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Behaviorally Selective Effects of Neuroactive Steroids on Plus-Maze Anxiety in Mice

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RODGERS, R. J. AND N. J. T. JOHNSON. *Behaviorally selective effects of neuroactive steroids on plus-maze anxiety in mice.* PHARMACOL BIOCHEM BEHAV **59**(1) 221–232, 1998.—A number of A-ring–reduced metabolites of deoxycorticosterone and progesterone, known to exert agonist activity at the $GABA_A$ receptor complex, have been reported to reduce anxiety-related behavior in rodents. In the present study, the behavioral selectivity of these effects was assessed in an ethological version of the mouse elevated plus-maze paradigm. Anxiolytic-like profiles, characterised principally by reductions in open arm avoidance measures, were observed following systemic treatment with 5α-pregnan-3α, 21-diol-20-one (5α,3α-THDOC; 5.0 and 20.0 mg/kg), 5β-pregnan-3,20-dione (5β-DHP; 10–20 mg/kg), 5β-pregnan-3α-ol-20-one (pregnanolone; 20 mg/kg), and 5α-pregnan-3α-ol-20-one (allopregnanolone; 10–20 mg/kg). In contrast, 5α-pregnan-3,20-dione (5α-DHP; 10.0–20.0 mg/kg) and 5α-pregnan-3β-ol-20-one (2.5–20.0 mg/kg) were without effect under present test conditions. Detailed behavioral analysis further showed that the antianxiety effects of 5α,3α-THDOC, 5α-DHP, pregnanolone and allopregnanolone were not associated with changes in general activity levels. In addition, profile comparisons revealed that the anxiolytic steroids tend to produce a narrower range of behavioral effects than diazepam (1.0 mg/kg) and, in particular, do not reliably decrease measures of risk assessment. It is concluded that neuroactive steroids produce anxioselective effects in the mouse plus-maze and that their profile of action can at least partially be distinguished from that of a well-characterised benzodiazepine. © 1998 Elsevier Science Inc.

Anxiety Behavioral selectivity Plus-maze Neuroactive steroids Diazepam Mice

THE classical endocrine/neuroendocrine effects of steroids are mediated via specific intracellular receptors that regulate protein synthesis, and generally have a time-lag of days–months (33,48). However, it has been known for over half a century that, in addition to such genomic actions, steroids such as progesterone, deoxycorticosterone, and their reduced metabolites can produce very much more rapid effects in the CNS (49). Stemming from initial research on the sedative/anaesthetic effects of the synthetic steroid anaesthetic, alphaxalone [e.g. (22)], it is now widely believed that these neuroactive steroids exert many of their central effects through positive allosteric modulation of the $GABA_A$ receptor complex (26,27,30,41,43). For example, the naturally occurring ring A-reduced metabolites of progesterone, 5α-pregnan-3α-ol-20-one (allopregnanolone), and deoxycorticosterone, 5α-pregnan-3α, 21-diol-20-one (5α,3α-THDOC), display high affinity for the $GABA_A$ receptor complex, enhance GABA and benzodiazepine binding, and potentiate Cl^- ion conductance [e.g. (31,37)]. However, despite many functional similarities, these steroids do not to share a common site of action with benzodiazepines or barbiturates but, rather, appear to act through unique (and perhaps multiple) extracellular receptors (26,39).

Recent research has implicated neuroactive steroids in diverse physiological and behavioral processes, including arousal (28,35), CNS excitability (18), pain sensitivity (19), feeding (7), aggression (24), sociosexual behavior (25), memory (34), and drug withdrawal (6,15). However, in view of the effects of these agents on GABA function, it is perhaps not surprising that most behavioral research in this area has focussed on stress, fear, and anxiety. In this context, exposure to stress has been found to elevate brain concentrations of several neuroactive steroids (2,3), while steroids (such as allopregnanolone and 5α,3α-THDOC) have, in turn, been found to reduce the effects of stressors on GABA receptor function (10), forebrain release of acetyl choline (14), and dopamine (21,38), plasma corticosterone levels (40), and pain perception (24,47). These and similar findings have prompted speculation that neuroactive steroids may function as endogenous anxiolytics produced in response to stress [e.g. (21,27,41)].

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Consistent with this hypothesis, anxiolytic-like effects have been reported for 5α,3α-THDOC in light/dark exploration, punished drinking, and elevated plus-maze paradigms (1,11). Antianxiety activity has also been found with pregnanolone and/or allopregnanolone in rat Geller-Seifter, defensive burying, ultrasonic distress vocalization, and plus-maze procedures (4,42,52,54), as well as mouse Vogel, open-field, light/dark exploration, and plus-maze tests (52,53). Importantly, however, the behavioral selectivity of many of these findings has not yet been clearly established. Thus, earlier reports frequently confound anxiety and activity testing, employ inappropriate indices of general activity, and/or suggest that observed anxiolytic-like effects, may be secondary to changes in general activity levels.

The aim of the present study was, therefore, to assess in detail the effects of a range of neuroactive steroids in an ethological version of the murine plus-maze paradigm, which permits comprehensive behavioral profiling [e.g. (8,9,13,23,44–46). Steroids selected for study were 5α-pregnan-3α, 21-diol-20-one (5α,3α-THDOC), 5α- and 5β-pregnan-3,20-dione (5α- and 5β-dihydroprogesterone; 5α- and 5β-DHP), 5β-pregnan-3α-ol-20-one (pregnanolone), 5α-pregnan-3α-ol-20-one (allopregnanolone), and 5α-pregnan-3β-ol-20-one (3β isomer of allopregnanolone). For comparative purposes, diazepam was also included in the present series.

METHOD

Subjects

Subjects were 12–14-week-old adult male DBA/2 mice (Biomedical Services, University of Leeds), housed 10 per cage (cage size: 45 × 28 × 13 cm) for at least 4 weeks prior to testing. Animals were maintained in a temperature (21 ± 1°C)- and humidity (50 ± 5%)-controlled environment under a 12-h reversed light–dark cycle (lights off: 0700 h). Food and drinking water were freely available with the exception of brief test periods. All mice were experimentally naïve and, at testing, weighed 21–36 g.

Drugs

All compounds were purchased from Sigma UK Ltd (Poole, Dorset, UK): diazepam; 5α-pregnan-3α, 21-diol-20-one (5α,3α-tetrahydrodeoxycorticosterone; 5α,3α-THDOC); 5α-pregnan-3, 20-dione (5α-dihydroprogesterone; 5α-DHP); 5β-pregnan-3, 20-dione (5β-dihydroprogesterone, 5β-DHP); 5β-pregnan-3α-ol-20-one (pregnanolone; 5β,3α-P); 5α-pregnan-3α-ol-20-one (allopregnanolone; 5α,3α-P); and 5α-pregnan-3β-ol-20-one (5α, 3β-P). Diazepam was ultrasonically suspended in saline to which Tween 80 (2 drops/10 ml) had been added. 5α,3α-THDOC and pregnanolone were suspended in 0.5% methyl cellulose, while all other steroids were solubilized in 25% 2-hydroxy-β-cyclodextrin. Corresponding vehicles were used for control injections. Compounds were prepared freshly on test days and administered IP (10 ml/kg) either 30 (diazepam) or 10 min (steroids) prior to testing.

Apparatus

The elevated plus-maze used was a modification of the apparatus validated for NIH Swiss mice by Lister (29) and consisted of two open (30 × 5 × 0.25 cm) and two closed arms (30 × 5 × 15 cm) emanating from a common central platform (5 × 5 cm). The entire apparatus was elevated to a height of 60 cm above floor level. The maze floor was constructed of black Plexiglas and the side/end walls of the enclosed arms of clear Plexiglas. As previously reported [e.g. (46)], 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 encouraged by testing under dim red light (4 × 60 W indirect).

Procedure

To facilitate adaptation, mice were transported to the dimly lit laboratory at least 1 h prior to testing. All testing was conducted during the dark phase of the LD cycle (1000–1400 h), with animals randomly allocated to treatment conditions (n = 10–13) 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 from the room in which the maze was situated. A standard 5-min test duration was employed and, between subjects, the maze was thoroughly cleaned with damp and dry towels. Behavior was recorded by videocamera (positioned above the maze at an angle of approximately 50°) with the signal relayed to a monitor and VCR in an adjacent laboratory. Videotapes were later scored blind by a highly trained observer (intrarater reliability ⩾0.9) employing ethological analysis software (Hindsight v1.4) developed by Dr. Scott Weiss in our laboratory. Using separate behavior and location keys, this package permits the real-time scoring of videotapes by direct keyboard entry to a PC. In addition to conventional measures, scoring incorporated a variety of specific behavioral acts and postures (13,23).

Conventional parameters comprised the frequency of open and closed arm entries (arm entry defined as all four paws into an arm), total arm entries, and the amount of time spent by the animals in open, center, and closed sections of the maze. These data were also used to calculate percent open entries [i.e., (open/total) × 100] and percent time spent in the open, center, and closed zones of the maze [i.e., (time/300) × 100)]. In addition to these parameters, the following ethologically derived measures were recorded; frequency of rearing, stretched attend postures (SAP; exploratory posture where the mouse stretches forward and then retracts to its original 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 returning/doubling back into the same arm), as well as the duration (s) of rearing, sniffing (olfactory exploration of the maze floor and walls, and occasional air sampling), grooming (species-typical sequence beginning with snout, progressing to ears, and ending with whole body groom), and flatback approach behavior (exploratory locomotion where the animal stretches to its full length and slowly moves forward). In view of the importance of thigmotactic cues in plus-maze exploration (51), stretched attend postures, head dipping, sniffing, and flatback approach were further differentiated as "protected" (i.e., occurring on/from the relative security of the closed arms/center platform) or "unprotected" (i.e., occurring on/from open arms). Data for the latter measures are, therefore, given both as total scores and "percent protected" scores [(protected/total) × 100]. A recent factor analytic study (46) on the plus-maze behavior of DBA/2 mice has shown that the above measures can be accommodated by six factors: anxiety (e.g., open entries, % open entries, % open time, and the % protected forms of head dipping, SAP, sniff, and flatback approach); activity (e.g., closed entries); risk assessment (e.g., total SAP); decision making (e.g., % center time); vertical activity (e.g., rearing); and directed exploration (e.g., total head dipping).

Six experiments were conducted. Diazepam: vehicle, 1.0 mg/kg; 5α,3α-THDOC: vehicle, 2.5, 5.0, 10.0, and 20.0 mg/kg; 5α and 5β-DHP: vehicle, 10.0, 20.0 mg/kg; 5β,3α-P (pregnanolone): vehicle, 2.5, 5.0, 10.0, and 20.0 mg/kg; 5α,3α-P (allopregnanolone): vehicle, 2.5, 5.0, 10.0, and 20.0 mg/kg; 5α, 3β-P: vehicle, 2.5, 5.0, 10.0, and 20.0 mg/kg. Doses, vehicles, and treatment times were selected on the basis of existing literature and pilot studies.

Statistical Analysis

Data were analysed either by single-factor (treatment) or two-factor (treatment, location; repeated measures on second factor) analyses of variance (ANOVA). Following significant ANOVA, Dunnett's *t*-tests were used to compare treatment groups with vehicle control.

RESULTS

Diazepam

Results and ANOVA details are summarized in Table 1. Diazepam treatment increased percent open arm entries and percent open time, reduced percent closed time, but did not affect percent center time. This alteration in the spatiotemporal distribution of behavior was confirmed by two-way ANOVA, $F(2, 48) = 5.87$, $p < 0.01$, indicating that diazepam abolished the preference shown by control mice for closed arms > center platform > open arms. Although treatment did not affect either open or total arm entries, a significant reduction in closed arm entries was observed. On the ethological measures, diazepam reduced rear frequency and duration, closed arm returns, total SAP, total flatback approach, and percent protected sniffing. An increase in percent protected flatback approach was the only other significant effect noted.

5α,3α-THDOC

Data are summarized in Fig. 1 and Table 2 . ANOVA revealed significant treatment effects on open arm entries, $F(4, 43) = 3.03$, $p < 0.05$, percent open entries, $F(4, 43) = 3.16$, $p < 0.05$, and percent open time, $F(4, 43) = 2.53$, $p < 0.05$. Although the apparent increases in percent open entries (5.0 and 10.0 mg/kg) failed to reach an acceptable level of statistical significance, open arm entries per se were significantly increased at 20.0 mg/kg ($p < 0.01$), while percent open time was increased at 5.0 and 20.0 mg/kg ($p < 0.05$; Fig. 1). A two-way ANOVA on time spent in different areas of the maze yielded an interaction term for treatment × location that closely approached significance, $F(8,86) = 1.95$, $p = 0.062$. Further analysis revealed that the control profile (closed > center > open) was altered such that at 2.5 mg/kg, closed = center > open; 5.0 and 10.0 mg/kg, closed > center = open; 20.0 mg/kg, closed = center = open. Treatment with 5α,3α-THDOC did not significantly alter total arm entries, closed arm entries, percent closed arm time, or percent center time (Table 2).

On the ethological measures, significant steroid effects were observed only for rear duration ($F = 3.33$, $p < 0.05$), and the percent protected forms of head dipping ($F = 3.02$, $p < 0.05$) and SAP ($F = 2.96$, $p < 0.05$). Follow-up tests failed to reveal any significant differences between treatment and control conditions for rear duration, but did confirm (Fig. 1) significant reductions in protected head dipping (20.0 mg/kg; $p < 0.05$) and protected SAP (5.0 mg/kg & 20.0 mg/kg; $p < 0.05$). No other significant steroid effects were observed (Table 2).

5α- and 5β-DHP

Data are summarized in Figs. 2 and 3 and Table 3. ANOVA showed significant treatment effects for open arm entries, $F(4, 43) = 2.84$, $p < 0.05$, percent open arm time, $F(4, 43) = 4.15$,

TABLE 1

EFFECTS OF DIAZEPAM (1.0 mg/kg) ON THE BEHAVIOR OF MALE DBA/2 MICE IN THE ELEVATED PLUS-MAZE TEST

Behavior	Vehicle	Diazepam	$F(1, 24)$
Total arm entries	15.4 ± 1.5	13.8 ± 2.2	0.36, NS
Open arm entries	5.8 ± 0.8	7.2 ± 1.5	0.69, NS
Closed arm entries	9.5 ± 1.0	6.5 ± 1.1	4.41, $p < 0.05$
% Open arm entries	38.0 ± 2.8	56.4 ± 5.2	9.77, $p < 0.005$
% Open arm time	16.3 ± 2.9	38.7 ± 9.0	5.63, $p < 0.03$
% Centre platform time	23.7 ± 1.6	23.0 ± 3.1	0.05, NS
% Closed arm time	59.9 ± 3.1	38.3 ± 7.3	7.33, $p < 0.02$
Rear frequency	15.6 ± 2.4	8.5 ± 1.7	5.86, $p < 0.03$
Rear duration (s)	27.9 ± 5.4	8.3 ± 2.0	11.59, $p < 0.003$
Head-dips	3.8 ± 0.9	5.5 ± 1.1	1.45, NS
SAP	12.1 ± 1.3	8.6 ± 1.1	4.14, $p = 0.053$
Closed arm returns	1.2 ± 0.4	0.3 ± 0.2	4.57, $p < 0.05$
Sniffing (s)	112.2 ± 7.9	135.1 ± 8.4	3.96, NS
Flatback approach (s)	7.0 ± 1.3	3.2 ± 0.6	7.20, $p < 0.02$
Grooming (s)	25.5 ± 5.3	17.3 ± 4.2	1.45, NS
%p Dips	42.7 ± 10.4	41.8 ± 10.2	0.00, NS
%p SAP	69.2 ± 4.8	68.8 ± 7.0	0.00, NS
%p Sniff	87.0 ± 3.0	63.9 ± 9.4	5.40, $p < 0.03$
%p Flatback approach	67.2 ± 9.9	93.7 ± 3.7	5.45, $p < 0.03$

Data presented are mean values ± SEM. SAP = stretched attend postures. %p = percent protected. (s) = seconds. NS = nonsignificant.

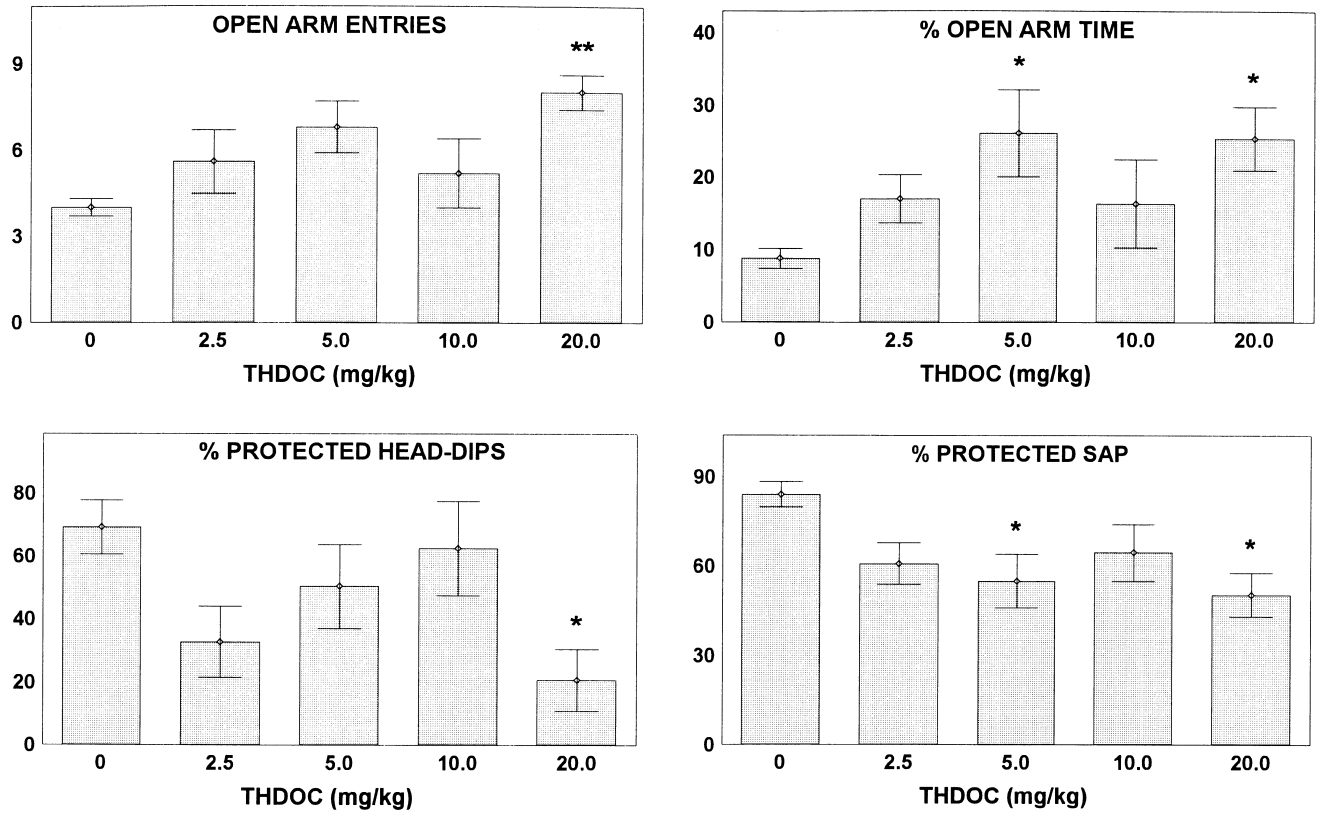

FIG. 1. Effects of 5α, 3α-THDOC (2.5–20.0 mg/kg) on behaviors displayed by DBA/2 mice in the elevated plus-maze. *$p < 0.05$, **$p < 0.01$ vs. vehicle control. See Table 2 for complementary data.

TABLE 2

EFFECTS OF 5α, 3α-THDOC (2.5–20.0 mg/kg) ON THE BEHAVIOR OF MALE DBA/2 MICE IN THE ELEVATED PLUS-MAZE

	5α,3α-THDOC (mg/kg)					
Behavior	Vehicle	2.5	5.0	10.0	20.0	$F(4, 43)$
Total arm entries	13.1 ± 1.2	16.0 ± 1.7	15.2 ± 1.3	16.2 ± 1.4	18.1 ± 1.8	1.43, NS
Closed arm entries	9.1 ± 1.0	10.4 ± 0.9	8.4 ± 1.0	11.0 ± 1.2	10.1 ± 1.4	0.93, NS
% Open arm entries	31.0 ± 1.7	31.8 ± 4.7	45.1 ± 4.3	30.6 ± 6.8	45.3 ± 2.5	3.16, $p < 0.05$*
% Centre platform time	35.4 ± 2.9	36.4 ± 3.7	26.3 ± 3.1	28.0 ± 3.1	30.7 ± 3.8	1.84, NS
% Closed arm time	55.8 ± 2.9	46.5 ± 4.3	47.6 ± 5.1	55.6 ± 6.0	44.0 ± 4.1	1.42, NS
Rear frequency	15.3 ± 1.9	19.9 ± 2.0	17.3 ± 1.8	20.2 ± 2.9	12.9 ± 2.0	2.06, NS
Rear duration (s)	25.0 ± 3.9	34.1 ± 3.3	26.4 ± 2.6	32.5 ± 6.3	15.6 ± 2.8	3.33, $p < 0.05$*
Head-dips	2.8 ± 0.4	3.3 ± 0.9	5.0 ± 1.2	3.8 ± 1.4	5.6 ± 0.9	1.28, NS
SAP	15.4 ± 1.0	16.9 ± 1.1	13.5 ± 1.6	13.2 ± 1.6	13.8 ± 2.4	0.97, NS
Closed arm returns	0.8 ± 0.4	0.8 ± 0.6	0.8 ± 0.4	1.1 ± 0.6	0.3 ± 0.2	0.36, NS
Sniffing (s)	96.0 ± 4.5	83.2 ± 8.3	93.9 ± 9.2	102.1 ± 6.0	111.0 ± 8.0	1.88, NS
Flatback approach (s)	7.5 ± 1.3	7.0 ± 1.4	5.6 ± 1.4	6.4 ± 2.0	3.4 ± 0.9	1.24, NS
Grooming (s)	13.6 ± 5.1	9.4 ± 2.4	11.4 ± 5.6	3.3 ± 1.2	1.2 ± 0.8	1.95, NS
%p Sniff	97.1 ± 0.7	93.6 ± 2.0	84.2 ± 5.5	92.3 ± 5.3	84.5 ± 5.1	1.97, NS
%p Flatback approach	67.0 ± 9.1	44.0 ± 10.2	64.5 ± 7.9	59.1 ± 13.1	57.5 ± 10.3	0.83, NS

Data presented are mean values ± SEM. SAP = stretched attend posture. %p = percent protected. NS = nonsignificant.
*No significant differences vs. vehicle control (Dunnetts). See Fig. 1 for complementary data.

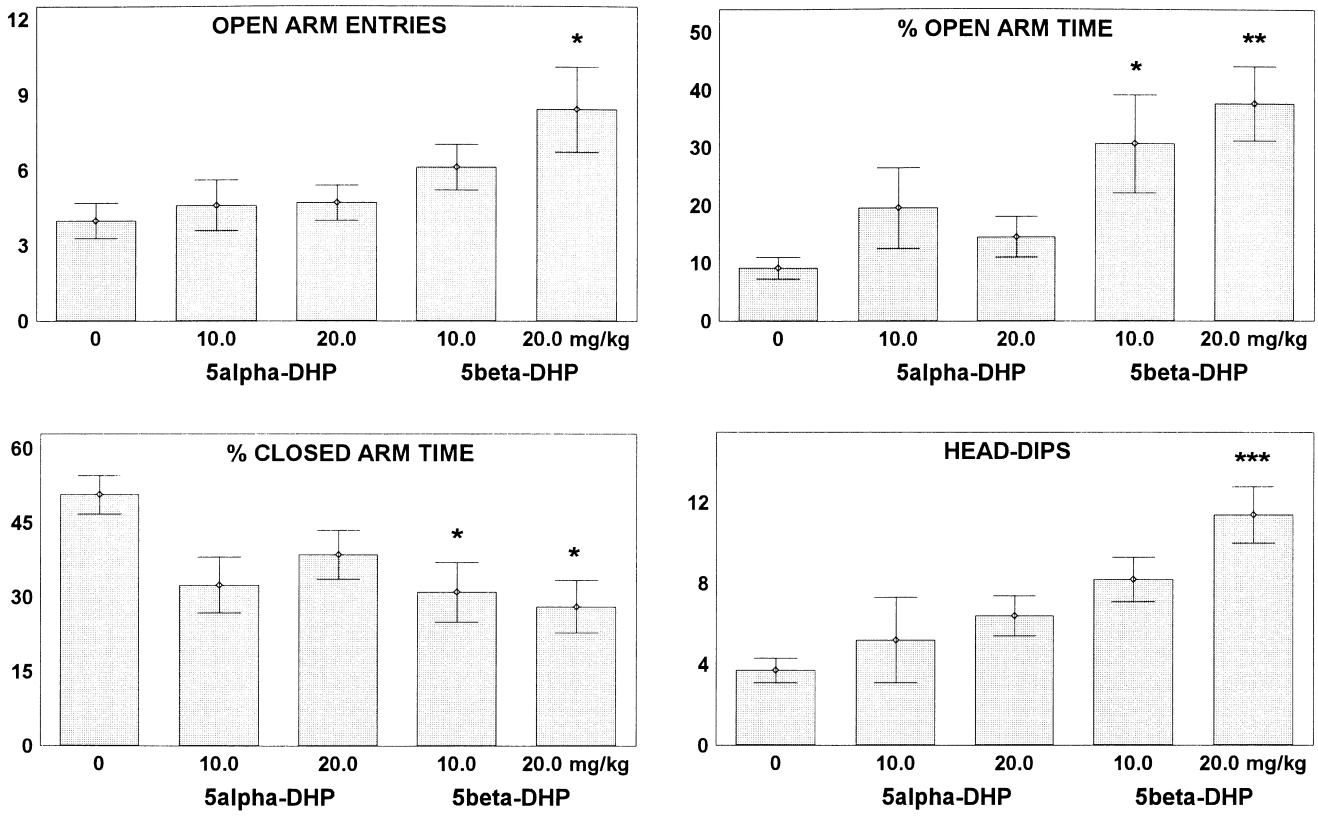

FIG. 2. Effects of 5α-DHP and 5β-DHP (10.0–20.0 mg/kg) on open arm entries, percent open arm time, percent closed arm time and head-dips in the elevated plus-maze. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vs. vehicle control. See Table 3 for complementary data.

$p < 0.01$, and percent closed arm time, $F(4, 43) = 3.13$, $p < 0.05$. Follow-up tests revealed that these effects were due solely to the 5β-isomer (Fig. 2) which, at 20.0 mg/kg, increased open arm entries ($p < 0.05$) and, at both doses, increased percent open time (10 mg/kg; $p < 0.05$; 20.0 mg/kg; $p < 0.01$) and reduced percent closed time ($p < 0.05$). These alterations in the spatiotemporal distribution of behavior were confirmed by a significant treatment × location interaction, $F(8, 86) = 2.95$, $p < 0.01$. Thus, the control profile of closed = center > open was changed by both doses of 5β- (but not 5α-) DHP, such that mice no longer displayed a significant preference for any maze location. Neither steroid isomer significantly altered total arm entries, closed arm entries, or percent center time (Table 3).

ANOVA indicated that three ethological measures were significantly affected by treatment: total head dips, $F = 5.34$, $p < 0.001$; percent protected head dips, $F = 3.48$, $p < 0.05$; percent

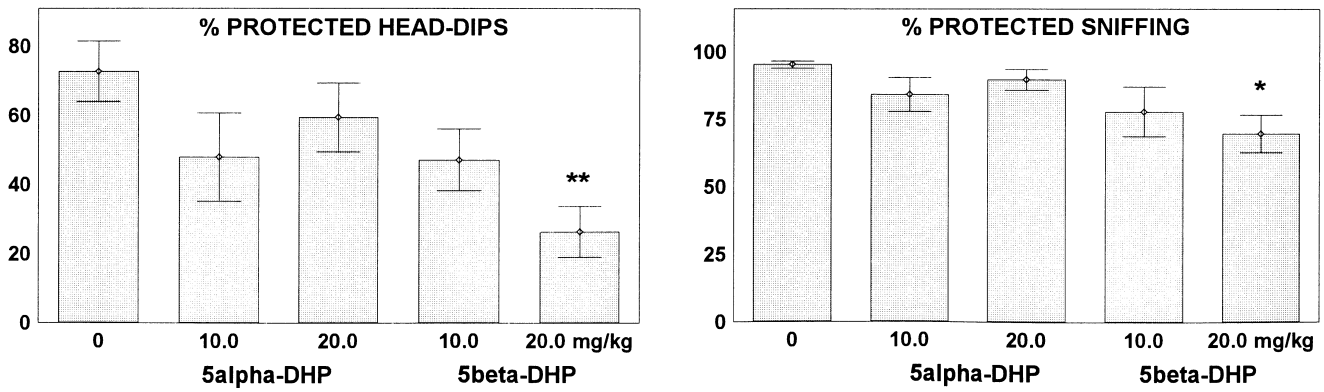

FIG. 3. Effects of 5α-DHP and 5β-DHP (10.0–20.0 mg/kg) on the percent protected forms of head dipping and sniffing in the elevated plus-maze. *$p < 0.05$, **$p < 0.01$ vs. vehicle control. See Table 4 for complementary data.

TABLE 3

EFFECTS OF 5α- AND 5β-DHP (10–20 mg/kg) ON THE BEHAVIOR OF MALE DBA/2 MICE IN THE ELEVATED PLUS-MAZE

		5α-DHP (mg/kg)		5β-DHP (mg/kg)		
Behavior	Vehicle	10.0	20.0	10.0	20.0	$F(4, 43)$
Total arm entries	13.6 ± 1.2	11.2 ± 2.1	13.7 ± 1.5	14.0 ± 1.8	19.2 ± 3.7	1.70, NS
Closed arm entries	9.6 ± 1.1	6.7 ± 1.4	9.0 ± 1.2	7.9 ± 1.4	10.8 ± 2.2	1.03, NS
% Open arm entries	30.2 ± 6.3	37.6 ± 6.2	34.7 ± 4.2	48.6 ± 7.2	47.8 ± 7.2	1.69, NS
% Centre platform time	40.1 ± 3.4	47.9 ± 7.7	46.8 ± 5.7	38.3 ± 4.1	34.2 ± 4.3	1.26, NS
Rear frequency	12.8 ± 2.1	10.8 ± 2.9	11.6 ± 2.2	9.9 ± 2.9	13.7 ± 3.4	0.31, NS
Rear duration (s)	19.4 ± 3.6	13.8 ± 3.5	15.7 ± 3.5	12.8 ± 4.3	12.8 ± 3.0	0.62, NS
SAP	13.5 ± 1.5	10.1 ± 1.6	11.0 ± 1.3	13.0 ± 2.3	14.5 ± 1.6	1.17, NS
Closed arm returns	1.4 ± 0.3	0.8 ± 0.4	1.1 ± 0.5	0.3 ± 0.2	0.9 ± 0.6	0.80, NS
Sniffing (s)	126.1 ± 5.1	142.1 ± 13.5	139.6 ± 5.4	130.8 ± 8.9	126.5 ± 9.9	0.70, NS
Flatback approach (s)	5.2 ± 1.2	3.9 ± 1.0	4.3 ± 0.8	4.0 ± 1.0	5.1 ± 2.1	0.21, NS
Grooming (s)	9.1 ± 4.2	5.8 ± 3.0	7.0 ± 2.6	3.9 ± 1.6	3.0 ± 1.2	0.77, NS
%p SAP	81.6 ± 4.9	74.8 ± 10.9	76.0 ± 7.1	67.5 ± 8.1	56.8 ± 9.6	1.38, NS
%p Flatback approach	65.8 ± 10.4	70.8 ± 14.0	66.3 ± 13.9	52.7 ± 11.9	46.8 ± 12.5	0.63, NS

Data presented are mean values ± SEM. SAP = stretched attend posture. %p = percent protected. NS = nonsignificant.
See Figs. 2 and 3 for complementary data.

protected sniffing, $F = 2.90$, $p < 0.05$. Again, these effects were seen only with 5β-DHP (20 mg/kg) and comprised increases in head dips ($p < 0.001$), and decreases in percent protected forms of head dipping ($p < 0.01$) and sniffing ($p < 0.05$) (Fig. 3). Other measures were not affected by either isomer (Table 3).

Pregnanolone (5β,3α-P)

ANOVA revealed significant effects of pregnanolone on total arm entries, $F(4, 41) = 3.23$, $p < 0.05$, open arm entries, $F(4, 41) = 2.59$, $p = 0.05$, and percent open arm time, $F(4, 41) = 3.47$, $p < 0.05$. Further analysis (Fig. 4) indicated significant increases ($p < 0.05$) in all these measures at the top dose tested (20.0 mg/kg). A two-way ANOVA on percent time measures yielded a significant treatment × location interaction, $F(8, 82) = 2.15$, $p < 0.05$, supporting a treatment-induced alteration in the spatiotemporal distribution of behavior. Thus, unlike controls (closed = center > open), animals treated with 20 mg/kg did not differentiate between areas of the maze. Pregnanolone did not alter closed arm entries, or percent time spent on either the center platform or closed arms (Table 4).

None of the ethological measures was significantly affected by pregnanolone treatment (Table 4). Although significant F-values were obtained for rear frequency, percent protected SAP, closed arm returns, and percent protected sniffing, follow-up tests failed to reveal significant differences between control and any steroid dose. Nevertheless, clear dose-dependent trends towards reductions in the percent protected forms of SAP and sniffing were apparent.

Allopregnanolone (5α,3α-P)

Analysis indicated significant effects of allopregnanolone on open arm entries, $F(4, 49) = 2.60$, $p < 0.05$, and percent open arm time, $F(4, 49) = 3.25$, $p < 0.05$. As shown in Fig. 5, these effects were due to significant increases in open arm entries at 5.0 and 10.0 mg/kg ($p < 0.05$), and a significant increase in percent open time at 10.0 mg/kg ($p < 0.01$). A two-way ANOVA on percent time measures, $F(8, 98) = 2.38$, $p < 0.05$, confirmed that allopregnanolone (10 and 20 mg/kg) abolished the spatial preference shown by control animals (i.e., closed > center > open). No significant treatment effects were observed for total arm entries, closed arm entries, percent open arm entries (though note apparent increase at 10.0 mg/kg), percent closed time, or percent center time (Table 5).

Allopregnanolone also had significant effects on several ethological measures: total head dips, $F = 3.06$, $p < 0.05$, and total flatback approach, $F = 3.01$, $p < 0.05$, with an increase in the former at 20.0 mg/kg ($p < 0.05$) and a dose-dependent decrease in the latter that reached significance ($p < 0.05$) at both 10.0 and 20.0 mg/kg (Fig. 5). In addition, the F-value for percent protected sniffing closely approached significance ($F_{crit0.05} = 2.56$, $F_{obs} = 2.33$), with evidence of a reduction in this parameter at 10.0 mg/kg (significant vs. vehicle, $p < 0.05$) . No other measures were significantly altered (Table 5).

5α,3α-P

Data are summarized in Table 6. No significant treatment effects were observed on either conventional or ethological measures. This lack of activity of 5α,3α-P was further supported by a two-way ANOVA, which, despite demonstrating that mice had an overall spatiotemporal preference, $F(2, 86) = 120.0$, $p < 0.001$ (closed > center > open), failed to show a significant treatment × location interaction, $F(8, 86) = 1.23$, NS.

DISCUSSION

Although recent findings have provided empirical support for the suggestion that neuroactive steroids may function as endogenous anxiolytics (21,27,41), some doubts remain concerning the behavioral selectivity of such effects. In particular, much of the existing literature either pays little attention to possible motoric influences or, alternatively, confounds such

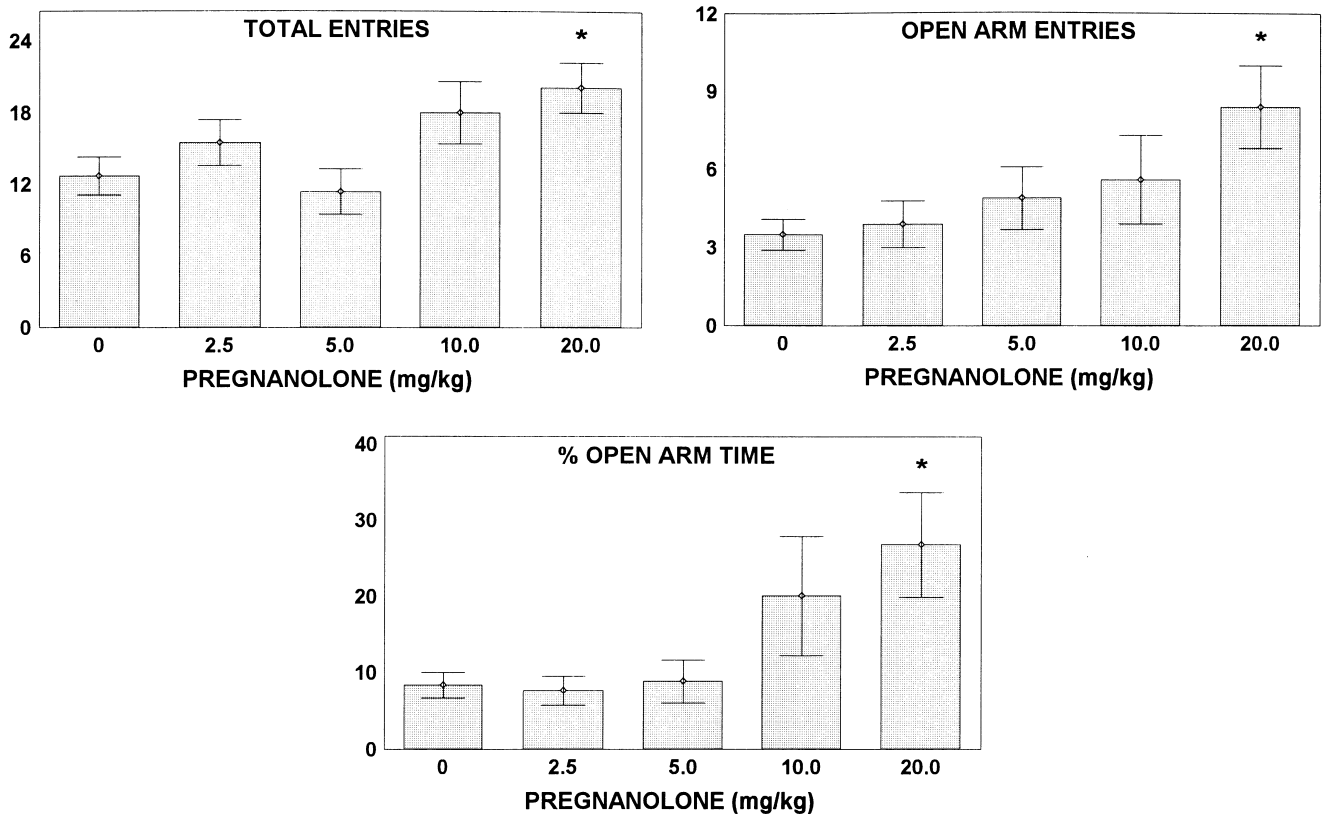

FIG. 4. Effects of pregnanolone (2.5–20.0 mg/kg) on behaviors displayed by DBA/2 mice in the elevated plus-maze. *$p < 0.05$ vs. vehicle control. See Table 4 for complementary data.

TABLE 4

EFFECTS OF PREGNANOLONE (5β, 3α-P; 2.5–20.0 mg/kg) ON THE BEHAVIOR OF MALE DBA/2 MICE IN THE ELEVATED PLUS-MAZE

	Pregnanolone (mg/kg)					
Behavior	Vehicle	2.5	5.0	10.0	20.0	$F(4, 41)$
Closed arm entries	9.2 ± 1.6	11.6 ± 1.5	6.6 ± 1.2	12.4 ± 2.5	11.7 ± 1.3	2.05, NS
% Open arm entries	32.5 ± 6.5	24.7 ± 4.3	45.3 ± 8.0	32.4 ± 8.9	40.5 ± 5.6	1.46, NS
% Centre platform time	41.3 ± 5.2	35.3 ± 2.5	48.3 ± 7.7	31.4 ± 3.6	31.9 ± 2.9	2.19, NS
% Closed arm time	50.2 ± 4.6	57.1 ± 3.7	42.8 ± 7.5	48.5 ± 7.9	41.3 ± 4.7	1.27, NS
Rear frequency	12.6 ± 1.9	18.2 ± 2.1	9.0 ± 2.2	15.5 ± 1.7	15.8 ± 1.8	3.22, $p < 0.05$*
Rear duration (s)	22.3 ± 4.2	28.4 ± 3.5	13.1 ± 4.3	22.3 ± 3.3	21.3 ± 1.7	2.43, NS
Head-dips	5.7 ± 1.1	6.2 ± 1.3	4.8 ± 1.0	7.0 ± 1.1	8.7 ± 1.7	1.35, NS
SAP	13.9 ± 1.9	18.2 ± 1.7	18.8 ± 2.1	12.5 ± 2.0	15.1 ± 1.4	2.19, NS
Closed arm returns	0.9 ± 0.4	2.2 ± 0.7	0.7 ± 0.4	1.3 ± 0.5	0.2 ± 0.1	2.67, $p < 0.05$*
Sniffing (s)	97.4 ± 6.1	99.8 ± 4.2	112.6 ± 9.1	99.4 ± 10.1	95.7 ± 8.8	0.75, NS
Flatback approach (s)	6.1 ± 1.2	3.9 ± 1.4	5.1 ± 1.3	2.9 ± 0.9	1.7 ± 0.4	2.38, NS
Grooming (s)	14.3 ± 3.4	8.0 ± 2.9	17.2 ± 8.3	7.8 ± 3.6	4.1 ± 2.0	1.38, NS
%p Dips	61.6 ± 10.1	59.6 ± 8.4	71.2 ± 6.7	39.5 ± 11.7	43.2 ± 9.1	1.95, NS
%p SAP	77.2 ± 6.5	82.6 ± 4.5	82.7 ± 7.1	67.2 ± 11.6	52.4 ± 8.5	2.82, $p < 0.05$*
%p Sniff	96.1 ± 1.2	98.4 ± 0.6	95.0 ± 2.1	86.4 ± 6.2	84.1 ± 5.9	2.93, $p < 0.05$*
%p Flatback approach	73.8 ± 12.7	76.6 ± 13.0	77.6 ± 12.9	87.2 ± 8.3	62.8 ± 17.0	0.34, NS

Data presented are mean values ± SEM. SAP = stretched attend posture. %p = percent protected. NS = nonsignificant.
*No significant differences vs. vehicle control (Dunnetts). See Fig. 4 for complementary data.

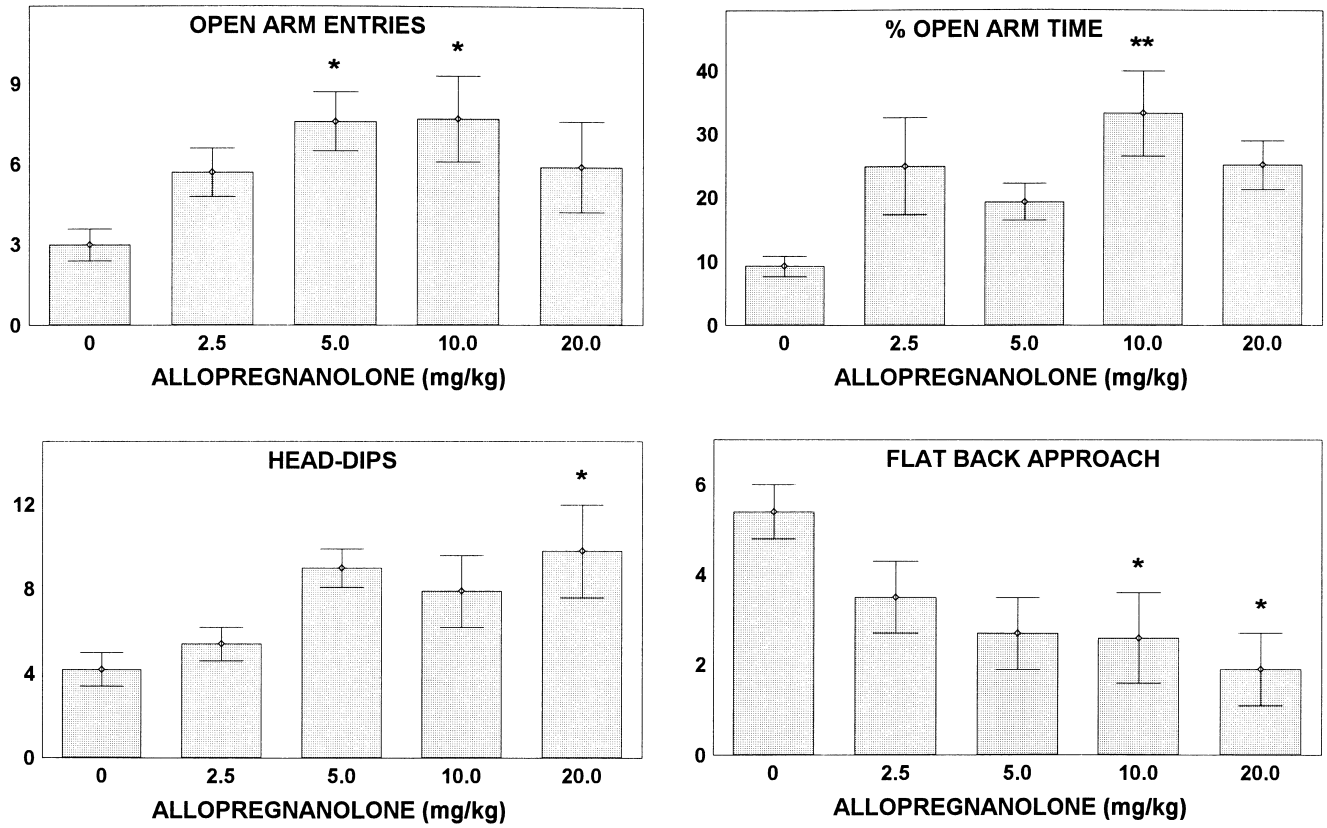

FIG. 5. Effects of allopregnanolone (2.5–20.0 mg/kg) on behaviors displayed by DBA/2 mice in the elevated plus-maze. *$p < 0.05$, **$p < 0.01$ vs. vehicle control. See Table 5 for complementary data.

TABLE 5

EFFECTS OF ALLOPREGNANOLONE (5α, 3α-P; 2.5–20.0 mg/kg) ON THE BEHAVIOR OF MALE DBA/2 MICE IN THE ELEVATED PLUS-MAZE

	Allopregnanolone (mg/kg)					
Behavior	Vehicle	2.5	5.0	10.0	20.0	$F(4, 49)$
Total arm entries	10.9 ± 1.0	13.2 ± 1.3	16.6 ± 2.1	14.9 ± 3.1	19.2 ± 4.4	1.49, NS
Closed arm entries	7.9 ± 0.9	7.5 ± 1.3	9.0 ± 1.5	7.2 ± 2.4	13.3 ± 3.2	1.58, NS
% Open arm entries	26.8 ± 3.6	46.1 ± 7.3	46.2 ± 4.4	53.4 ± 8.3	42.9 ± 9.9	2.15, NS
% Centre platform time	29.0 ± 3.8	34.2 ± 3.5	39.1 ± 4.1	24.8 ± 4.4	32.8 ± 5.9	1.52, NS
% Closed arm time	61.8 ± 4.6	40.8 ± 5.1	41.5 ± 4.3	41.9 ± 8.9	42.0 ± 7.6	2.22, NS
Rear frequency	14.3 ± 1.6	10.9 ± 2.0	13.0 ± 2.1	12.3 ± 3.2	11.9 ± 3.9	0.24, NS
Rear duration (s)	20.4 ± 3.0	14.3 ± 3.5	16.1 ± 3.4	15.2 ± 4.4	11.5 ± 3.3	0.85, NS
SAP	11.9 ± 1.9	9.4 ± 1.7	8.1 ± 1.2	7.3 ± 1.7	5.7 ± 1.3	2.15, NS
Closed arm returns	1.3 ± 0.7	0.7 ± 0.2	0.5 ± 0.4	0.2 ± 0.1	0.4 ± 0.2	1.12, NS
Sniffing (s)	130.1 ± 8.3	126.7 ± 7.9	122.0 ± 13.4	114.7 ± 10.0	136.3 ± 17.2	0.50, NS
Grooming (s)	18.0 ± 4.0	16.9 ± 7.6	24.7 ± 11.2	8.4 ± 2.8	4.0 ± 1.3	1.63, NS
%p Dips	62.0 ± 11.4	39.0 ± 9.2	38.5 ± 6.1	30.4 ± 10.4	39.4 ± 11.3	1.55, NS
%p SAP	80.4 ± 4.2	65.2 ± 7.6	64.3 ± 8.8	56.5 ± 9.5	65.8 ± 13.5	1.08, NS
%p Sniff	94.8 ± 1.1	80.5 ± 7.6	86.8 ± 3.9	74.9 ± 6.2	83.5 ± 3.7	2.33, NS
%p Flatback approach	70.1 ± 8.7	77.0 ± 6.2	85.6 ± 7.8	87.7 ± 5.9	92.1 ± 5.9	1.40, NS

Data presented are mean values ± SEM. SAP = stretched attend posture. %p = percent protected. NS = nonsignificant. See Fig. 5 for complementary data.

TABLE 6

EFFECTS OF 5α,3β-P (2.5–20.0 mg/kg) ON THE BEHAVIOR OF MALE DBA/2 MICE ON THE ELEVATED PLUS-MAZE

	5α,3β-P (mg/kg)					
Behavior	Vehicle	2.5	5.0	10.0	20.0	$F(4, 49)$
Total arm entries	13.4 ± 1.6	13.0 ± 1.5	12.2 ± 1.7	13.0 ± 1.1	10.3 ± 2.2	0.53, NS
Open arm entries	4.3 ± 0.9	4.1 ± 0.6	2.8 ± 0.5	3.2 ± 0.4	3.8 ± 1.1	0.71, NS
Closed arm entries	9.1 ± 0.9	8.9 ± 0.9	9.4 ± 1.4	9.8 ± 0.8	6.6 ± 1.3	1.32, NS
% Open arm entries	29.3 ± 4.2	30.9 ± 2.4	21.5 ± 4.2	24.2 ± 2.1	29.5 ± 6.6	0.97, NS
% Open arm time	9.6 ± 2.2	8.6 ± 1.7	5.8 ± 1.1	9.4 ± 1.9	11.2 ± 4.1	0.73, NS
% Centre platform time	32.1 ± 2.9	43.9 ± 6.1	34.8 ± 3.6	31.7 ± 2.8	31.5 ± 6.0	1.38, NS
% Closed arm time	58.3 ± 3.2	47.5 ± 5.5	59.4 ± 3.9	58.9 ± 2.8	57.4 ± 6.5	1.21, NS
Rear frequency	16.7 ± 0.9	15.5 ± 2.9	12.8 ± 2.5	17.1 ± 2.4	11.4 ± 3.0	1.02, NS
Rear duration (s)	24.5 ± 2.6	21.9 ± 4.7	19.1 ± 3.8	26.9 ± 4.7	17.7 ± 5.3	0.74, NS
Head-dips	5.0 ± 0.9	6.8 ± 1.6	4.5 ± 0.8	2.1 ± 0.6	7.4 ± 2.0	2.67, $p < 0.05$*
SAP	15.1 ± 1.5	14.6 ± 2.1	11.3 ± 1.6	15.0 ± 1.6	10.6 ± 2.4	1.37, NS
Closed arm returns	0.8 ± 0.4	1.1 ± 0.3	1.4 ± 0.3	2.1 ± 0.7	0.3 ± 0.2	2.57, NS
Sniffing (s)	128.5 ± 7.1	137.9 ± 9.1	149.9 ± 9.1	144.2 ± 7.7	138.3 ± 10.8	0.85, NS
Flatback approach (s)	6.2 ± 0.6	8.2 ± 2.0	7.4 ± 2.1	7.2 ± 1.7	5.7 ± 1.6	0.35, NS
Grooming (s)	16.8 ± 6.3	7.4 ± 2.3	6.8 ± 3.8	9.9 ± 2.9	10.9 ± 3.6	1.00, NS
%p Dips	48.7 ± 12.5	74.5 ± 6.8	53.3 ± 7.8	61.0 ± 14.1	67.0 ± 10.3	1.06, NS
%p SAP	79.0 ± 7.5	74.5 ± 5.6	83.9 ± 4.2	75.9 ± 6.0	78.2 ± 10.0	0.28, NS
%p Sniff	95.1 ± 1.3	97.1 ± 0.8	97.9 ± 0.7	96.1 ± 1.0	91.1 ± 4.1	1.84, NS
%p Flatback approach	72.8 ± 10.3	73.3 ± 10.4	77.7 ± 7.0	60.5 ± 11.1	66.6 ± 16.3	0.38, NS

Data presented are mean values ± SEM. SAP = stretched attend posture. %p = percent protected. NS = nonsignificant.
*No significant differences vs. vehicle (Dunnetts).

assessment by running the same subjects in unrelated activity tests either immediately before or after anxiety testing. Even when locomotor activity is assessed concurrently with anxiety (i.e., in the same test), the measures used are not always the most appropriate. For example, in the elevated plus-maze paradigm, conclusions regarding behavioral selectivity are frequently based upon treatment effects on total arm entries. However, factor analyses have consistently shown that, in both rats and mice, the total entry score is a contaminated measure, loading on both the "activity" and "anxiety" factors, whereas the closed arm entry score is a relatively pure index of general activity (12,16,17,32,46).

Consistent with previous research from this laboratory (9,13,23) and elsewhere (45), diazepam produced a behavioral profile consistent with anxiety reduction. This conclusion is supported by significant changes in measures related to "anxiety" (46), i.e., increased percent open entries/percent open time, and reduced percent closed time, percent protected sniffing, and closed arm returns. The absence of treatment effects on the protected forms of head dipping and SAP may be attributed to the relatively low control scores for these measures in this study. Diazepam also abolished the spatiotemporal preference shown by control animals, and reduced total SAP (the primary index of "risk assessment" in the plus-maze). Although these effects were observed in the absence of an alteration in total arm entries, significant reductions in rearing, closed arm entries, and total flatback approach were observed. As suggested in previous studies [e.g. (9,13)], the effect of diazepam on rearing most probably relates to the significant reduction in time spent in those parts of the maze (closed arms) where rearing normally occurs. However, the observed reductions in closed arm entries and flatback approach ["activity" measures; (46)] would indicate that the robust anxiolytic-like profile of diazepam in this study was associated with a weak, though significant, inhibition of locomotor activity. Nevertheless, it should be noted that the reduction in closed entries was fully compensated by the increase in open entries (i.e., no treatment effect on total arm entries), thereby confirming the diazepam-induced spatial redistribution of behavior on the maze. Furthermore, direct comparison with the results of an earlier diazepam study from this laboratory (23) would support a more potent action of the benzodiazepine under present test conditions.

Although 5α,3α-THDOC has been found to exert anxiolytic-like effects in the mouse light/dark exploration test as well as rat punished drinking and plus-maze paradigms, these and other reports suggest the possibility of a locomotor confound especially at higher doses (1,11,24). Our results demonstrate for the first time that 5α,3α-THDOC has anxiolytic-like effects in the murine plus-maze and, further, show that this action is not secondary to changes in general activity. Thus, in the absence of effects on closed arm entries, treatment with this steroid increased open arm entries and percent open arm time, and decreased the percent protected forms of head dipping and SAP. Interestingly, however, these effects were observed at nonconsecutive doses (5 and 20 mg/kg), a finding that clearly demands further investigation. It is also noteworthy that 5α,3α-THDOC did not affect risk assessment (or rearing), thereby differentiating its profile from that of diazepam.

Prior to the formation of pregnanolone and allopregnanolone, progesterone is 5α- and 5β-reduced to yield the intermediate metabolites, 5α-DHP and 5β-DHP (43). Although limited to two doses only, present results show that 5α-DHP was without effect in the murine plus-maze, whereas 5β-DHP produced dose-dependent anxiolytic-like effects: both doses increased percent open arm entries and percent open time, re-

duced percent closed arm time, and the protected forms of head dipping and sniffing, and eliminated the typical spatiotemporal preference shown by mice in this test. In addition, the higher dose increased total head dips, an effect often (though nonsignificantly in the present study) seen with benzodiazepines (8,9,13,23). However, while these effects were seen to be anxioselective in that neither dose produced significant changes in closed arm entries, it is again notable that (as for 5α,3α-THDOC) risk assessment was unaffected by steroid treatment. The present results differ from those obtained in the rat defensive burying paradigm in which only 5α-DHP was found to have anxiolytic-like effects (42). However, the effective dose in the latter study tended to suppress locomotor activity and, furthermore, these authors employed a 4-h injection-test interval, which may be expected to result in significant in vivo bioconversion (e.g., to allopregnanolone), an effect unlikely to have occurred with the 10-min injection-test interval used in the current study.

Of all the neuroactive steroids, pregnanolone (5β,3α-P) and allopregnanolone (5α,3α-P) have been the most extensively studied for antianxiety activity, with positive effects for both reported in rodent Geller-Seifter and/or Vogel conflict, defensive burying, light/dark exploration, and plus-maze tests (4,42,52,53). Allopregnanolone has additionally been found to have anxiolytic-like effects in the mouse open-field (53) and rat pup ultrasonic distress vocalization (54) tests. However, these reports not only differ with respect to the relative efficacy/potency of the two steroids but also suggest that their anxiolytic activity may be confounded by general locomotor stimulation [e.g. (52)]. In the present study, the highest dose of pregnanolone tested (20 mg/kg) produced a significant increase in percent open arm time coupled with an elimination of the usual spatiotemporal preference for closed arms vs. other areas of the maze. Furthermore, although nonsignificant, anxiolytic-like trends were also apparent on a range of other measures (i.e., an increase in open arm entries and reductions in all percent protected scores). While these findings agree well with a previous report on the anxiolytic-like effects of pregnanolone in the mouse plus-maze (52), the concomitant increase in total arm entries might be taken as evidence of a nonspecific behavioral effect. However, as treatment did not affect closed arm entries (suggesting that the increase in total entries was largely due to an increase in open entries) or, indeed, any other active behaviors (including risk assessment), the observed profile is consistent with a behaviorally selective action of pregnanolone on plus-maze anxiety. As pregnanolone is formed from 5β-DHP, via the action of 3α-hydroxysteroid reductase (43), it is of further interest to note the somewhat greater efficacy and potency of 5β-DHP under present test conditions.

Allopregnanolone (5α,3α-P) also produced anxiolytic-like effects under present test conditions. Thus, open arm entries, percent open time, and head dipping were increased at 5–10 mg/kg, while the normal spatiotemporal preference pattern was abolished at 10–20 mg/kg. Similar to the profile obtained with pregnanolone, nonsignificant anxiolytic trends were also apparent on the percent protected forms of head dipping, SAP, and sniffing. Furthermore, the apparent inhibitory effect of allopregnanolone on total SAP did not reach an acceptable level of statistical significance, thus confirming the general finding of this series that anxiolytic neuroactive steroids do not produce a benzodiazepine-like inhibition of risk assessment. Although flatback approach was reduced at 5–20 mg/kg, the absence of changes in closed or total arm entries would suggest that the anxiolytic effects of allopregnanolone in the murine plus-maze are not secondary to locomotor changes. In contrast to previous work on the mouse plus-maze and light/dark tests (52), but in agreement with studies on the rat defensive burying paradigm (42), profile comparisons would suggest superior efficacy and potency of allopregnanolone compared with pregnanolone. Finally, from the viewpoint of structure–activity requirements for GABA facilitation (26), it is important to note that, as in other studies (4,42,52,53), 5α,3β-P was completely devoid of behavioral activity over the dose range studied (2.5–20.0 mg/kg).

In conclusion, the present series of experiments has revealed anxiolytic-like effects for a range of 5α- and 5β-reduced progestines (5α,3α-THDOC, 5β-DHP, 5α,3α-P, and 5β,3α-P) in the murine elevated plus-maze paradigm. Detailed ethological analysis further showed that these antianxiety effects cannot readily be attributed to alterations in general activity levels, while the failure of the 3β-isomer of allopregnanolone to produce behavioral change is fully consistent with structural requirements for agonist-like activity at the GABA receptor complex. Our results also suggest that, while producing overlapping profiles, these anxiolytic steroids differ from diazepam in that they produce a narrower range of behavioral effects and, in particular, do not reliably inhibit risk assessment. This is a potentially important distinction because inhibition of risk assessment is a consistent effect of benzodiazepines, not only in the murine plus-maze but also in a number of other test situations (5,20,36,50). It is clear that future studies in this area should assess whether (a) anxiety reduction in the plus-maze is maintained following chronic steroid administration, and (b) cessation of chronic treatment is associated with withdrawal anxiogenesis in this paradigm. Furthermore, in view of reported stress-induced elevations in brain levels of neuroactive steroids (2,3), it will important to determine whether such changes occur in response to plus-maze exposure per se and, if so, whether the behavioral significance of such release can be revealed through the use of selective steroid antagonists.

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