a second experiment was conducted in which the effects of repeated L/D testing were examined in mice that had either received a single saline injection 30 min prior to testing or had been exposed to the test without prior injection. Results showed that handling/injection had minimal influence on L/D test behavioural profiles in either test-naïve or test-experienced mice. Furthermore, although prior experience per se resulted in some behavioural alterations, these changes did not include any of the major indices of anxiety. Together, these findings indicate the apparent absence of experientially-induced alterations in baseline L/D anxiety scores cannot be attributed to a confounding influence of pre-trial injection stress. Importantly, they also confirm that the experientially-induced loss of an anxiolytic response to CDP (Section 2.4.1) cannot simply be an artefact of altered baseline anxiety-like behaviour.

In summary, the results of the present study suggest that, in a manner similar to that reported for the elevated plus-maze (and, more recently, the four-plate test), a single undrugged experience of the L/D test largely abolishes the normal behavioural response of Swiss-Webster mice to an anxiolytic dose of chlordiazepoxide. Importantly, the absence of a major effect of prior test experience on behavioural baselines would seem to argue against explanations based upon simple habituation or sensitisation to the test apparatus/procedure. Whether the observed changes in drug responsivity reflect a pharmacologically-relevant alteration in the nature of the emotional reaction to the test (as suggested for parallel findings in the elevated plus-maze [28,39]), is clearly a matter for further investigation. Dose-response studies will be required to determine whether test-experienced mice are completely insensitive or merely less sensitive to the anxiolytic effects of benzodiazepines, and it will also be of interest to assess whether prior test experience influences responses to non-benzodiazepine anxiolytics in this model. Furthermore, parametric manipulation of test–retest (inter-trial) intervals should facilitate identification of the optimal conditions required to avoid potential confounds arising from prior experience. Given the sensitivity of exploratory models to a range of organismic and procedural factors [54], and the variability of these factors across laboratories [37], we should perhaps caution against too hasty a generalisation of present findings to other mouse strains and test conditions. Nevertheless, in view of the mounting evidence that certain anxiety models may be particularly sensitive to repeated testing, investigators should at least be aware of the potential confounds arising from prior experience of the L/D paradigm.

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