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Behavioral Effects of Diazepam in the Murine Plus-Maze: Flumazenil Antagonism of Enhanced Head Dipping But Not the Disinhibition of Open-Arm Avoidance

A. DALVI AND R. J. RODGERS

Ethopharmacology Laboratory, School of Psychology, University of Leeds, Leeds LS2 9JT, UK

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DALVI, A. AND R. J. RODGERS. *Behavioral effects of diazepam in the murine plus-maze: flumazenil antagonism of enhanced head-dipping but not the disinhibition of open arm avoidance.* PHARMACOL BIOCHEM BEHAV **62**(4) 727–734; 1999.—Although it is widely believed that benzodiazepines reduce anxiety through positive allosteric modulation of the $GABA_A$–chloride channel complex, this is not the only mechanism through which agents of this class can modify CNS function. Furthermore, a significant number of reports of apparent flumazenil blockade of diazepam anxiolysis in animal models have paid limited attention to possible intrinsic behavioral actions of the antagonist per se. In the present study, ethological methods were employed to assess in detail the effects of diazepam, flumazenil, and their combination on the behavior of male DBA/2 mice in the elevated plus-maze paradigm. In two experiments, diazepam (1.5 mg/kg) alone reduced open-arm avoidance and increased head dipping, whereas flumazenil (10–40 mg/kg) alone was without significant behavioral effect. However, with the sole exception of head dipping, prior administration of flumazenil (10 and 40 mg/kg) failed to block the behavioral effects of diazepam under present test conditions. These findings imply that the anxiolytic effects of diazepam in the mouse plus-maze are not mediated through flumazenil-sensitive benzodiazepine receptors and that alternate mechanisms must be considered. © 1999 Elsevier Science Inc.

Anxiety Plus-maze Benzodiazepines Diazepam Flumazenil Animal models Mice

THE benzodiazepine receptor antagonist, flumazenil (Ro15-1788), produces conflicting effects on anxiety in humans and animals. Findings ranging from anxiogenesis (26) through no effect (23,62) to anxiolysis (49) have been reported in human volunteer studies, with a similarly inconsistent pattern evident in clinical work with anxiety disorder patients (56,62,71). The profile of flumazenil in animal models of anxiety is also notoriously inconsistent. Thus, while apparently devoid of intrinsic activity in the defensive burying (29), fear-potentiated startle (6), light–dark (4,19), and Y-maze (50) paradigms, comparable doses of the antagonist are anxiogenic in the open field (42); either anxiogenic (91) or inactive (3,18,63,68,69,90) in conflict procedures; either anxiogenic (34,35) or inactive (7,70) in the social interaction test; either anxiolytic (46) or inactive (38) in the ultrasonic vocalisation test; and either anxiogenic (52,68) or inactive (2,5,14,31,60,68,72,81,82,85) in the elevated plus-maze test.

In view of this pattern of results, the highly variable intrinsic effects of flumazenil cannot readily be attributed to differences in dosage, species, or model used. However, several authors have suggested that they may be accounted for by variation in benzodiazepine receptor conformation at testing and/or situation-dependent release of positive or negative endogenous modulators [e.g., (32,56,66)]. This possibility has potentially serious implications for research in which flumazenil antagonism is considered definitive evidence for benzodiazepine receptor involvement in the behavioral effects of agents such as diazepam. More specifically, although flumazenil has been found to block the antianxiety effects of diazepam in diverse animal models (6,8,19,25,45,46,53,61,72,73,86,

Requests for reprints should be addressed to Dr. R. J. Rodgers, University of Leeds, Ethopharmacology Laboratory, School of Psychology, Leeds LS2 9JT, UK.

92,94), these studies have paid limited attention to the possible contribution of intrinsic behavioral effects of the antagonist. The salience of this point is further highlighted by the failure of behaviorally inactive doses of flumazenil to block the anti-anxiety effects of benzodiazepines in certain animal tests [e.g., (40)], or to influence benzodiazepine-induced effects on anxiety, episodic memory, and alertness in normal human volunteers (21,26,39,43). Furthermore, unpublished pilot observations in our own laboratory have suggested that the anxiolytic effects of diazepam in mice may also be resistant to flumazenil antagonism.

In view of the theoretical importance of this issue, the aim of the present study was to assess both the intrinsic activity of flumazenil and its ability to antagonize the effects of diazepam in a well-validated model of anxiety. The elevated plus-maze was considered a particularly appropriate model for this work in view of its well-known sensitivity to anxiolytic agents believed to act via the $GABA_A$-receptor complex (i.e., benzodiazepines, barbiturates, GABA agonists, neurosteroids) [e.g., (17,22,77,84,87)]. Ethological methods were used to record behavior, thereby generating very much more comprehensive profiles of drug action than is possible using conventional scoring techniques [e.g., (76,79)].

METHOD

Subjects

Subjects were 12–16-week-old adult male DBA/2 mice (Biomedical Services, University of Leeds), group housed (9–10 per cage: cage size: 45 × 28 × 13 cm), and maintained in a temperature (21 ± 1°C)- and humidity (50 ± 5%)-controlled environment under a 12 L:12 R cycle (lights off: 0700 h). Food and drinking water were available ad lib with the exception of brief test periods. All subjects were experimentally naive and, apart from routine husbandry, were not specifically handled prior to testing.

Drugs

Drugs used were diazepam (Sigma, Poole, UK) and flumazenil (Ro15-1788; Hoffmann–La Roche; Basel). Both compounds were ultrasonically dispersed in physiological saline to which Tween 80 (2 drops/10 ml) had been added. Drugs were prepared freshly on test days and administered IP in a volume of 10 ml/kg. In Experiment 1 (dose–response study), compounds were administered 30 min prior to testing with control animals receiving the saline/Tween80 vehicle. In Experiment 2 (interaction study), diazepam and flumazenil were administered 30 and 35 min prior to testing, respectively, with control animals receiving two vehicle injections according to the same administration schedule.

Apparatus

The elevated plus-maze used was a modified version of that validated for NIH Swiss mice by Lister (54). It consisted of two opposing open (30 × 5 × 0.25 cm) and two opposing closed arms (30 × 5 × 15 cm), extending from a common central platform (5 × 5 cm) and elevated to a height of 60 cm above floor level. The maze floor was constructed of black Plexiglas, and the walls of the enclosed arms of clear Plexiglas. As previously reported [e.g., (22,79)], a slight raised edge (0.25 cm) around the perimeter of the open arms provided additional grip for the animals, while open-arm activity was further promoted by testing under dim red light (4 × 60 W indirect).

Procedure

To facilitate adaptation to new surroundings, mice were transported to the dimly lit laboratory at least 1 h prior to testing. All experimental sessions were conducted during the dark phase of the LD cycle (1000–1400 h), with animals randomly allocated 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 [e.g., (52,54,64)] and, between subjects, the maze was thoroughly cleaned with damp and dry towels. All test sessions were videorecorded by a camera positioned above and at ca. 50° to the maze.

Videotapes were later scored blind by a highly trained observer (intrarater reliability ≥0.90) using the ethological analysis package 'Hindsight *v*1.4' developed by Dr Scott Weiss (now at Cerebrus Ltd, UK). Using separate behavior and location keys, this software permits the real-time scoring of videotapes by direct keyboard entry to a PC. Measures scored from videotape were the conventional spatiotemporal measures, together with a variety of specific behaviors related to the murine defensive repertoire [e.g., (79)]. Conventional parameters comprised the frequency of open and closed-arm entries (arm entry defined as all four paws into an arm; arm exit defined as two paws onto the central square), total arm entries, and the amount of time spent by the animals in open, central, and closed sections of the maze. These data were also used to compute percent open entries [i.e., (open entries/total entries) × 100] and percent time spent in the different zones of the maze [i.e., (time/300) × 100]. In addition, the following ethologically derived measures were recorded; frequency of rearing, stretched attend postures (exploratory posture where the mouse extends forward and then retreats to its initial position without locomoting forward), head dipping (exploratory head/shoulder movement over sides of maze), and closed-arm returns (exiting from an arm with only two paws, and then turning back into the same arm); duration of rearing, grooming (species-typical sequence commencing with snout, advancing to ears, and concluding with full body groom), flatback approach behavior (exploratory locomotion where the animal extends to its full length and moves forward), and immobility (no visible movement). In view of the importance of thigmotactic cues in plus-maze exploration (93), stretched attend postures, head dipping, and flatback approach were differentiated as "protected" (i.e., occurring on/from the relative security of the closed arms/central platform), or "unprotected" (i.e., occurring on/from open arms). Data for stretched attend postures, head dipping, and flatback approach are given both as total scores and "percent protected" scores [(protected/total) × 100].

In Experiment 1 (dose–response study), animals were randomly assigned to vehicle, diazepam 1.5 mg/kg, flumazenil 10, 20, or 40 mg/kg conditions and, in Experiment 2 (interaction study), to vehicle-vehicle, vehicle-diazepam 1.5 mg/kg, flumazenil 10 mg/kg-vehicle, flumazenil 10 mg/kg-diazepam 1.5 mg/kg, flumazenil 40 mg/kg-vehicle, or flumazenil 40 mg/kg-diazepam 1.5 mg/kg conditions. Sample sizes of $n = 10$ were used throughout. Doses and injection-test intervals were selected on the basis of existing literature and pilot studies.

Statistical Analysis

Data from Experiment 1 were analyzed either by single-factor (treatment) or two-factor (treatment, location; repeated

measures on second factor) analyses of variance (ANOVA), followed where appropriate by Dunnett's *t*-tests. Data from Experiment 2 were analyzed by two-factor (diazepam, flumazenil) or three-factor (diazepam, flumazenil, location; repeated measures on location) multivariate analysis of variance (MANOVA); pairwise comparisons were conducted by Newman–Keuls tests, a method recommended even in the absence of an overall significant *F*-test (44,95).

Ethics

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

RESULTS

Experiment 1

The effects of diazepam (1.5 mg/kg) and flumazenil (10–40 mg/kg) on plus-maze behavior are summarized in Table 1 and Fig. 1. While total arm entries and closed-arm entries were not altered by drug treatment, ANOVA ($df = 4, 45$) indicated significant treatment effects on open-arm entries ($F = 3.79$, $p < 0.025$), percent open-arm entries ($F = 2.88$, $p < 0.05$), and percent open-arm time ($F = 3.11$, $p < 0.025$). Further analyses confirmed that diazepam significantly increased open-arm entries ($p < 0.05$) and percent open entries ($p < 0.01$), and produced an increase in percent open time that closely approached significance (Fig. 1). In addition, subjects showed an overall rank order preference for time spent on different maze sections of closed > center > open, $F(2, 98) = 42.32$, $p < 0.005$. Although ANOVA suggested that this profile was not significantly altered by treatment, $F(10, 98) = 1.46$, NS (Fig. 1) (percent time chart) this clearly indicates that diazepam-treated animals no longer differentiated between the closed arms and central platform. For the ethological measures, ANOVA (Table 1) indicated significant effects for rear frequency ($F = 5.17$, $p < 0.005$) and percent protected stretched-attend postures ($F = 3.64$, $p < 0.025$), together with an effect for total head dipping that closely approached significance ($F_{obt} = 2.48$, $F_{crit0.05} = 2.61$). However, while follow-up tests confirmed that diazepam increased head dipping ($p < 0.05$), no significant drug-vehicle differences were detected for rear frequency or percent protected stretched attend postures. In contrast to the effects observed with diazepam, flumazenil did not significantly alter any behavior over the dose range tested.

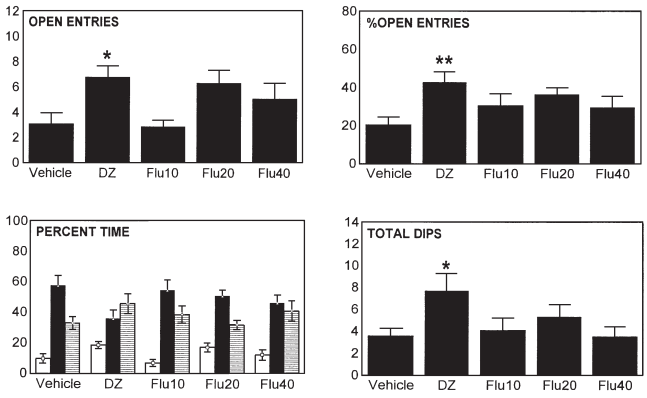

FIG. 1. Effects of diazepam (1.5 mg/kg) and flumazenil (10–40 mg/kg) on the behavior of male DBA/2 mice in the elevated plus-maze. Data are presented as mean values ± SEM. For the percent time chart: clear bars, open; black bars, closed; hatched bars, centre. DZ, diazepam, Flu, flumazenil. See Table 1 for complementary data. *$p < 0.05$, **$p < 0.01$ vs. vehicle.

Experiment 2

Descriptive statistics and MANOVA results are summarized in Table 2. Although no significant effects were obtained for total arm entries or closed-arm entries, MANOVA revealed significant main effects for diazepam ($df = 1, 54$) on all conventional anxiety indices, with follow-up tests showing that, relative to other treatment conditions, animals receiving diazepam displayed anxiolytic-like increases in open entries, percent open entries, and percent open time, together with a concomitant reduction in percent closed time (all $p < 0.005$). However, there were no main effects for flumazenil ($df = 2, 54$) on these parameters, nor any significant diazepam × flumazenil interactions. For the spatiotemporal preference measure, controls showed a rank-order preference for maze section of closed > center > open, $F(2, 108) = 26.29$, $p < 0.005$,

TABLE 1

EFFECTS OF DIAZEPAM (1.5 mg/kg) AND FLUMAZENIL (10.0–40.0 mg/kg) ON THE BEHAVIOR OF MALE DBA/2 MICE IN THE ELEVATED PLUS-MAZE

Behavior	Vehicle	DZ	Flu10	Flu20	Flu40	
Total entries	12.8 ± 2.0	16.4 ± 2.0	9.8 ± 1.4	16.7 ± 1.5	15.4 ± 1.9	F = 2.55, NS
Rear frequency	10.0 ± 2.2	5.7 ± 1.3	7.3 ± 1.5	15.8 ± 1.9	7.8 ± 1.4	F = 5.17, P < 0.005
Reartime(s)	13.0 ± 3.3	7.6 ± 2.3	15.1 ± 4.9	19.6 ± 3.2	9.0 ± 1.5	F = 2.23, NS
Closed entries	9.7 ± 1.4	9.6 ± 1.5	6.9 ± 1.2	10.3 ± 0.8	10.3 ± 1.2	F = 1.22, NS
% Protected dips	85.7 ± 6.0	53.8 ± 8.5	74.5 ± 5.0	60.1 ± 10.9	70.8 ± 13.5	F = 1.77, NS
Total SAP	14.0 ± 2.1	14.8 ± 1.9	16.1 ± 3.2	18.4 ± 2.0	16.3 ± 2.2	F = 0.54, NS
% Protected SAP	69.6 ± 8.2	45.7 ± 8.5	83.4 ± 6.1	47.1 ± 7.2	57.7 ± 10.9	F = 3.64, P < 0.025
Closed arm returns	0.6 ± 0.3	0.4 ± 0.2	0.7 ± 0.3	0.8 ± 0.3	0.1 ± 0.1	F = 1.13, NS
Total flatback(s)	1.8 ± 0.8	0.1 ± 0.1	1.2 ± 0.5	1.0 ± 0.7	1.7 ± 1.0	F = 1.00, NS
Total groom(s)	22.9 ± 12.3	10.1 ± 2.8	27.4 ± 8.0	5.8 ± 2.7	16.8 ± 5.4	F =1.41, NS
Immobility(s)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A

SAPS, stretched attend postures; DZ, diazepam; Flu, flumazenil; N/A, not appropriate.
Data are presented as mean values ± SEM. See Fig. 1 for complementary data.

and diazepam significantly altered this profile, $F(2, 108) = 5.35$, $p < 0.01$, such that closed > center = open. However, there was no evidence of a significant main effect for flumazenil on spatial preference, $F(4, 108) = 0.48$, NS, nor did the antagonist influence diazepam's effect on this measure, $F(4, 108) = 0.46$, NS.

MANOVA also revealed significant main effects for diazepam on rear frequency, reartime, flatback approach, and grooming (Table 2). Follow-up tests indicated that diazepam treatment produced reductions in all four measures (rear frequency, $p < 0.05$; rear time, $p < 0.025$; flatback approach, $p < 0.05$; grooming, $p < 0.05$). There were no significant main effects for flumazenil on these measures, nor any significant flumazenil × diazepam interactions. However, analysis did yield a significant diazepam × flumazenil interaction for total head dips (Fig. 2). Post hoc analysis indicated that diazepam significantly increased this measure ($p < 0.005$) and that, in the absence of intrinsic activity, both doses of flumazenil blocked this increase ($p < 0.005$). No significant main effects or interactions were obtained for total stretched-attend postures, percent protected head dips, percent protected stretched attend postures, closed-arm returns, or immobility (Table 2).

DISCUSSION

The elevated plus-maze is one of the most extensively used models for the investigation of drug effects on anxiety-related behavior in laboratory rodents (75–77), and is based on the natural tendency of animals to avoid open spaces (93). The primary indices of plus-maze anxiety (i.e., % open-arm entries, % open-arm time) reflect this natural tendency and are bidirectionally sensitive to anti- and proanxiety manipulations [e.g., (77)]. Although locomotor activity is often assessed by total arm entries, early factor analytic studies challenged this approach and pointed to closed arm entries as a more valid index [e.g., (30,54)]. This important distinction has been further emphasized in more recent research, which, by recording specific behavioral acts and postures in addition to conventional parameters, has also revealed the existence of other behavioral dimensions in patterns of plus-maze exploration (20,27,28,59,79). For example, the structure of plus-maze behavior in DBA/2 mice comprises not only independent factors related to open-arm avoidance and locomotor activity but also factors associated with risk assessment, vertical activity, decision making, and directed exploration (79). It has been argued that the adoption of this more comprehensive approach to behavioral profiling in the plus-maze has certain advantages over conventional scoring, including enhanced pharmacological sensitivity and an improved basis for determining the behavioral selectivity of treatment effects (75–77).

The principal aim of the present study was to examine the influence of flumazenil on behavioral changes induced by diazepam in the murine elevated plus-maze test. In accord with previous research in this area (6,8,19,25,45,48,53,61,72,73,86,

TABLE 2

EFFECTS OF DIAZEPAM (1.5 mg/kg) AND FLUMAZENIL (10.0–40.0 mg/kg), ALONE AND IN COMBINATION, ON THE BEHAVIOR OF MALE DBA/2 MICE IN THE ELEVATED PLUS-MAZE

Behavior	V-V	V-DZ	Flu10-V	Flu10-DZ	Flu40-V	Flu40-DZ	Main effect Flumazenil	Main effect Diazepam	Interaction
Total entries	16.1 ± 1.4	22.6 ± 2.1	15.0 ± 2.7	17.5 ± 3.5	15.4 ± 1.8	16.2 ± 3.0	$F = 1.14$, NS	$F = 2.48$, NS	$F = 0.65$, NS
Rear frequency	10.2 ± 1.6	5.5 ± 1.6	10.3 ± 2.2	9.2 ± 2.1	9.5 ± 1.3	6.5 ± 1.3	$F = 0.74$, NS	$F = 4.28$, $p < 0.05$	$F = 0.54$, NS
Reartime(s)	13.9 ± 2.2	4.6 ± 1.5	12.9 ± 3.0	10.0 ± 3.3	12.7 ± 3.3	8.3 ± 2.3	$F = 0.33$, NS	$F = 6.26$, $p < 0.025$	$F = 0.77$, NS
Open entries	5.4 ± 0.8	10.3 ± 1.4	4.6 ± 1.0	7.9 ± 1.9	5.2 ± 0.9	7.6 ± 1.9	$F = 0.78$, NS	$F = 9.17$, $p < 0.005$	$F = 0.38$, NS
Closed entries	10.7 ± 1.2	12.3 ± 1.4	10.4 ± 2.1	9.6 ± 1.8	10.2 ± 1.1	8.6 ± 1.8	$F = 0.88$, NS	$F = 0.04$, NS	$F = 0.54$, NS
% Open entries	34.3 ± 4.4	45.6 ± 1.5	30.6 ± 4.6	44.5 ± 5.0	31.8 ± 4.9	53.1 ± 10.2	$F = 1.22$, NS	$F = 9.92$, $p < 0.005$	$F = 0.38$, NS
% Open time	13.3 ± 2.0	28.0 ± 3.8	15.2 ± 4.5	20.2 ± 5.2	12.3 ± 2.3	24.3 ± 5.5	$F = 0.28$, NS	$F = 9.63$, $p < 0.005$	$F = 0.71$, NS
% Closed time	53.9 ± 3.3	43.5 ± 3.2	50.9 ± 6.6	37.7 ± 7.4	55.1 ± 2.8	37.4 ± 7.9	$F = 0.30$, NS	$F = 8.70$, $p < 0.005$	$F = 0.20$, NS
% Centre time	32.7 ± 2.4	28.5 ± 2.9	33.9 ± 7.5	42.1 ± 8.9	32.6 ± 2.8	38.2 ± 7.6	$F = 0.74$, NS	$F = 0.41$, NS	$F = 0.57$, NS
Total dips	4.4 ± 0.7	12.9 ± 1.3	3.6 ± 0.9	6.4 ± 1.6	3.5 ± 0.6	5.7 ± 1.4	$F = 7.55$, $p < 0.005$	$F = 23.01$, $p < 0.005$	$F = 4.46$, $p < 0.025$
% Protected dips	53.9 ± 10.9	29.5 ± 7.5	57.6 ± 12.3	59.3 ± 10.7	74.7 ± 8.3	58.1 ± 10.5	$F = 3.21$, NS	$F = 2.56$, NS	$F = 0.87$, NS
Total SAP	19.7 ± 1.9	12.4 ± 1.3	15.5 ± 2.8	15.4 ± 1.9	17.1 ± 2.0	14.4 ± 2.7	$F = 0.04$, NS	$F = 3.52$, NS	$F = 1.35$, NS
% Protected SAP	57.9 ± 6.4	30.8 ± 6.4	57.2 ± 8.7	45.4 ± 7.8	50.2 ± 9.8	53.0 ± 12.1	F = 0.44, NS	F = 2.82, NS	F = 1.45, NS
Closed arm returns	0.4 ± 0.2	0.5 ± 0.2	1.0 ± 0.5	0.1 ± 0.1	0.7 ± 0.4	0.9 ± 0.3	$F = 0.50$, NS	$F = 0.59$, NS	$F = 1.55$, NS
Total flatback(s)	2.0 ± 0.4	1.4 ± 0.8	2.4 ± 0.8	0.9 ± 0.4	1.8 ± 0.5	0.7 ± 0.5	$F = 0.32$, NS	$F = 4.27$, $p < 0.05$	$F = 0.28$, NS
Total groom(s)	9.8 ± 3.4	10.0 ± 3.4	8.7 ± 2.5	2.3 ± 1.0	17.3 ± 4.2	7.6 ± 3.3	$F = 2.55$, NS	$F = 4.31$, $p < 0.05$	$F = 1.26$, NS
Immobility(s)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A	N/A	N/A

SAP, stretched attend postures; V, vehicle; Flu, flumazenil; DZ, diazepam; N/A, not appropriate.
See Fig. 2 for complementary data.

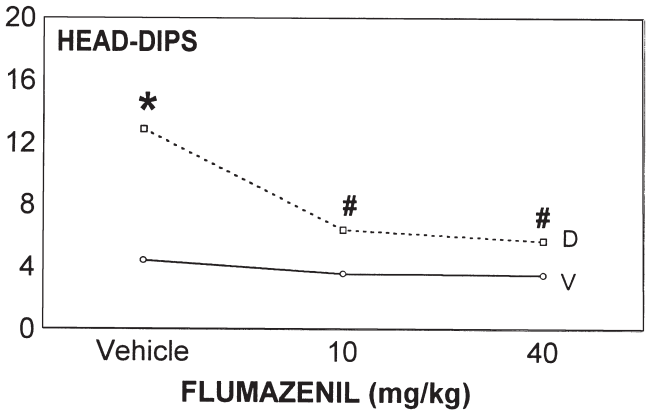

FIG. 2. Flumazenil antagonism of diazepam-induced stimulation of head dipping in mice tested on the elevated plus-maze. See Table 2 for complementary data. V, vehicle; D, diazepam (1.5 mg/kg). $^{*}p < 0.005$ vs. vehicle; $^{\#}p < 0.005$ vs. diazepam alone.

92,94), an optimal anxiolytic dose of diazepam was selected on the basis of earlier dose–response studies conducted in the same mouse strain and model [e.g., (16,22,48)]. Particular attention was paid to possible intrinsic behavioral effects of flumazenil under present test conditions as any such effects could compromise the interpretation of drug interaction studies. Thus, Experiment 1 assessed the effects of flumazenil (10–40 mg/kg) given alone, with diazepam (1.5 mg/kg) used as a positive control, while the design of Experiment 2 incorporated groups receiving each agent alone as well as groups receiving combined treatment. The results of Experiment 1 confirmed that, in the absence of effect on closed-arm entries, diazepam increased open entries, percent open entries, and head dipping. Although these results are consistent with numerous reports on the effects of benzodiazepines in the plus-maze [review: (77)], the absence of a statistically significant effect on percent open time indicates a somewhat milder anxiolytic profile for diazepam than that observed in previous studies from this laboratory [e.g., (15,16,22,48)]. This conclusion is supported by the lack of effect of diazepam on stretched-attend postures (the primary measure of risk assessment), which are normally reduced by benzodiazepine treatment. The data also revealed that, despite some trends (particularly on open-arm entries), flumazenil (10–40 mg/kg) per se is behaviorally inactive under present test conditions. In this context, it is important to note that the profile of flumazenil not only contrasts with that of diazepam, but also with the potent anxiogenic effects previously observed with the β-carboline, FG 7142, under identical test conditions (78). Although others have also failed to find evidence of intrinsic effects of flumazenil in the plus-maze (2,5,14,31,60,65,72,85,94), several groups have reported anxiogenic-like activity for the antagonist in this (52,66) and other [e.g., (34,35,42,91)] models. Although the latter findings could potentially compromise interpretation of studies reporting flumazenil antagonism of diazepam-induced anxiolysis (6,8,19,25,38,45,46,53,55,61,72,73, 92,94), the neutral profile of the antagonist under present test conditions indicates that any noted interactions with diazepam cannot be attributed to opposing intrinsic behavioral actions.

To our knowledge, Experiment 2 is the first to have assessed the ability of flumazenil to antagonize the anxiolytic effects of diazepam in the mouse plus-maze paradigm. Confirming the results of the first experiment, flumazenil (10 and 40 mg/kg) had no significant behavioral effects when administered alone, whereas diazepam (1.5 mg/kg) alone increased open entries, percent open entries, and percent open time, and reduced percent closed time, grooming, flatback approach, and rearing. The absence of a diazepam effect on closed-arm entries again supports an anxioselective action, while the overall behavioral profile is more typical of previous findings from this laboratory (15,16,22,48) than the relatively mild diazepam effect observed in Experiment 1. Despite this good baseline, flumazenil completely failed to block the anxiolytic effects of diazepam, a result consistent with earlier unpublished findings in our laboratory using flumazenil at doses of 10 and 20 mg/kg. However, it is vitally important to note that, since both doses blocked the observed diazepam-induced stimulation of exploratory head dipping, the antagonist was not completely ineffective under present test conditions. Although this particular behavioral effect of diazepam (and chlordiazepoxide) has been repeatedly observed in mice [e.g., (10,13,15,19,45)], it should not be interpreted as evidence of an anxiolytic action. Thus, in DBA/2 mice, total head dipping loads on a factor independent of measures related to "anxiety," "risk assessment," and "locomotor activity" (74), and is not increased by other classes of other anxiolytic compound, e.g., 5-HT_{1A} receptor antagonists [e.g., (11,12)]. The implication of present findings is that, while the effects of diazepam on exploratory head dipping in the murine plus-maze are mediated by flumazenil-sensitive benzodiazepine receptors, its effects on plus-maze anxiety are not. Despite biochemical evidence that diazepam increases the binding of [^{3}H]GABA to the GABA receptor in a flumazenil-reversible manner [for review: (88)], and behavioral evidence that flumazenil can block the anxiolytic effects of diazepam in several animal models of anxiety, the current failure of the antagonist to significantly counter behavioral effects of diazepam is not without precedent. For example, flumazenil does not influence benzodiazepine effects on anxiety, episodic memory, or motor sedation in healthy human volunteers (21,26,39,43), and has recently been reported as ineffective in blocking the antianxiety effects of midazolam microinjected directly into the dorsal raphé nucleus of rats (40).

Several possible explanations for current results may be considered. First, the doses of flumazenil employed and/or the present treatment protocol may have been inappropriate. However, these seem highly improbable explanations given the demonstrable efficacy of flumazenil (10–40 mg/kg) in blocking diazepam-induced stimulation of head dipping (see Fig. 2), and the range of doses (5–50 mg/kg) found to antagonize diazepam anxiolysis in previous studies (6,8,19,25,38,45,46,53, 55,61,72,73,92,94). Furthermore, detailed analysis of this literature indicates that the interval between flumazenil administration and testing ranged 10–60 min, while the interinjection interval ranged 0–30 min with the antagonist sometimes given before, sometimes after, and sometimes simultaneously with diazepam. Indeed, some of the earliest work on the benzodiazepine receptor antagonist properties of flumazenil found the order of drug administration to be irrelevant to successful blockade/reversal of diazepam effects in rats and mice (45), while time-course studies revealed a 1–2-h duration of action in both species (8). Secondly, it may be relevant that mice show significantly higher plasma and brain levels of diazepam metabolites compared with rats (57,58), and that these metabolites have anxiolytic efficacy both in humans (41) and mice (13,24). As much of the published literature on flumazenil antagonism of diazepam anxiolysis has been based on rats, the present negative findings may reflect a hitherto unrecognized

species difference. However, this possibility is negated by reports that flumazenil blocks benzodiazepine effects in the mouse ultrasonic distress vocalisation (61), four-plate (25), and light/dark exploration (19) models. Nevertheless, as none of these studies employed DBA/2 mice, it might still be argued that present results reflect an influence of genetic strain on response to flumazenil and/or its ability to block benzodiazepine receptor-mediated effects. It is, therefore, pertinent that, in previous work from this laboratory using male DBA/2 mice of a similar age, flumazenil has been shown to completely antagonize defeat-induced analgesia as well as the analgetic effects of benzodiazepine receptor inverse agonists such as FG7142 and DMCM (80).

In view of the above analysis, the possibility must be entertained that the effects of diazepam in the murine plus-maze are largely mediated either by flumazenil-insensitive $GABA_A$ receptors or by non-GABAergic mechanisms. The potential involvement of flumazenil-insensitive $GABA_A$ receptors would be supported by the finding that recombinant $GABA_A$ receptors, comprising $\alpha_1\beta_1\gamma_1$ subunits, are labeled by [^{3}H]flunitrazepam but not [^{3}H]flumazenil (9). Although native $GABA_A$ receptors that are diazepam sensitive/flumazenil insensitive have yet to be identified, it is conceivable that future studies in molecular biology will yield such data (83). Alternatively, it is well known that benzodiazepines influence a range of non-GABAergic mechanisms [e.g., excitatory amino acids, cholecystokinin, adenosine, voltage-dependent ion currents, or membrane fluidity; (47,51,67)], any one of which could potentially play a role in the antianxiety effects of diazepam. Furthermore, diazepam binds equally well to neuronal and nonneuronal benzodiazepine sites, whereas flumazenil binds only to the former [e.g., (74)]. Because ligands selective for the nonneuronal site have also been shown to alter anxiety in humans (1) and animals [e.g., (33,36,37,65,89)], these sites may also have functional relevance in the present context. Given these multiple possibilities, further work is required in order to establish the precise mechanism(s) whereby diazepam reduces plus-maze anxiety in mice and to assess the potential generality of present findings to other murine models of anxiety.

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