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Anxiolytic effects of valproate and diazepam in mice are differentially sensitive to picrotoxin antagonism

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Abstract

Although it is widely believed that the anxiolytic effects of benzodiazepines are mediated through facilitation of $GABA_A$ receptor function, behavioural studies have to date provided rather weak support for this hypothesis. In particular, considerable inconsistency has been noted both for the effects of GABAergic manipulations in animal models of anxiety and the ability of $GABA_A$ receptor antagonists to block the anxiolytic effects of diazepam (DZ) and chlordiazepoxide. In view of the sensitivity of the murine plus-maze to the anxiety-modulating effects of GABAergic agents as well as classical benzodiazepines, the current study examined the extent to which the anxiolytic actions of valproic acid (VPA) and DZ in this test involve picrotoxin (PX)-sensitive receptor mechanisms. Subjects were male DBA/2 mice, test duration was 5 min, and ethological scoring methods were employed. Our results show that, while devoid of intrinsic behavioural effects under present test conditions, PX (0.25–0.5 mg/kg) selectively antagonised the anxiolytic-like (but not other) effects of VPA (400 mg/kg). In contrast, the same doses of PX failed to block any of the behavioural changes induced by DZ (1.5 mg/kg), including disinhibition of open arm exploration. These data suggest that the plus-maze anxiolytic effects of DZ in DBA/2 mice are not mediated through PX-sensitive $GABA_A$ receptors. Further studies will be required to assess the generality of present findings to other mouse strains, species and behavioural paradigms.

Keywords: Anxiety; Plus-maze; GABA; Diazepam; Valproate; Picrotoxin; Mice

1. Introduction

It is now well established that benzodiazepines, such as chlordiazepoxide and diazepam (DZ), enhance central GABAergic neurotransmission through positive allosteric modulation of the $GABA_A$ receptor complex (for reviews: Rabow et al., 1995; Sieghart, 1995). However, the equivocal effects of specific $GABA_A$ receptor manipulations in animal models of anxiety (e.g. Sanger, 1985; Shephard, 1986, 1987) have led some authors to question the relevance of this particular molecular mechanism to the anxiolytic properties of benzodiazepines (e.g. Agmo et al., 1991; Bartholini, 1985; Dalvi and Rodgers, 1996; Dalvi and Rodgers, 1999; Paredes and Agmo, 1992; Polc, 1991; Shephard, 1986, 1987).

While some GABAergic compounds, such as valproic acid (VPA, GABA-T inhibitor) and picrotoxin (PX, non-competitive $GABA_A$ receptor antagonist), produce consistent anti- and proanxiety effects across a wide range of animal models (e.g. Barros et al., 1992; Cannizzaro et al., 1987; Corbett et al., 1991; Corda and Biggio, 1986; Dalvi and Rodgers, 1996; de Angelis, 1992; File and Lister, 1984; Prado de Carvalho et al., 1983; Quintero et al., 1985a,b; Sanger, 1985; Shephard, 1987; Shephard and Hamilton, 1989; Shimada et al., 1992; Simiand et al., 1984; Soubrie et al., 1979; Stutzmann et al., 1987), others are either limited in efficacy or produce nonselective behavioural effects. Among the latter compounds, GABA-T inhibitors, amino-oxyacetic acid (AOAA) and vigabatrin, as well as $GABA_A$ receptor agonists, muscimol, isoguvacine and 4,5,6,7-tetrahydroisoxazolo[4,5]pyridin-3-ol (THIP) have generally been found to exert anxiolytic-like effects in unconditioned models such as the social interaction and plus-maze tests (Corbett et al., 1991; Dalvi and Rodgers, 1996; Nastiti et al., 1991; Rodgers and Dalvi, 1997; Sayin et al., 1992; Sherif et al., 1994; Sherif

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and Oreland, 1994; Vivian et al., 1997), but to produce relatively nonspecific effects in conditioned models such as Geller and Vogel conflict (Agmo et al., 1991; Cook and Sepinwall, 1980; Corbett et al., 1991; Gardner and Piper, 1982; Hodges and Green, 1984; Hodges et al., 1981; Rasmussen et al., 1981; Shephard et al., 1990; Thiebot et al., 1979). Similar inconsistency has been found with respect to the anxiogenic effects of competitive $GABA_A$ antagonists (e.g. bicuculline and SR95531) (Agmo et al., 1991; Corda and Biggio, 1986; Dalvi and Rodgers, 1996; Nastiti et al., 1991; Toal et al., 1991; Zakusov et al., 1977) and the anxiolytic effects of GABA uptake inhibitors (e.g. CI-966 and No-711) (Agmo et al., 1991; Dalvi and Rodgers, 1996; Giusti et al., 1990). Relative to bicuculline and SR95531, the superior efficacy of PX in animal models of anxiety may be associated with the fact that this compound is a noncompetitive antagonist, whereas the former agents are competitive antagonists. Furthermore, bicuculline is known to be unstable in vivo and to show poor blood–brain penetration, while SR95531 is less selective than bicuculline for the $GABA_A$ receptor (Luque et al., 1994). In contrast, PX is known to penetrate the blood–brain barrier with an onset of action of approximately 30 min (Sieghart, 1995).

In addition to the variable anxiety-modulating effects of GABAergic agents per se, attempts to block the anxiolytic effects of benzodiazepines with $GABA_A$ receptor antagonists have also met with limited success. Thus, while PX and bicuculline have generally been reported to block the antianxiety effects of GABA agonists (Quintero et al., 1985a,b; Rasmussen et al., 1981; Shephard and Hamilton, 1989; Shephard et al., 1985; Vivian et al., 1997), the literature is strongly divided on the question of whether these agents are also able to block the anxiolytic effects of benzodiazepines. More specifically, in studies largely dominated by the use of diverse conditioning models, both positive (e.g. Billingsley and Kubena, 1978; Gomita et al., 1991; Miyamoto et al., 1983; Stein et al., 1977; Treit, 1987; Vellucci and Webster, 1984; Zakusov et al., 1977) and negative (e.g. Agmo et al., 1991; Buckland et al., 1986; Cook and Sepinwall, 1980; Liljequist and Engel, 1984a; Miyamoto et al., 1983; Quintero et al., 1985a,b; Shephard and Broadhurst, 1982; Toal et al., 1991) results have been reported following the coadministration of benzodiazepines and $GABA_A$ receptor antagonists. It is pertinent to note also that, in many of the instances where successful antagonism of benzodiazepine anxiolysis has been reported, interpretation is compromised by a failure to control for possible intrinsic behavioural activity of the $GABA_A$ antagonists (Agmo et al., 1991; Sanger, 1985).

The above review suggests that the conflicting effects of GABAergic manipulations in animal models of anxiety may at least partially be attributable to the type of test employed. As unconditioned behavioural models appear to fare somewhat better in supporting a role for GABA in anxiety (i.e. sensitivity to GABA-T inhibition, GABA reuptake inhibitors, $GABA_A$ receptor agonists and antagonists; see above), it is surprising that tests such as the elevated plus-maze have not been more extensively employed in drug interaction studies. In this context, previous research from our laboratory has established the sensitivity of the murine plus-maze not only to the anxiolytic effects of benzodiazepines (Cole and Rodgers, 1993, 1995; Dalvi and Rodgers, 1996, 1999; Johnson and Rodgers, 1996; Rodgers and Johnson, 1995), but also to the anxiety-modulating effects of GABAergic agonists and antagonists (Dalvi and Rodgers, 1996; Rodgers et al., 1995; Rodgers and Dalvi, 1997). In order to examine whether the $GABA_A$–benzodiazepine receptor complex mediates the anxiolytic properties of DZ, the present report determined whether the anxiolytic effects of VPA and DZ in the plus-maze are commonly mediated via PX-sensitive effector mechanisms. Ethological scoring methods were used to provide comprehensive behavioural profiles.

2. General method

2.1. Subjects

Subjects were 12–16-week-old adult male DBA/2 mice (Biomedical Services, University of Leeds), group-housed (10 per cage: cage size: $45 \times 28 \times 13$ cm) for at least 4 weeks prior to testing. Animals were maintained in a temperature- ($21 \pm 1^{\circ}C$) and humidity- ($50 \pm 5\%$) controlled environment under a 12 h reversed light–dark cycle (lights off: 07.00 h). Food and drinking water were available ad libitum with the exception of the brief test periods. All subjects were experimentally naive.

2.2. Drugs

Drugs used were DZ, VPA and PX (all obtained from Sigma, UK). VPA and PX were dissolved in distilled water and physiological saline, respectively. DZ was ultrasonically dispersed in physiological saline to which Tween 80 (2 drops/10 ml) had been added. Compounds were prepared freshly on test days and administered ip in a volume of 10 ml/kg. In both experiments, agonists and antagonists were administered 30 and 35 min prior to testing, respectively, with controls receiving two vehicle injections according to the same administration schedule.

2.3. Apparatus

The elevated plus-maze was based on that described by (Lister, 1987) and consisted of two opposing open ($30 \times 5 \times 0.25$ cm) and two opposing closed arms ($30 \times 5 \times 15$ cm). These arms extended from a common central platform (5×5 cm) and the entire apparatus was elevated to

a height of 60 cm above floor level. The maze floor was made of black Plexiglas and the walls of clear Plexiglas. As previously reported (e.g. Lee and Rodgers, 1991; Rodgers and Dalvi, 1997), a slight raised edge (0.25 cm) around the perimeter of the open arms provided grip for the animals, and open arm exploration was further encouraged by testing under dim red light (4×60 W indirect).

2.4. Procedure

To facilitate adaptation to novel surroundings, mice were transported to the dimly lit laboratory at least 1 h prior to testing. All experimental sessions were conducted during the mid-dark phase of the LD cycle (10.00–14.00 h), with animals randomly assigned to treatment conditions and tested in counterbalanced order. Testing commenced by placing an animal on the central platform of the maze facing an open arm, following which the experimenter withdrew to an adjacent laboratory. A standard 5 min test duration was employed (Lee and Rodgers, 1991; Lister, 1987; Pellow et al., 1985) and, between subjects, the maze was thoroughly cleaned with damp and dry towels. All test sessions were video-recorded by a camera positioned above and at c. 50° to the maze. Videotapes were subsequently scored blind by a highly trained observer (intra-rater reliability ≥ 0.90) using ethological software (Hindsight v. 1.4) developed by Dr. Scott Weiss (now at Cerebrus, UK). Using separate behaviour and location keys, this software allows the real-time scoring of videotapes by direct keyboard entry to a PC.

2.5. Behavioural analysis

Measures scored from videotape were the conventional spatiotemporal parameters (e.g. Lister, 1987; Pellow et al., 1985), together with a number of specific behavioural acts and postures displayed by rodents on elevated mazes (e.g. Anseloni and Brandao, 1997; Cole and Rodgers, 1993; Cruz et al., 1994; Espejo, 1997; Holmes and Rodgers, 1998; Shepherd et al., 1994). In this context, factor analysis of the behaviour of male DBA/2 mice in the plus-maze (Rodgers and Johnson, 1995) has produced six main factors (anxiety, locomotor activity, risk assessment, decision-making, vertical activity and directed exploration) accounting for 76% of the total variance. For clarity, current results are presented in accordance with this behavioural structure, with the measures reported representing the highest loading elements (≥ 0.8) under each factor.

An arm entry was defined as all four paws into an arm, rearing as vertical movement against the walls of the enclosed arms, stretched attend posture (SAP) as forward elongation of the body followed by retraction to initial position, and head-dipping as an exploratory head/shoulder movement over the sides of the maze (Dalvi and Rodgers, 1999; Rodgers and Johnson, 1995). In view of the importance of thigmotactic cues in plus-maze exploration (Rodgers et al., 1997; Treit et al., 1993), the closed arms and central platform were together designated as protected zones, i.e. affording thigmotactic contact with vertical surfaces. In accordance with the factor structure identified for male DBA/2 mice

Table 1
Effects of VPA (400 mg/kg) and PX (0.25–0.5 mg/kg), alone and in combination, on the behaviour of male DBA/2 mice in the elevated plus-maze

Behaviour	S–DW	S–VPA	PX0.25–DW	PX0.25–VPA	PX0.5–DW	PX0.5–VPA
Anxiety						
% Open entries	24.1 ± 3.9	**55.1 ± 6.8**	27.9 ± 6.0	36.7 ± 7.1 *	32.9 ± 5.7 *	32.6 ± 6.4
% Open time	6.3 ± 1.4	**50.1 ± 9.2**	11.1 ± 3.6	23.0 ± 5.9 *	11.4 ± 3.8	35 ± 5.5
% Protected SAP	82.6 ± 4.8	**16.9 ± 6.7**	64.7 ± 7.9	45.4 ± 13.9	78.7 ± 6.6 *	38.2 ± 8.8
Locomotor activity						
Closed entries	9.1 ± 1.1	5.5 ± 1.3	9.3 ± 1.3	11.0 ± 2.4	8.4 ± 1.7	16.9 ± 3.3 *
Total entries	12.0 ± 1.3	12.3 ± 2.3	12.9 ± 1.6	18.7 ± 4.1	11.1 ± 2.0	24.0 ± 3.4
Risk assessment						
Total SAP	20.6 ± 3.4	**9.0 ± 2.3**	21.2 ± 2.5	10.4 ± 2.3	23.0 ± 2.6	16.0 ± 2.4
Decision-making						
% Centre time	36.0 ± 3.5	**9.8 ± 1.6**	43.3 ± 6.3	28.2 ± 10.2	41.1 ± 7.1	38.2 ± 7.6
Vertical activity						
Rear frequency	7.6 ± 1.5	2.5 ± 0.6	6.7 ± 1.5	3.9 ± 1.0	5.4 ± 1.4	6.8 ± 1.6
Rear time (s)	8.9 ± 2.1	**2.0 ± 0.7**	6.8 ± 2.0	2.2 ± 0.7	5.8 ± 1.6	3.7 ± 0.9
Directed exploration						
Total dips	1.5 ± 0.4	**13.5 ± 2.4**	1.1 ± 0.4	10.7 ± 2.5	4.5 ± 1.9	16.5 ± 2.6

See Fig. 1 for complementary data. Significant effects for S–VPA vs. S–DW emboldened.
SAP, stretched attend postures; S, saline; DW, distilled water; PX, picrotoxin; VPA, valproic acid; N/A, not appropriate.
* Significant interaction effects for PX–VPA vs. S–VPA.

(Rodgers and Johnson, 1995), *anxiety measures* comprised % open arm entries ([open entries/total entries] × 100), % open arm time ([time spent on open arms/session duration] × 100), and % protected SAP (%pSAP; [protected SAP/total SAP] × 100). The frequency of closed and total arm entries were used as measures of *locomotor activity*, while the total frequency of SAP was employed as the primary measure of *risk assessment*. Percent time spent on the central platform ([centre time/session duration] × 100) was used as an index of *decision-making*, rear frequency and duration as measures of *vertical activity*, and total head-dipping as a measure of *directed exploration*.

Two experiments were conducted, in which the influence of PX (PX 0.25 and 0.5 mg/kg) on the anxiolytic effects of VPA (400 mg/kg; Experiment 1) and DZ (1.5 mg/kg; Experiment 2) were assessed. Doses and injection-test intervals were selected on the basis of previous studies conducted in our laboratory (e.g. Cole and Rodgers, 1995; Dalvi and Rodgers, 1996, 1999; Johnson and Rodgers, 1996; Rodgers and Dalvi, 1997; Rodgers and Johnson, 1998). Each experiment comprised six treatment conditions ($n = 10–15$), i.e. vehicle + vehicle, vehicle + VPA or DZ, 0.25PX + vehicle, 0.5PX + vehicle, 0.25PX + VPA or DZ, and 0.5PX + VPA or DZ.

2.6. Statistical analysis

Data were analysed by two-factor (treatment 1, treatment 2) multivariate analysis of variance (MANOVA). Where indicated, pairwise comparisons were conducted by Newman–Keuls tests, a method endorsed even in the absence of an overall significant F value (Howell, 1992; Wilcox, 1987).

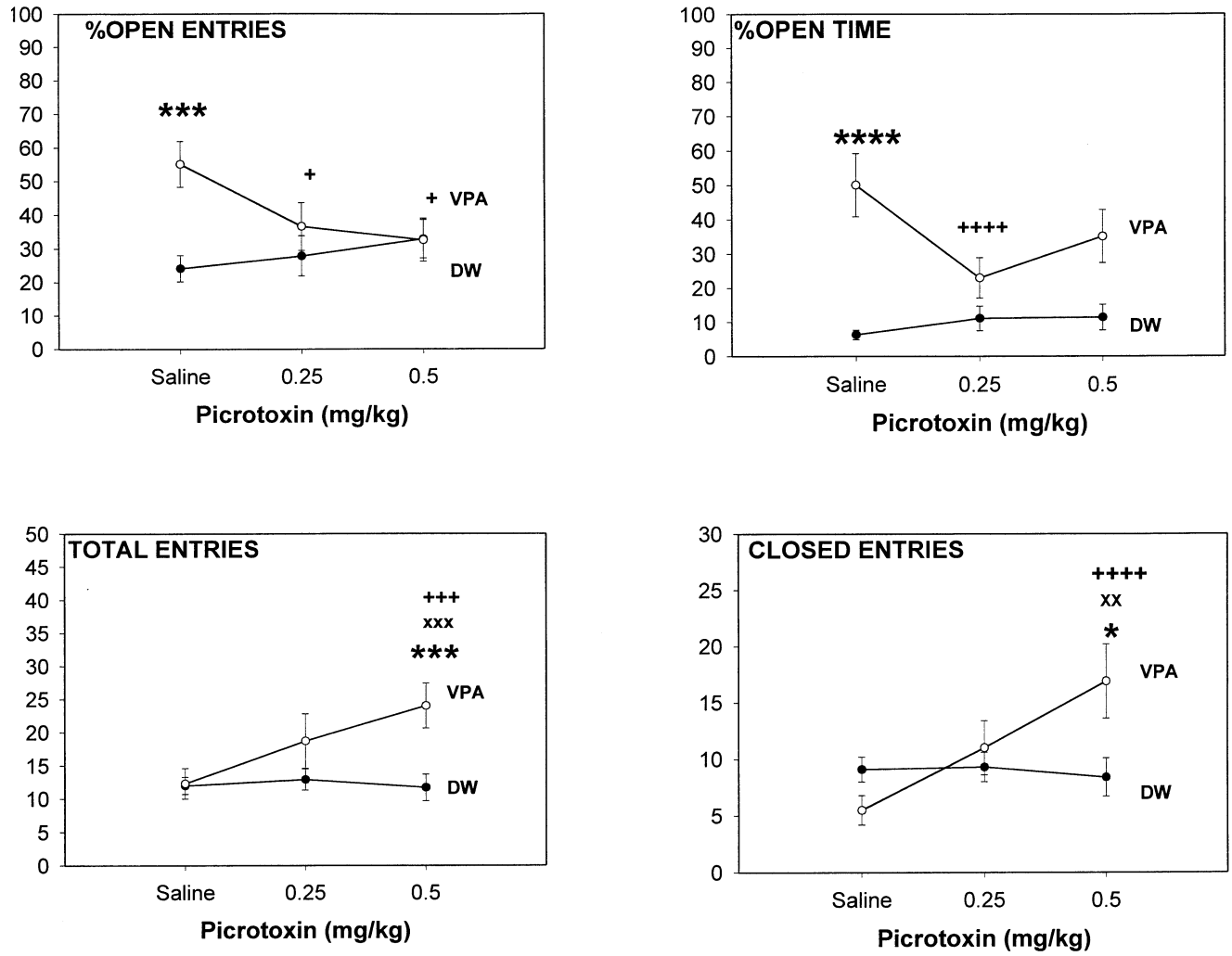

Fig. 1. Interaction effects of PX and VPA on plus-maze behaviour in DBA/2 mice. DW, distilled water; VPA, sodium valproate. Data are presented as mean ± S.E.M. $^{*}P < .05$, $^{***}P < .01$, $^{****}P < .005$ vs. control; $^{+}P < .05$, $^{++++}P < .005$ vs. VPA alone; $^{xx}P < .025$, $^{xxx}P < .01$ vs. correspondingly dosed PX alone group.

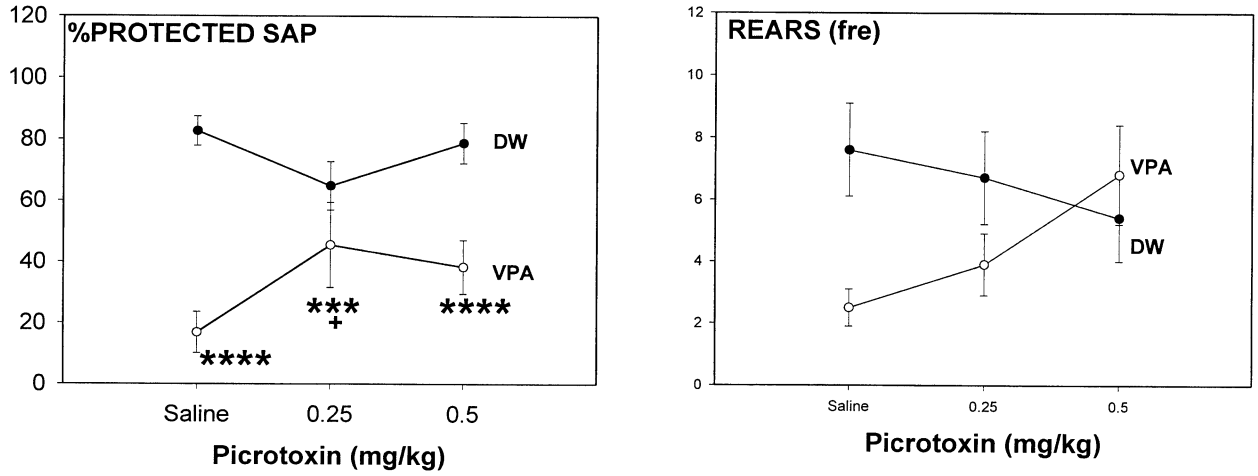

Fig. 2. Interaction effects of PX and VPA on plus-maze behaviour in DBA/2 mice. Data are presented as mean ± S.E.M. For key, see Fig. 1.

2.7. Ethics

The present experiments were licensed by the Home Office under the Animals (Scientific Procedures) Act 1986.

3. Results

3.1. Experiment 1

Results are summarised in Table 1, with significant VPA × PX interactions presented graphically in Figs. 1 and 2.

3.1.1. Anxiety

Significant VPA × PX interactions (Figs. 1 and 2) were obtained for % open arm entries [$F(2,54) = 3.61$, $P < .05$], % open arm time [$F(2,54) = 3.71$, $P < .05$] and % protected SAP [$F(2,54) = 3.69$, $P < .05$]. VPA alone increased % open entries ($P < .01$) and % open time ($P < .005$), and reduced % protected SAP ($P < .005$). Although devoid of intrinsic activity, PX significantly antagonised these behavioural effects of the GABA-T inhibitor: relative to VPA alone: % open entries (0.25 mg/kg, $P < .025$; 0.5 mg/kg; $P < .05$), % open time (0.25 mg/kg, $P < .005$) and % protected SAP (0.25 mg/kg, $P < .05$).

Table 2
Effects of DZ (1.5 mg/kg) and PX (0.25–0.5 mg/kg), alone and in combination, on the behaviour of male DBA/2 mice in the elevated plus-maze

Behaviour	S–V	S–DZ	PX0.25–V	PX0.25–DZ	PX0.5–S	PX0.5–DZ
Anxiety						
% Open entries	24.4 ± 2.1	**42.5 ± 4.1**	25.3 ± 2.9	41.4 ± 3.0	35.2 ± 8.9	42.4 ± 4.9
% Open time	8.5 ± 2.1	**23.6 ± 3.3**	9.8 ± 2.1	26.6 ± 4.1	7.7 ± 2.1	15.4 ± 3.2
% Protected SAP	76.8 ± 5.3	**48.2 ± 7.6**	74.1 ± 6.8	46.3 ± 7.7	80.2 ± 4.3	69.5 ± 7.6
Locomotor activity						
Closed entries	9.1 ± 0.9	9.5 ± 0.9	10.0 ± 1.2	10.9 ± 0.9	7.2 ± 1.6	7.5 ± 1.2
Total entries	12.2 ± 1.3	**17.3 ± 1.6**	13.6 ± 1.6	19.3 ± 1.7	10.4 ± 2.2	12.5 ± 2.1
Risk assessment						
Total SAP	17.5 ± 1.9	13.6 ± 1.5	20.6 ± 2.2	16.8 ± 1.9	14.4 ± 2.5	16.0 ± 2.4
Decision-making						
% Centre time	39.6 ± 4.6	**32.4 ± 2.2**	44.8 ± 5.3	34.0 ± 3.7	49.9 ± 6.6	44.7 ± 4.7
Vertical activity						
Rear frequency	7.1 ± 1.2	4.6 ± 0.8	8.4 ± 1.4	7.3 ± 1.0	4.2 ± 1.5	3.5 ± 1.3
Rear time (s)	7.6 ± 1.3	**3.4 ± 0.8**	9.7 ± 2.2	5.2 ± 0.9	4.1 ± 1.5	2.8 ± 1.0
Directed exploration						
Total dips	3.7 ± 0.7	10.3 ± 1.6	3.6 ± 0.5	8.8 ± 1.3	3.6 ± 0.8	8.5 ± 1.3

See Fig. 2 for complementary data. Significant effects for S–DZ vs. S–V emboldened. SAP, stretched attend postures; S, saline; V, vehicle; PX, picrotoxin; DZ, diazepam; N/A, not appropriate.

3.1.2. Locomotor activity

MANOVA revealed a significant VPA × PX interaction for closed arm entries [$F(2,54) = 4.80$, $P < .01$], together with an interaction for total entries that closely approached significance ($F_{obt} = 2.82$; $F_{crit0.05} = 3.18$) (Fig. 1). Further analysis revealed that, in the absence of significant intrinsic activity for either compound, cotreatment with VPA and PX 0.5 mg/kg (but not 0.25 mg/kg) significantly increased closed arm entries relative to control ($P < .05$), VPA alone ($P < .005$) and 0.5 mg/kg PX alone ($P < .05$). A similar pattern of interaction was also evident for total arm entries.

3.1.3. Vertical activity

Although a significant VPA × PX interaction (Fig. 2) was obtained for rear frequency [$F(2,54) = 3.19$, $P < .05$], no significant pairwise contrasts were evident. This discrepancy may be due to the nonsignificant trend towards a decrease in this measure with VPA alone ($P < .09$). Consistent with this interpretation, VPA alone significantly reduced rear duration [$F(1,54) = 14.15$, $P < .005$], an effect that was unaltered by coadministration of PX [$F(2,54) = 1.33$, NS].

3.1.4. Decision-making, risk assessment and directed exploration

Although VPA alone significantly decreased % centre time [$F(1,54) = 14.20$, $P < .005$], reduced total SAP [$F(1,54) = 20.67$, $P < .005$] and enhanced total head-dipping [$F(1,54) = 50.47$, $P < .005$], none of these effects was altered by PX [$F(2,54) = 0.43$, 0.44 and 0.25, respectively].

3.2. Experiment 2

Results are summarised in Table 2.

3.2.1. Anxiety

MANOVA revealed significant main effects for DZ on the primary indices of plus-maze anxiety: % open arm entries [increased: $F(1,84) = 12.94$, $P < .005$], % open arm tine [increased: $F(1,84) = 29.81$, $P < .005$] and % protected SAP [reduced: $F(1,84) = 16.10$, $P < .001$]. However, PX had no significant intrinsic effects on these parameters and, furthermore, completely failed to alter the DZ-induced changes [$F(2,84) = 0.75$, 1.31 and 1.08, respectively].

3.2.2. Locomotor activity and vertical activity

DZ significantly enhanced total arm entries [$F(1,84) = 9.05$, $P < .005$] and reduced rear duration [$F(1,84) = 9.23$, $P < .005$], but neither effect was influenced by coadministration of PX [total entries: $F(2,84) = 0.58$, NS; rear duration: $F(2,84) = 0.85$, NS]. DZ did not significantly alter either closed arm entries [$F(1,84) = 0.36$, NS] or rear frequency [$F(1,84) = 2.10$, NS], nor was there any evidence of DZ × PX interactions on these parameters [$F(2,84) = 0.05$ and 0.31, NS, respectively].

3.2.3. Decision-making and directed exploration

DZ significantly reduced % centre time [$F(1,84) = 4.13$, $P < .05$], and increased total head-dipping [$F(1,84) = 37.21$, $P < .005$]. Neither effect was altered by PX [$F(2,84) = 0.19$ and 0.35, NS, respectively].

3.2.4. Risk assessment

No significant drug main effects or interactions were obtained for total SAP [DZ: $F(1,84) = 1.47$, NS; PX: $F(1,84) = 1.76$, NS, interaction: $F(2,84) = 1.15$, NS].

4. Discussion

Previous research on the involvement of $GABA_A$ receptor mechanisms in the anxiolytic activity of benzodiazepines has produced highly inconsistent results, with evidence both for (Billingsley and Kubena, 1978; Gomita et al., 1991; Miyamoto et al., 1983; Stein et al., 1977; Treit, 1987; Vellucci and Webster, 1984; Zakusov et al., 1977) and against (Agmo et al., 1991, Buckland et al., 1986; Cook and Sepinwall, 1980; Liljequist and Engel, 1984a; Miyamoto et al., 1983; Quintero et al., 1985a,b; Rodgers and Cole, 1994; Toal et al., 1991) selective antagonism of benzodiazepine anxiolysis by PX and/or bicuculline. As noted, this variable pattern may be due to the use of potentially inappropriate behavioural tests (i.e. tests insensitive to direct GABAergic manipulations per se) and/or failure to control for intrinsic behavioural effects of the $GABA_A$ receptor antagonists. In view of these findings, the aim of the current study was to exploit the established benzodiazepine *and* GABAergic sensitivity of the murine plus-maze test (see Introduction) to determine whether the anxiety-modulating effects of VPA (a GABA-T inhibitor) and DZ are commonly mediated via PX-sensitive receptors. To this end, two experiments examined the effects of subthreshold (i.e. nonanxiogenic) doses of PX on the behavioural profiles of VPA and DZ under identical test conditions. Although the doses of PX used were selected on the basis of previously published work from our laboratory (Dalvi and Rodgers, 1996), each study incorporated additional controls for possible intrinsic behavioural effects of the antagonist.

VPA is the only GABA-T inhibitor to consistently produce reductions in anxiety across a range of animal models (e.g. Barros et al., 1992; Cannizzaro et al., 1987; Corbett et al., 1991; Dalvi and Rodgers, 1996; de Angelis, 1992; Sanger, 1985; Shephard, 1986, 1987; Shephard and Hamilton, 1989; Simiand et al., 1984). However, as this compound is known to additionally modulate a variety of non-GABAergic mechanisms (Balfour and Bryson, 1995; Löscher, 1993), its anxiolytic action cannot simply be assumed to reflect an increased synaptic availability of GABA. In this context, the results of Experiment 1 concur with earlier findings in rat (Corbett et al., 1991) and mouse (Dalvi and Rodgers, 1996) plus-maze paradigms in showing

that VPA (400 mg/kg) exerted a robust anxiolytic-like action under present test conditions, with significant increases in % open arm entries and % open arm time paralleled by a reduction in % protected SAP. Importantly, the lack of an effect of VPA on closed arm entries (see also: Dalvi and Rodgers, 1996) confirms that the observed changes in open arm activity were not secondary to a more general alteration in locomotor activity. Furthermore, our results show that the noncompetitive $GABA_A$ receptor antagonist, PX (0.25–0.50 mg/kg), significantly blocked the effects of VPA on all three indices of plus-maze anxiety. This finding concurs with earlier reports of PX antagonism of the anticonflict (Liljequist and Engel, 1984b; Shephard et al., 1990; Vellucci and Webster, 1984) and antineophobia (Shephard et al., 1985) effects of VPA. However, it is pertinent to note that whereas neither dose of PX currently used was found to alter anxiety measures, the doses employed in previous studies (1.5–2.0 mg/kg) were either intrinsically active, i.e. anxiogenic (Shephard et al., 1985), or not actually tested for intrinsic behavioural activity (Liljequist and Engel, 1984b; Shephard et al., 1990b).

Consistent with previous findings in our laboratory (Dalvi and Rodgers, 1996), VPA alone also significantly reduced indices of decision-making (% centre time), risk assessment (total SAP) and vertical activity (rear duration), while stimulating directed exploration (head-dipping). However, the observation that none of these effects was altered by coadministration of PX not only confirms the specificity of VPA/PX interactions on anxiety parameters, but also implies the involvement of non-GABAergic mechanisms (e.g. Balfour and Bryson, 1995; Löscher, 1993) in the additional behavioural effects of VPA. Intriguingly, while devoid of effects when given alone, the combined administration of 0.5 mg/kg PX and VPA significantly increased locomotor activity (closed and total arm entries). Although present data do not permit a clear explanation of this finding, intrinsically inactive doses of these two compounds have, when coadministered, been found to reduce eating latency in food-deprived rats (Shephard et al., 1985). As a similar behavioural effect was seen with a lower dose of VPA alone, the authors suggested that the synergism apparent at a higher dose of VPA may actually reflect partial PX antagonism, resulting in a behavioural profile similar to that seen with the lower dose per se. Although neither the current experiment nor previous studies (Dalvi and Rodgers, 1996) have found evidence of a locomotor stimulant effect for VPA, benzodiazepine anxiolytics are known to produce biphasic effects on novelty-induced ambulation, increasing and decreasing it at low and high doses, respectively (e.g. Marriott and Smith, 1972; Treit, 1985). It is thus conceivable that the absence of locomotor stimulation with VPA (400 mg/kg) represents a moderate dose profile and that the observed increase in closed entries produced by cotreatment with PX mimics an effect that might be seen with a lower (subthreshold anxiolytic) dose of VPA. However, it should also be noted that, as VPA has been reported to possess direct agonist properties at the PX binding site (Agmo et al., 1991; Ticku and Davis, 1981), it is at least structurally plausible that the behavioural synergism seen in the present study resulted from pharmacological potentiation at PX sites distinct from those involved in anxiety modulation. In this context, it is particularly important to note that, despite the observed effect of combined VPA and PX treatment on locomotor activity, this interaction was found only with the high dose (0.5 mg/kg) of PX whereas the lower dose of PX was actually more effective in blocking the anxiolytic effects of VPA.

Consistent with a large literature (for review: Rodgers and Cole, 1994), Experiment 2 confirmed the sensitivity of the plus-maze paradigm to the benzodiazepine receptor agonist, DZ. Indeed, paralleling the findings of an earlier comparative study (Dalvi and Rodgers, 1996), the overall behavioural profile obtained with 1.5 mg/kg DZ was remarkably similar to (but somewhat weaker than) that observed with 400 mg/kg VPA. Significant increases in % open arm entries and % open arm time, coupled with a marked reduction in % protected SAP, confirmed the anti-anxiety effects of DZ under present test conditions. Furthermore, in agreement with previous findings from this laboratory (e.g. Cole and Rodgers, 1995; Johnson and Rodgers, 1996; Rodgers and Johnson, 1998), these anxiolytic effects of DZ were accompanied by significant increases in head-dipping (directed exploration; see also Griebel et al., 1996; Shepherd et al., 1994), as well as reductions in % centre time (decision-making) and rear duration (vertical activity). However, in contrast to this earlier work and the profile obtained with VPA (Experiment 1), DZ failed to significantly reduce total SAP (risk assessment). This unexpected finding may be related to the high baseline level of anxiety shown by control animals in the current studies (e.g. low % open time scores) and the generally more potent effects of VPA (400 mg/kg) on all anxiety measures. Although the inhibition of rear duration (but not frequency) might be indicative of a myorelaxant/sedative action of DZ, this is not supported by the increases in total arm entries and head-dipping and is more likely a function of response competition. Furthermore, while the enhancement of total arm entries might suggest a nonspecific locomotor stimulant effect, the absence of a DZ effect on closed arm entries (the primary measure of plus-maze locomotion: e.g. Cruz et al., 1994; Espejo, 1997; Fernandes and File, 1996; Rodgers and Johnson, 1995) indicates that the increased total entry score simply reflects the selective increase in open arm activity. In this context, it should perhaps be emphasised that total entries co-load on "locomotor activity" and "anxiety," whereas closed entries load highly and selectively on locomotor activity (Rodgers and Johnson, 1995).

Against this background, the anxiolytic action of DZ (1.5 mg/kg) in the plus-maze was not blocked by either dose of PX (0.25–0.50 mg/kg). Although this $GABA_A$ antagonist has been reported to block the effects of DZ on

periaqueductal gray aversion (Gomita et al., 1991) and defensive burying (Treit, 1987), it is pertinent to note that negative results have also been obtained in more traditional conflict paradigms (Agmo et al., 1991; Liljequist and Engel, 1984a). Although the reason for this discrepancy is unclear, current findings are strengthened by the observation that none of the other behavioural effects of DZ (e.g. enhanced head-dipping and reduced rearing) were altered by coadministration of PX. However, it should be noted that PX blocked the anxiolytic effects of VPA under identical test conditions (Experiment 1) and shows good blood–brain penetration at the injection-test interval employed in the current study (e.g. DeFeudis, 1979; Sieghart, 1995). In view of the theoretical significance of present findings, it is important to emphasise the cross-study similarity in behavioural profiles for the control groups as well as the absence of intrinsic behavioural effects of the antagonist.

While confirming the anxiolytic activity of VPA and DZ in the murine plus-maze, the present results clearly point to the involvement of different substrates in the mediation of these effects. Several possible explanations for this differential pattern of PX antagonism may be considered. Although DZ may produce its antianxiety effects at PX-insensitive $GABA_A$ receptor subtypes, this explanation seems unlikely in view of the finding that all natural $GABA_A$ receptors recognise both GABA and PX (Whiting et al., 1995). Perhaps a more feasible hypothesis is that DZ reduces anxiety via nonneuronal binding sites that are not coupled to $GABA_A$ receptors, while VPA and PX exert their effects via neuronally located $GABA_A$ receptors. For example, reduced platelet (i.e. nonneuronal) benzodiazepine receptor density has been reported in patients with generalised anxiety disorder (Weizman et al., 1987) and posttraumatic stress disorder (Gavish et al., 1996), while benzodiazepine receptor ligands selective for nonneuronal benzodiazepine receptors have been shown to alter anxiety in both humans (Ansseau et al., 1991) and animals (File and Lister, 1983; File and Pellow, 1985a,b; Myslobodsky et al., 1983; Pellow and File, 1996). These data not only indicate that anxiety can be modulated via DZ-sensitive, nonneuronal, benzodiazepine receptors but also offer a potential explanation for the lack of effect of flumazenil on the anxiolytic effects of DZ under present test conditions (Dalvi and Rodgers, 1999). Finally, the possibility must be entertained that the anxiety-reducing effects of DZ are mediated via one or a combination of other non-GABAergic mechanisms known to be benzodiazepine-sensitive (e.g. excitatory amino acids, cholecystokinin, adenosine, voltage-dependent ion currents, membrane fluidity; Ishizawa et al., 1997; Kurishingal, 1994; Polc, 1991).

Current findings, together with our earlier report that flumazenil does not block the anxiolytic effects of DZ in DBA/2 mice exposed to the plus-maze (Dalvi and Rodgers, 1999), raise important questions about the precise molecular mechanisms through which benzodiazepines reduce anxiety in this test. Further research will clearly be required to determine the generality of present observations to other mouse strains, other species and other anxiety models. Of direct relevance in this context is a recent report (Crestani et al., 1999) on the phenotyping of transgenic mice ($\gamma 2^{+/-}$) with significantly impaired $GABA_A$ receptor clustering/[^{3}H]flumazenil binding in cortex and hippocampus. Despite displaying markedly enhanced basal levels of anxiety in several models, including the plus-maze, these animals continued to show an excellent anxiolytic response to DZ.

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