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Effects of diazepam on behavioural and antinociceptive responses to the elevated plus-maze in male mice depend upon treatment regimen and prior maze experience

R.J. Rodgers[1], C. Lee[2], and J.K. Shepherd[1]

[1]Department of Psychology, University of Leeds, Leeds LS2 9JT, UK
[2]Department of Psychology, University of Bradford, Bradford BD7 1DP, UK

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Abstract. Recent studies have shown that brief exposure to an elevated plus-maze (EPM) produces non-opioid antinociception in male mice. The present experiments were designed to assess the effects of diazepam on this phenomenon. When acutely administered, low doses (0.5–1.0 mg/kg) of diazepam failed to produce an anxiolytic profile and exerted rather inconsistent effects on EPM-induced elevations in tail-flick latencies. In EPM-experienced mice, chronic treatment with higher doses of diazepam (2–4 mg/kg, 8 days) produced a weak anxiolytic action and inhibited the early phase of EPM antinociception only. However, in EPM-naive mice, 8-day diazepam pretreatment exerted a marked anxiolytic effect and completely eliminated the antinociceptive response to the maze. Together, these data support the view that anxiety is a key factor in certain forms of adaptive pain inhibition and suggest a possible mediational role for benzodiazepine receptors. Our findings also show that prior exposure to the EPM, rather than chronic handling/injection, greatly reduces the anti-anxiety effect of diazepam. Furthermore, since re-exposure to the maze, per se, decreased time spent on the open arms and central platform, a shift in behavioural baseline ("retest anxiogenesis") may have contributed to the weak behavioural effects of diazepam in test-experienced animals. Importantly, as chronic treatment with diazepam did not influence this anxiogenic-like retest profile, our data suggest that a single prior experience of the EPM may radically alter the nature of the anxiety reaction provoked by this test.

Key words: Elevated plus-maze – Anxiety – Antinociception – Diazepam – Prior experience – Treatment regimen – Mice

Pain inhibition is an adaptive component of the defensive repertoire of many species (Bolles and Fanselow 1980; Amir 1986; Amit and Galina 1986; Fanselow 1986; Rodgers and Randall 1987c), with both opioid and non-opioid substrates implicated (for reviews: Tricklebank and Curzon 1984; Kelly 1986; Rodgers and Randall 1988a). Extensive behavioural and pharmacological studies on non-opioid antinociception in defeated male mice (Rodgers and Randall 1986a) have suggested a relationship to mechanisms of anxiety. For example, this form of adaptive pain inhibition appears to be anticipatory in nature, since it may be induced by exposure to the territorial scent of an aggressive male conspecific (Rodgers and Randall 1986b; Kavaliers and Innes 1988). Furthermore, the reaction is blocked by a range of traditional (e.g. diazepam) and novel (e.g. buspirone, ondansetron) anti-anxiety agents (Rodgers and Randall 1987a, b, 1988b; Kavaliers and Innes 1988; Rodgers and Shepherd 1989a, b, 1990 a, b).

The proposed involvement of anxiety in non-opioid antinociception has more recently been supported by the observation of pain inhibition in mice exposed to the elevated plus-maze (EPM) test (Lee and Rodgers 1990). This animal model of anxiety is based upon the natural aversion of rodents to heights and open spaces (Montgomery 1958), has been validated for both rats (Pellow et al. 1985) and mice (Lister 1987), and is bidirectionally sensitive to pharmacological manipulations (e.g. Handley and Mithani 1984; Pellow and File 1986; Critchley and Handley 1987; Baldwin and File 1988; Rago et al. 1988; Moser 1989; Soderpalm et al. 1989; Benjamin et al. 1990). We have found that a 5-min exposure to the EPM results in a long-lasting elevation in tail-flick latencies in male mice (Lee and Rodgers 1990), with similar findings recently reported for rats (Taukulis and Goggin 1990). In mice, this response is not blocked by naltrexone or chronic morphine treatment, indicating probable non-opioid mediation (Lee and Rodgers 1990). Consistent with the "anxiety" hypothesis of non-opioid antinociception, naltrexone actually produced an anxiogenic-like profile and an associated enhancement of the EPM-induced elevation in tail-flick latencies.

In view of these findings, and earlier work on defeat

Offprint requests to: R.J. Rodgers

analgesia (Rodgers and Randall 1987a, b), the present studies were designed to assess the effects of diazepam on EPM behaviour and antinociception. As pilot observations confirmed significant behavioural suppression with > 1 mg/kg diazepam (e.g. Pellow et al. 1985), both acute and chronic treatment regimens were employed.

Materials and methods

Animals. Ten-to-15-week-old male DBA/2 mice (Bantin & Kingman, Hull, UK and Biomedical Services, University of Leeds), weighing 25–32 g, were used. Animals were group-housed (ten per cage; cage size: 45 × 28 × 13 cm) in a temperature-controlled room (24 ± 1 °C) in which a 12 h reversed light-dark (LD) cycle was operative (lights on: 19.00 hours). Food and water were freely available. With the exception of experiment 2, which involved repeated testing on the plus-maze, naive animals were used throughout this series.

Apparatus. Nociceptive latencies were determined by traditional (radiant heat) tail-flick assay (Socrel Tail-Flick Apparatus, Ugo Basile, Italy), with temperature adjusted to give control latencies (TFL) of 2–3 s. A cut-off of 8 s was employed.

The EPM was a modification of the apparatus validated for the mouse by Lister (1987). Two open (30 × 5 cm) and two enclosed (30 × 5 × 15 cm) arms extended from a central platform (5 × 5 cm), making the shape of a plus-sign, and the entire apparatus was elevated to a height of 45 cm above floor-level. The central platform and maze floor were constructed from black Plexiglas, while the side walls of the closed arms were made of clear Plexiglas. As previously reported (Lee and Rodgers 1990), grip on the open arms was provided by inclusion of a slight lip (height: 0.25 cm) and open arm activity was facilitated by testing under dim red light (2 × 60 W).

Drugs. Diazepam (Roche Products Ltd, UK) was ultrasonically dispersed in distilled water to which Tween 80 (2 drops per 10 ml) had been added; a corresponding water/Tween mixture served as vehicle control. All injections were performed intraperitoneally (IP) in a volume of 10 ml/kg. Treatments were coded, with codes broken only after complete data analysis.

Procedure. All testing was conducted under dim red light during the mid-portion of the dark phase of the LD cycle. Mice were randomly allocated to treatment conditions and tested in a counterbalanced order. In the EPM test, animals were individually placed onto the central platform facing an open arm and removed following a 5 min test period (Montgomery 1958; Pellow et al. 1985; Lister 1987; Soderpalm et al. 1989; Moser 1989; Lee and Rodgers 1990). To reduce any lingering olfactory cues, the apparatus was wiped with a clean damp cloth between successive tests. Test sessions were recorded by a vertically-mounted videocamera, linked to a monitor and VCR in an adjacent laboratory. Behaviours scored from videotape were: number of rears, number of open and closed arm entries, (plus total arm entries) and time spent on the various sections of the maze (open, closed, centre platform; Lee and Rodgers 1990). Arms entries were defined as entry of all four paws into the arm. Distribution of behaviour on the maze was additionally calculated as "percent total" both for frequency and duration measures.

Baseline TFL were established either immediately prior to EPM exposure (experiment 2, day 1) or immediately prior to injection (excluding "injection only" days in experiments 2 and 3). Further TFL measurements were recorded at 0, 5 and 10 min following EPM exposure. Methodologically, it is important to note that baseline TFL measurement has minimal impact upon EPM behaviour; under present test conditions, and irrespective of prior TFL assessment, group-housed DBA/2 mice normally spend 10–20% of their time on the open arms of the maze (unpublished observations). During the injection-test interval, and between post-EPM TFL measures, mice were returned to their home cages. This procedure which, per se, does not influence nociception (Lee and Rodgers 1990), was followed in order to minimize any confounding influence of exposure to an additional unfamiliar environment. Three experiments were performed in this series:

Effects of acute diazepam treatment on EPM behaviour and antinociception. Mice were randomly allocated to three treatment conditions ($n = 10$–11): vehicle, 0.5 or 1.0 mg/kg diazepam. Pilot studies had shown that higher acute doses of diazepam (2–4 mg/kg) produce marked behavioural suppression in this test. Animals were injected immediately following determination of baseline TFL and, 30 min later, were exposed to the EPM for 5 min.

Effects of chronic diazepam treatment on EPM behaviour and antinociception (maze pretest). On day 1, untreated mice were pretested on the EPM in order to equate three groups ($n = 10$–11) in terms of initial antinociceptive responsivity to the maze. TFL were taken both before (baseline) and after maze exposure. Over the next 8 days (days 2–9), and in the testing laboratory, animals received daily injections of vehicle, 2.0 or 4.0 mg/kg diazepam. Thirty minutes following the final injection, animals were again exposed to the EPM. TFL measurements were taken immediately prior to the final injection (baseline) and at 0, 5 and 10 min post-EPM.

Effects of chronic diazepam treatment on EPM behaviour and antinociception (no maze pretest). This study essentially replicated the design of experiment 2, with the important omission of the day 1 maze pretest. Mice were randomly assigned to three treatment conditions ($n = 10$) and, in the testing laboratory, received daily injections of vehicle, 2.0 or 4.0 mg/kg diazepam for 8 days. TFL were taken immediately prior to the final injection (baseline) and, 30 min later, animals were exposed to the EPM for the first and only time. TFL were re-established at 0, 5 and 10 min post-EPM.

Statistics. Data (TFL and behaviour) were initially subjected to single factor, or two-factor (repeated measures on second factor), analyses of variance (ANOVA). Follow-up comparisons, both within and between groups, were performed using the appropriate error variance terms from the ANOVAs.

Results

Acute diazepam

Behavioural data are summarized in Table 1 and Fig. 1. On general activity measures, ANOVA revealed that although diazepam tended to increase total rearing, this effect did not reach statistical significance ($F_{2,29} = 2.00$, NS). However, the total number of arm entries was influenced by drug treatment ($F_{2,29} = 4.42$, $P < 0.05$), an effect attributable to an increase at 0.5 mg/kg ($P < 0.01$). Further analysis indicated that all groups had a distinct preference for closed versus open arms (entries: $F_{1,29} = 71.29$, $P < 0.01$), a profile that appeared to be enhanced by diazepam ($F_{2,29} = 3.16$; $F_{\text{crit } 0.05} = 3.32$). This suggestion was confirmed by between-group comparisons (versus vehicle control) which revealed that, despite an absence of drug effect on open arm entries, both doses increased closed arm entries ($P < 0.01$).

Analysis of percent number of arm entries and percent time on arms confirmed the strong preference for closed versus open arms ($F_{1,29} = 60.82$, $P < 0.01$ and $F_{1,29} = 110.4$, $P < 0.01$), but failed to reveal an significant effect of diazepam ($F_{2,29} = 0.79$, NS and $F_{2,29} = 0.5$, NS). For

Table 1. Effects of acute diazepam (0.5–1.0 mg/kg, IP) on behaviours displayed by male DBA/2 mice on the elevated plus-maze. Data are presented as mean values ± SEM. See also Fig. 1

Behaviour	Vehicle	Diazepam	
		0.5 mg/kg	1.0 mg/kg
Total entries	13.64 ± 1.74	20.55 ± 1.81[a]	17.40 ± 1.38
Total rears	11.10 ± 2.51	17.10 ± 2.39	16.80 ± 2.32
Open arm entries	5.10 ± 0.97[b]	6.55 ± 1.06[b]	5.60 ± 0.70[b]
Closed arm entries	8.54 ± 1.09	14.00 ± 1.14[a]	11.80 ± 1.05[a]
% Open entries	36.55 ± 4.34[b]	30.10 ± 3.30[b]	31.70 ± 3.71[b]
% Closed entries	63.45 ± 4.34	69.90 ± 3.30	68.30 ± 3.71
n	11	11	10

[a] $P<0.01$ vs vehicle [b] $P<0.01$ vs closed arms

Table 2. Effects of acute diazepam (0.5–1.0 mg/kg, IP) on the antinociceptive effects of EPM exposure. Data are presented as mean tail-flick latencies (s, ± SEM)

Condition	Baseline	0	5	10 min
Vehicle	2.19 ± 0.09	2.94 ± 0.31[c]	3.02 ± 0.22[c]	2.79 ± 0.23[c]
0.5 mg/kg diazepam	2.09 ± 0.05	2.38 ± 0.11[b, d]	2.33 ± 0.10[d]	2.15 ± 0.09[d]
1.0 mg/kg diazepam	2.20 ± 0.09	2.45 ± 0.14[d]	2.48 ± 0.07[a, d]	2.59 ± 0.18[c]

[a] $P<0.05$
[b] $P<0.02$ } vs baseline
[c] $P<0.01$
[d] $P<0.01$ vs vehicle

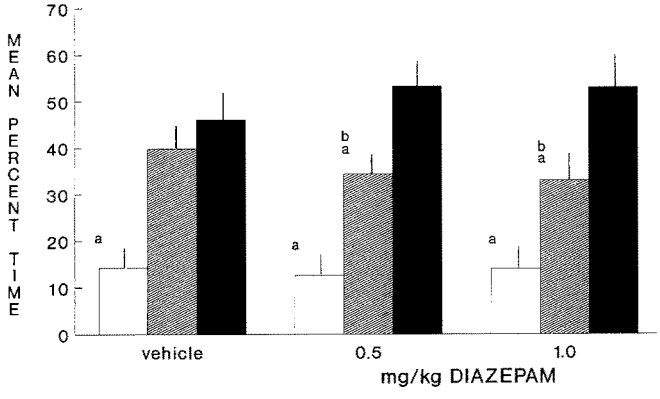

Fig. 1. Effects of acute diazepam treatment (0.5–1.0 mg/kg) on percent time spent (mean ± SEM) by male mice on different sections of an elevated plus-maze during a 5-min test. *Clear bars* open arms, *hatched bars* central platform; *black bars* closed arms; [a] $P<0.01$ versus closed arms; [b] $P<0.01$ versus open arms

percent time spent on maze sections (including central platform; Lee and Rodgers 1990; Fig. 1), ANOVA revealed a significant main effect ($F_{2,58}=48.19$, $P<0.05$) with controls displaying a rank-order preference of closed > centre = open. Although diazepam did not seem to alter this profile ($F_{4,58}=0.71$, NS), it is interesting to note that both doses subtly altered the rank-order preference to closed > centre > open (all comparisons, $P<0.01$).

The effects of acute diazepam treatment on the antinociceptive consequences of EPM exposure are summarized in Table 2. ANOVA revealed significant main effects for drug ($F_{2,29}=4.62$, $P<0.05$) and time ($F_{3,87}=8.8$, $P<0.01$), but no significant interaction ($F_{6,87}=1.87$, NS). Comparisons with baseline indicated that, in the vehicle control condition, tail-flick latencies were significantly elevated at all time-points following EPM exposure ($P<0.01$). This profile appeared to be attenuated by diazepam, with antinociception only evident in the 0.5 mg/kg group at time 0 ($P<0.02$), and in the 1.0 mg/kg group at 5 ($P<0.05$) and 10 ($P<0.01$) min post-EPM. Further comparisons (versus vehicle control) confirmed that, although groups were equivalent on baseline TFL scores, both doses of diazepam significantly reduced the degree of EPM antinociception ($P<0.01$ at all time points, except 1.0 mg/kg at 10 min).

Chronic diazepam (maze pretest)

Behavioural data are summarized in Table 3 and Fig. 2. For total arm entries, ANOVA failed to reveal a significant effect for drug history ($F_{2,29}=0.01$, NS), days ($F_{1,29}=1.62$ NS) or a drug × days interaction ($F_{2,29}=0.65$ NS). However, for rearing, significant effects were observed: drug history ($F_{2,29}=5.34$, $P<0.01$), days ($F_{1,29}=8.4$, $P<0.01$) and the interaction ($F_{2,29}=3.4$, $P<0.05$). Animals receiving diazepam (2 and 4 mg/kg) showed significantly reduced rearing in comparison to both vehicle control ($P<0.01$) and day 1 pretest ($P<0.01$) scores.

On day 1 pretest, all groups displayed a significantly greater number of closed (versus open) arm entries (day 1: $F_{1,29}=205.92$, $P<0.01$) with a similar profile evident on day 9 ($F_{1,29}=116.42$, $P<0.01$). Retesting on the maze did not alter this preference, with the frequency of open ($F_{1,29}=2.17$, NS) and closed ($F_{1,29}=0.86$, NS) arm entries remaining stable. Furthermore, chronic diazepam

Table 3. Effects of chronic diazepam (2–4 mg/kg, IP, 8 days) on behaviours displayed by male DBA/2 mice on the elevated plus-maze. Day 1 – prior to treatment; day 9 – following treatment. Data are presented as mean values ± SEM. See also Fig. 2

Behaviour	Day	Vehicle	Diazepam	
			2.0 mg/kg	4.0 mg/kg
Total entries	1	14.09 ± 0.77	14.50 ± 1.61	15.64 ± 1.64
	9	13.64 ± 1.76	13.80 ± 3.16	12.00 ± 2.34
Total rears	1	21.55 ± 2.67	19.60 ± 3.07	18.27 ± 2.55
	9	22.73 ± 2.20	10.40 ± 1.75[a, c]	10.64 ± 2.02[a, c]
Open arm entries	1	1.00 ± 0.49[d]	2.70 ± 1.10[d]	1.64 ± 0.49[d]
	9	0.45 ± 0.25[d]	2.20 ± 1.09[d]	1.27 ± 0.33[d]
Closed arm entries	1	13.09 ± 0.79	11.80 ± 1.11	14.00 ± 1.48
	9	13.18 ± 1.59	11.60 ± 2.13	10.73 ± 2.24
% Open entries	1	6.64 ± 3.12[d]	15.60 ± 5.47[d]	10.27 ± 2.79[d]
	9	2.27 ± 1.28[d]	10.50 ± 3.67[d]	13.73 ± 3.37[b, d]
% Closed entries	1	93.36 ± 3.12	84.40 ± 5.47	89.73 ± 2.79
	9	97.73 ± 1.28	89.50 ± 3.67	86.27 ± 3.37[b]
n		11	10	11

[a] $P<0.01$ vs day 1
[b] $P<0.05$ vs vehicle
[c] $P<0.01$ vs vehicle
[d] $P<0.01$ vs closed arms

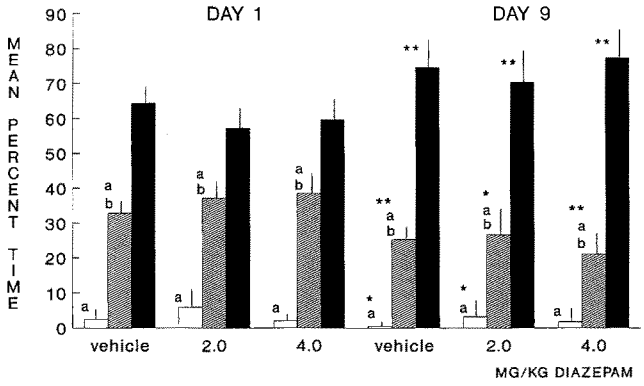

Fig. 2. Effects of chronic diazepam treatment (2–4 mg/kg, 8 days) on percent time spent (mean ± SEM) by male mice on different sections of an elevated plus-maze during a pretest (*day 1*) and following treatment (*day 9*). *Clear bars* open arms; *hatched bars* central platform; *black bars* closed arms; $*P<0.05$; $**P<0.01$ versus day 1; $^aP<0.01$ versus closed arms; $^bP<0.01$ versus open arms

treatment did not influence the pattern of arm entries ($F_{2,29}=1.28$, NS). Analysis of percent number of entries revealed essentially an identical picture, with the exception that ANOVA indicated a significant diazepam × arm interaction ($F_{2,29}=4.2$, $P<0.05$). Further analysis showed that 4 mg/kg diazepam significantly increased percent entries onto the open arms ($P<0.05$) and, reciprocally, decreased percent entries onto the closed arms ($P<0.05$).

Analysis of percent time spent on maze sections (see Fig. 2) revealed all groups to be equivalent on day 1, showing a rank order preference of closed arms > central platform > open arms ($F_{2,58}=263.3$, $P<0.01$). A similar rank order preference was seen on day 9 ($F_{2,58}=215.43$, $P<0.01$). However, prior maze experience did alter behaviour on day 9 in that, compared to pretest, controls spent significantly less time on the open arms ($F_{1,29}=4.18$, $P<0.05$). and central platform ($F_{1,29}=23.54$, $P<0.01$) and more time on the closed arms ($F_{1,29}=29.8$, $P<0.01$). No significant between-groups differences were noted on either test day (day 1: $F_{4,58}=1.49$, NS, day 9: $F_{4,58}=0.59$, NS). Thus, chronic diazepam treatment did not alter percent time spent on the various sections of the maze.

Analysis of day 1 TFL data (Table 4) revealed only a significant main effect for time ($F_{3,87}=9.47$, $P<0.01$), with subsequent comparisons confirming an elevation in TFL in all groups up to 10 min post-EPM exposure. No between-groups differences were apparent on either baseline or post-EPM measures. Between-days analysis indicated a general increase in TFL from day 1 to day 9 at all time-points ($F_{1,29}$: baseline = 16.73, $P<0.01$, 0 min = 10.91, $P<0.01$, 5 min = 8.50, $P<0.01$, 10 min = 28.24, $P<0.01$). For day 9 data, ANOVA revealed significant main effects for drug ($F_{2,29}=3.68$, $P<0.05$) and time ($F_{3,87}=7.87$, $P<0.01$), as well as a significant interaction ($F_{6,87}=2.2$, $P<0.05$). Elevated TFL were evident in the vehicle group at 0, 5 and 10 min post-EPM ($P<0.01$). In contrast, both diazepam groups showed increased TFL only at 10 min post-EPM (2 mg/kg – $P<0.01$; 4 mg/kg – $P<0.05$). Independent comparisons confirmed that, at 0 and 5 min post-EPM, these groups were significantly less analgetic than controls (see Table 4).

Chronic diazepam (no maze pretest)

Behavioural data are summarized in Table 5 and Fig. 3. Total rearing was significantly influenced by chronic diazepam treatment ($F_{2,27}=6.44$, $P<0.01$), an effect attributable to decreased rearing in mice receiving 4 mg/kg

Table 4. Effects of chronic diazepam (2–4 mg/kg, IP, 8 days) on the antinociceptive effects of EPM exposure. Day 1 – prior to treatment; day 9 – following treatment. Data are presented as mean tail-flick latencies (S, ±SEM)

Condition	Day	Baseline	0	5	10 min
Vehicle	1	1.63±0.10	1.92±0.11[c]	1.81±0.12[b]	1.79±0.08[a]
	9	1.87±0.08[f]	2.44±0.17[c, f]	2.14±0.09[c, f]	2.22±0.15[c, f]
2 mg/kg diazepam	1	1.54±0.07	1.89±0.09[c]	1.86±0.12[c]	1.78±0.10[c]
	9	1.88±0.07[g]	2.02±0.09[e]	1.95±0.07	2.22±0.12[c]
4 mg/kg diazepam	1	1.51±0.12	1.85±0.11[c]	1.70±0.08[b]	1.75±0.10[c]
	9	1.89±0.09[g]	1.95±0.04[e]	1.88±0.05[d]	2.06±0.08[a]

[a] $P<0.05$
[b] $P<0.02$ } vs baseline
[c] $P<0.01$
[d] $P<0.05$ vs vehicle
[e] $P<0.01$ vs vehicle
[f] $P<0.02$
[g] $P<0.01$ } vs day 1

Table 5. Effects of chronic diazepam (2–4 mg/kg, IP, 8 days) on behaviours displayed by male DBA/2 mice on the elevated plus maze (no day 1 pre-test). Data are expressed as mean values ± SEM. See also Fig. 3

Behaviour	Vehicle	Diazepam	
		2 mg/kg	4 mg/kg
Total entries	19.20±1.86	27.30±2.99[a]	23.80±2.06
Total rears	21.10±1.39	21.50±1.23	15.40±1.39[b]
Open arm entries	5.90±0.95[c]	14.10±1.92[b]	11.90±1.23[b]
Closed arm entries	13.30±0.97	13.00±1.43	11.90±1.28
% Open entries	28.79±2.82[c]	50.15±2.87[b]	50.30±3.13[b]
% Closed entries	71.21±2.82	49.85±2.87[b]	49.70±3.13[b]
n	10	10	10

[a] $P<0.025$
[b] $P<0.01$ } vs vehicle
[c] $P<0.01$ vs closed arms

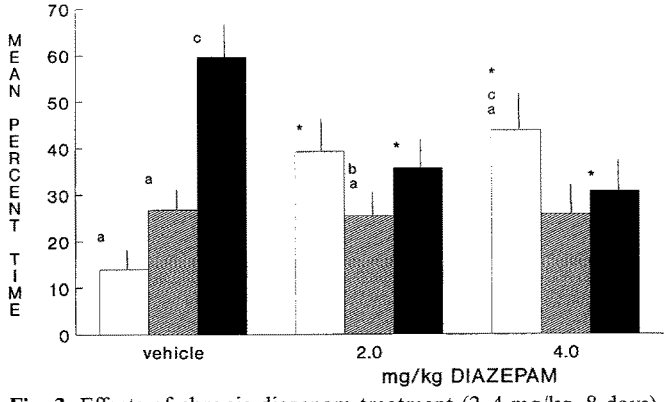

Fig. 3. Effects of chronic diazepam treatment (2–4 mg/kg, 8 days) on percent time spent (mean ± SEM) by male mice on different sections of an elevated plus-maze during a 5-min test (no prior maze experience). *Clear bars* open arms; *hatched bars* central platform; *black bars* closed arms; *$P<0.01$ versus vehicle; [a]$P<0.01$ versus closed arms; [b]$P<0.01$ versus open arms, [c]$P<0.001$ versus central platform

($P<0.01$). The effect of drug treatment on total arm entries also approached statistical significance ($F_{2,27}=2.95$; $F_{crit\ 0.05}=3.35$). Further comparisons revealed a significant increase in arm entries with 2 mg/kg diazepam ($P<0.025$). Analysis of open versus closed arm entries indicated a significant main effect for arm ($F_{1,27}=8.94$, $P<0.01$) and a significant diazepam × arm interaction ($F_{2,27}=14.45$, $P<0.01$); the main effect for diazepam approached, but failed to reach, statistical significance ($F_{2,27}=2.77$; $F_{crit\ 0.05}=3.35$). Further comparisons confirmed a preference for open arm entries in the vehicle condition ($P<0.01$), with no such preference displayed by either of the diazepam groups. Animals treated with both doses of diazepam showed a significant and preferential increase in open arm entries when compared to vehicle control ($P<0.01$). This pattern was confirmed by analysis of percent number of entries (Table 5).

Analysis of percent time spent on different maze sections (see Fig. 3) revealed a significant main effect for maze section ($F_{2,54}=10.36$, $P<0.01$) and a significant diazepam × maze section interaction ($F_{4,54}=13,18$, $P<0.01$). Control mice displayed a typical preference profile of closed arms > central platform > open arms ($P<0.01$). Diazepam 2 mg/kg abolished the preference for open versus closed arms, and increased time spent on the open arms compared with the central platform ($P<0.01$). Diazepam 4 mg/kg produced a preference for open arms compared to closed arms ($P<0.01$) and central platform ($P<0.001$), and abolished the preference for closed arms versus central platform. Independent group comparisons (versus vehicle control) confirmed that both diazepam doses increased percent time spent on the open arms ($F_{2,27}=14.98$, $P<0.01$) and decreased time spent on the closed arms ($F_{2,27}=17.19$, $P<0.01$),

Table 6. Effects of chronic diazepam (2–4 mg/kg, IP, 8 days, no pre-test) on the antinociceptive effects of EPM exposure. Data are presented as mean tail-flick latencies (S, ± SEM)

Condition	Baseline	0	5	10 min
Vehicle	2.53 ± 0.25	3.22 ± 0.29[a]	3.37 ± 0.42[a]	3.57 ± 0.52[a]
2 mg/kg diazepam	2.51 ± 0.20	2.15 ± 0.07	2.38 ± 0.13	2.28 ± 0.14
4 mg/kg diazepam	2.36 ± 0.18	2.35 ± 0.15	2.42 ± 0.14	2.49 ± 0.13[b]

[a] $P < 0.001$ vs baseline
[b] $P < 0.05$ vs vehicle

with no significant change in time spent on the central platform ($F_{2,27} = 0.08$, NS).

Analysis of TFL data (Table 6) indicated a significant main effect for diazepam ($F_{2,27} = 4.76$, $P < 0.025$) and a significant diazepam × time interaction ($F_{6,81} = 3.38$, $P < 0.01$). The main effect for time approached statistical significance ($F_{3,81} = 2.42$, $F_{\text{crit } 0.05} = 2.76$). Further analysis confirmed a significant elevation in TFL in the vehicle control group at all time points post-EPM ($P < 0.001$). Comparisons with baseline indicated that this antinociceptive effect of EPM exposure was completely abolished by chronic treatment with 2–4 mg/kg diazepam. The relatively modest (though significant) EPM-induced elevation in control TFL precluded detection of significant between-groups differences, with the exception of 4 mg/kg versus vehicle at 10 min post-exposure ($P < 0.05$).

Discussion

In confirmation of earlier reports, present findings indicate that mice display an aversion to the open arms of an elevated plus-maze (Lister 1987; Rago et al. 1988; Itoh et al. 1990; Lee and Rodgers 1990). It should, however, be emphasized that, in the current work and in contrast to normal procedure, animals were tested under dim red illumination. This modification was adopted on the basis of our previous observation that male DBA/2 mice show little or no open arm activity when tested under white light (Lee and Rodgers 1990). In contrast to reports by Pellow et al. (1985) and Lister (1987), which suggest that level of illumination has no effect upon EPM behaviour in rats or mice, more recent studies on both species would support our observation of suppressed open arm activity under brightly-lit conditions (Morato and Castrechini 1989; Benjamin et al. 1990). Nevertheless, the fact that DBA/2 mice still show a strong preference for the closed arms/central platform under red light suggests that sensory control of maze behaviour is predominantly non-visual (i.e. tactile and vibrissal senses).

In addition to the basic preference for closed versus open arms, our findings also confirm that animals spend a significant proportion of their time on the central platform of the maze (Lee and Rodgers 1990). It may be pertinent to note that, from this location, mice show high levels of exploratory head-dipping and "anxious" stretch attend/approach responses (Kaesermann 1986; Blanchard et al. 1990) towards the open arms. The results of all three experiments confirm the earlier finding that brief experience of the EPM results in a significant elevation in tail-flick latencies which persists for at least 10 min following testing (Lee and Rodgers 1990; Taukulis and Goggin 1990). Although this reaction is weak in comparison to other forms of environmental analgesia (e.g. footshock), the stimulus situation currently employed is relatively subtle and biologically meaningful. Furthermore, the fact that it is a statistically robust and fully replicable phenomenon would support the view that it has adaptive significance for the species concerned.

In view of the apparent non-opioid nature of EPM antinociception (Lee and Rodgers 1990), the present studies assessed the effects of diazepam on this particular form of adaptive pain inhibition. Previous work has shown, that while devoid of intrinsic effects on tail-flick responding, benzodiazepines significantly inhibit the antinociceptive effects of a range of environmental stimuli (e.g. Jackson et al. 1979; Drugan et al. 1984; Kinsheck et al. 1984; Willer and Ernst 1986; Fanselow and Helmstetter 1988), including social defeat (Rodgers and Randall 1987a, b, 1988b) and conspecific territorial scent (Kavaliers and Innes 1988) in male mice. If valid, the "anxiety" hypothesis of non-opioid antinociception would predict that diazepam should reduce both anxiety and pain inhibition in the EPM test.

In partial accord with this prediction, our results show that, when administered acutely, low doses of diazepam (0.5–1.0 mg/kg) significantly attenuate EPM-induced elevations in tail-flick latencies. However, this effect was neither complete nor strictly dose-dependent, i.e. pain inhibition was still evident immediately post-EPM in animals treated with 0.5 mg/kg and at 10 min post-EPM in animals receiving 1.0 mg/kg diazepam. Behaviourally, 0.5–1.0 mg/kg diazepam increased total arm entries and rearing, with a statistically significant increase in entries observed at the lower dose. This finding is consistent with the previously-reported low-dose activating effects of benzodiazepines (e.g. Marriott and Smith 1972; Simon and Soubrie 1979; Moser 1989). As more detailed analysis of the data failed to reveal any evidence of a significant reduction in anxiety with diazepam, it would appear that the stimulant and anxiolytic effects of this compound are unrelated. Indeed, the overall increase in arm entries disguised a specific increase in closed arm activity, a profile more consistent with an anxiogenic-like action. In favour of a weak and "paradoxical" anxiogenic-like effect of acute diazepam is the finding that, whereas controls spent equivalent time on the closed arms and central platform, drug-treated mice showed a clear preference for the closed arms (Fig. 1). Against such an interpretation, however, neither percent entries nor percent time on the arms was significantly altered by drug

treatment. This somewhat unexpected behavioural profile may account for the rather inconsistent effects of low acute doses of diazepam on EPM-induced antinociception. Certainly, the absence of an unambiguous anxiolytic drug profile would question the validity of this study as a test of the "anxiety" hypothesis of non-opioid antinociception.

Since marked behavioural suppression precluded the use of higher acute doses of diazepam, a second experiment was conducted in which diazepam was administered chronically over a period of 8 days. This procedure has been reported to result in the development of tolerance to the suppressant/sedative effects of benzodiazepines (e.g. Pellow et al. 1985; Johnston and File 1988; Shepherd and Rodgers 1989). To equate groups on degree of EPM antinociception, all animals were pretested on the maze and analysis of these (day 1) data confirmed that groups were indistinguishable both behaviourally and in terms of tail-flick response. However, when retested on the maze (day 9), animals that had received chronic diazepam (2–4 mg/kg) showed a significant inhibition of antinociception at 0 and 5 min post-exposure. However, the fact that elevated latencies were apparent in all treatment groups at 10 min post-EPM indicates that the inhibition of the response by diazepam was confined to the early phase of the response. Behavioural analysis revealed that development of tolerance to the behavioural suppressant actions of diazepam was incomplete. Thus, while total arm entries were unaffected, rearing (a more sensitive index) was still significantly inhibited by both doses following the 8-day treatment regimen. Although chronic diazepam treatment did not alter the basic preference for closed arms versus other sections of the maze (number of entries, percent time), analysis of percent number of entries revealed a selective increase in open arm activity (Table 3). Such a profile would be suggestive of a weak anxiolytic action only which, in turn, may explain the failure of diazepam to totally inhibit EPM antinociception.

It is important to note that in this experiment, and for reasons outlined above, all animals were pretested on the EPM i.e. on day 1. Furthermore, the design of the study required that animals be handled and injected on a daily basis for 9 days. In this context, it has recently been reported that prior experience of the EPM markedly reduces/abolishes the anxiolytic effect of chlordiazepoxide in mice (Lister 1987) and rats (File 1990; File et al. 1990). In addition, it has been found that daily handling/injection eliminates the anxiolytic effect of diazepam in the EPM (Brett and Pratt, 1990) and prevents the effects of diazepam on brain serotonin levels (Boix et al. 1990). As such, our present inability to demonstrate a pronounced anxiolytic effect for diazepam may have been due to prior maze experience and/or the chronic injection regimen employed. Another possible contributory factor to the weak anxiolytic effect of diazepam in this study is the between-days behavioural change observed in control animals. Although others have reported a stable EPM test-retest behavioural profile in intact rats and mice (Pellow et al. 1985; Lister 1987; File 1990; File et al. 1990), we have previously observed an apparent anxiogenic-like upon EPM retest (Lee and Rodgers 1990). A similar phenomenon is apparent in the present study where, on day 9 retest, control mice spent significantly less time on the open arms/central platform and, reciprocally, more time in the closed arms of the maze (Fig. 2). Importantly, Itoh et al. (1990) have recently shown that forced initial exposure to an open arm of the EPM results, on 24 h retest, in a significant reduction in open arm escape latency and increased time spent in the enclosed arms. In support of our findings, tail-flick latencies (baseline for all groups, and at all test points for controls) were significantly greater on day 9, suggesting a possible anticipatory anxiety reaction. Thus, enhanced "baseline" anxiety upon retest may have at least partially counteracted the anxiolytic action of diazepam. In this context, it is particularly important to note that chronic diazepam treatment did not significantly alter this retest "anxiogenesis". This finding is further addressed below.

In view of the above results, our final study examined the effects of chronic diazepam treatment in the absence of prior maze experience. Under these test conditions, diazepam (2–4 mg/kg) completely eliminated antinociception at all time-points post-EPM. Total arm entries were enhanced by the lower dose while rearing was suppressed at the higher dose. More detailed analysis indicated that, whereas controls displayed a greater number of closed versus open arm entries, both doses of diazepam eliminated this preference. Indeed, the drug significantly increased the percent number of open arm entries, an effect confirmed by changes in percent time spent in various sections of the maze. On this parameter, time spent on the central platform remained stable across treatment conditions while mirror-image effects were observed on time spent on open (increased) and closed (decreased) arms. Overall, this profile shows that, in the absence of prior maze experience, chronic diazepam treatment produces a very robust anxiolytic action.

Together, the results of the second and final experiments confirm previous reports that prior maze experience markedly attenuates/eliminates the anxiolytic effect of benzodiazepines (Lister 1987; File 1990; File et al. 1990). As both experiments involved equivalent daily handling and injection, our failure to observe a convincing anti-anxiety profile for diazepam in the second study cannot have been due to this aspect of experimental design. It should, perhaps, be noted that the handling-induced abolition of diazepam anxiolysis in rats (Brett and Pratt 1990) was observed after daily handling for 28 days whereas, in the current study, the chronic injection schedule lasted for 8 days only.

Recently, File et al. (1990) have suggested that a single behavioural experience of the EPM induces an adaptive change ("one-trial tolerance") similar, if not identical, to the effects produced by a period of chronic daily injections of chlordiazepoxide. They further propose that this change would not lead to alterations in the behavioural profile of controls, but would be revealed by a failure to respond to benzodiazepine treatment. Although our data clearly support a major reduction in benzodiazepine efficacy in test-experienced animals (Lister 1987; File 1990),

we have also confirmed our earlier observation (Lee and Rodgers 1990) that re-exposure to the EPM results in an anxiogenic-like behavioural profile in control animals. This shift in behavioural baseline would be consistent with test "sensitization" which, in turn, may reflect adaptive changes induced by initial maze exposure. Such a mechanism, perhaps by facilitating the subsequent development of tolerance to chronically-administered diazepam, could account both for the markedly reduced efficacy of diazepam in test-experienced mice and the apparent immunity of "retest anxiogenesis" to the effects of this compound. However, as tolerance develops more rapidly to the sedative than to the anxiolytic effects of benzodiazepines (File et al. 1990), our observation that rearing was still depressed following 8-day diazepam treatment undermines this proposal. Indeed, in direct contrast to the tolerance hypothesis, test-experienced animals were more sensitive to the inhibitory effects of diazepam on rearing than were test-naive animals (Tables 2 and 3).

In conclusion, present data confirm that exposure to the EPM induces antinociception in male mice, and show that this effect is attenuated by acute and chronic diazepam treatment. The most robust inhibition of EPM antinociception was observed in chronically-treated, test-naive subjects, an effect coincident with an unequivocal anti-anxiety action of diazepam. These findings contrast with the effects of opiate receptor manipulations (Lee and Rodgers 1990) and are consistent with the proposed involvement of anxiety in non-opioid forms of pain inhibition. It remains to be determined whether novel anxiolytics would also be effective in this model. Our data also show that prior experience of the EPM reduces the anxiolytic effect of diazepam, and suggest a major contribution of "retest anxiogenesis" to this phenomenon. Further studies are clearly indicated.

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