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ORIGINAL INVESTIGATION

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Distress vocalizations in maternally separated mouse pups: modulation via 5-HT_{1A}, 5-HT_{1B} and $GABA_A$ receptors

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Abstract *Rationale:* Young rodents emit ultrasonic vocalizations (USVs) when separated from their dams and littermates. Pharmacological agents that act on $GABA_A$ and/or 5-HT receptors and that alleviate anxiety in humans reduce the emission of these calls. *Objectives:* 1) to investigate specific 5-HT_1 receptor subtypes that modulate maternal separation-induced USVs in mice; 2) to assess the behavioral specificity of these effects; and 3) to compare 5-HT_1 agonists with a positive neurosteroid modulator of the $GABA_A$ receptor complex. *Methods:* Seven-day old CFW mouse pups were isolated from their littermates and placed onto a 20°C surface for 4 min. USVs between 30 and 80 kHz, grid crossing, and rectal temperature were measured in separate groups of mouse pups following subcutaneous administration of 5-HT_{1A} and 5-HT_{1B} receptor agonists and antagonists, the neurosteroid allopregnanolone, or the benzodiazepine midazolam. *Results:* The 5-HT_{1A} agonists (+)8-OH-DPAT (0.01–0.1 mg/kg) and flesinoxan (0.3–1.0 mg/kg), the selective 5-HT_{1B} agonist CP-94,253 (0.03–30.0 mg/kg), and the mixed 5-$HT_{1B/2C}$ receptor agonist TFMPP (0.1–10.0 mg/kg) dose-dependently reduced USVs. These effects were reversed by the 5-HT_{1A} receptor antagonist WAY 100,635 (0.1 mg/kg) or the 5-$HT_{1B/D}$ receptor antagonist GR 127935 (0.1 mg/kg). The effects of TFMPP were biphasic; low doses (i.e. 0.01 and 0.03 mg/kg) increased the rate of vocalization. Midazolam and allopregnanolone also reduced USVs. The highest doses of flesinoxan, (+)8-OH-DPAT, and allopregnanolone suppressed locomotion, whereas CP-94,253, TFMPP, and midazolam stimulated motor activity. *Conclusions:* These experiments confirm that agonists at the 5-HT_1 receptors and a positive allosteric modulator of the $GABA_A$ receptor complex decrease maternal separation-induced USVs in mice, with 5-HT_{1B} manipulations dissociating the effects on vocalizations from sedative effects.

Key words Anxiety · Serotonin · GABA · Neurosteroid · Ultrasonic vocalization · Motor activity

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Introduction

Preclinical research on the neuropharmacology of anxiety has primarily used rats as experimental animals. Classic tests for studying anxiolytic drugs, such as the Geller-Seifter procedure (Geller and Seifter 1960), punished-lick suppression (Vogel et al 1971), fear-potentiated startle (Davis 1979), or pentylenetetrazole drug discrimination (Lal and Shearman 1982) have been very useful to identify the GABA and serotonin (5-HT) neurotransmitter systems as important modulators of anxiety. It has been more difficult to determine the genetic and molecular neurobiological mechanisms of anxiety because the techniques such as gene "knockout", insertion, transgenics, and quantitative trait loci (QTL) analysis use mice as experimental animals (Belknap et al 1993; Buck et al 1997; Grisel et al 1997; Nelson 1997).

There are few experimental procedures for assessing anxiolytic drugs that are applicable to mice (Griebel 1995; Crawley and Paylor 1997). Procedures that have been developed to measure anxiety-like responses have focused on: exploratory movements into an open field (Boissier et al 1968; Christmas and Maxwell 1970), onto open arms of a plus maze (Lister 1987), into the light portion of a two-chambered apparatus (Crawley 1981);

ultrasonic vocalizations in response to a stressor (Gardner 1985); and defensive or flight behaviors towards a predator in the mouse defensive test battery (Griebel et al 1995). With the exception of the ultrasonic vocalizations, these procedures rely on locomotor behaviors as the sole index of anxiety-like responses. Furthermore, the rapid habituation of locomotor behaviors in open-field exploration and the elevated plus maze limits the external validity of these procedures to transient states (Crawley 1985; Crawley et al 1997). The numerous reports that correlate the absence of a particular gene with anxiety (e.g., Ogawa et al 1997; Heisler et al 1998; Ramboz et al 1998; Kash et al 1999) are limited by the sole use of procedures that depend on locomotor behavior. Recently, Brunner et al. (1999) utilized maternal separation-induced USVs as part of a battery to examine the development of anxiety-like responses in 5-HT_{1B} knockout mice. Continuing to develop experimental procedures to study anxiety-like responses in mice that measure behaviors other than locomotor behaviors is essential to preclinical research on anxiety.

Infant mice, like the young of all mammalian species including humans, exhibit distress-like reactions when they are separated from their dam and littermates (Noirot 1966; 1972; Sales and Pye 1974). Their distress is signaled by the emission of 30–80 kHz ultrasonic vocalizations (USVs) that can be heard by the dam and trigger retrieval (Hofer and Shair 1987). Other neonatal rodents including rats, hamsters, and gerbils also emit maternal separation-induced USVs (Sales and Pye 1974; Dempster et al 1991). The predictive pharmacological profile and the species generality are major advantages of the maternal separation procedure (Miczek et al. 1995). USVs are an objective behavioral endpoint that is easily quantifiable, automated, and requires no conditioning procedure. Specificity of drug action can be assessed concurrently by additional behavioral measures and the rate of USV production can be modified by alterations in the testing environment.

Drugs that reduce anxiety in humans attenuate the emission of ultrasonic vocalizations in rat pups, leading to the use of this reaction as a screen for potentially anxiolytic compounds (Gardner 1985). Pharmacological modulation of maternal-separation induced USVs has been most frequently demonstrated in rats by modulators of the $GABA_A$ receptor complex and also by serotonergic agents (for review, see Lister 1990; Miczek et al 1995). Increased activity at the $GABA_A$ receptor by muscimol (Vivian et al 1997), diazepam (Insel et al. 1986; Gardner et al. 1987; Mos and Olivier 1989; Carden and Hofer 1990), reduces the emission of USVs in a manner that is reversible by the appropriate antagonist. Additionally, the neurosteroid allopregnanolone has been shown to reduce USVs (Zimmerberg et al. 1994;Vivian et al. 1997). Using mouse pups, Benton and Nastiti (1988) and Nastiti et al. (1991a, 1991b) demonstrated that muscimol and benzodiazepines reduce USVs.

Increasing serotonin levels by administration of the 5-HT precursor, 5-hydroxytryptophan or the SSRI paroxetine (Winslow and Insel 1990), also reduces separation-induced USVs in rat pups. Of the various 5-HT receptor subtypes, the 1A and 1B receptors are currently the most actively studied targets for modulating maternal separation-induced USVs. The prototypic 5-HT_{1A} receptor agonist 8-OH-DPAT systematically and dose-dependently reduces rat pup USVs in a manner that is reversible by the antagonist WAY 100635 (Hard and Engel 1988; Mos and Olivier 1989; Olivier et al 1998). Two other agents with action at the 5-HT_{1B} receptor site, eltoprazine and TFMPP, also reduce USVs (Mos and Olivier 1989). In contrast to the effects of 5-HT_{1A} and 5-HT_{1B} agonists, 5-HT_2 agonists either tend to increase or to have no effect on USVs, and 5-HT_2 antagonists increase the rate of calling (Mos and Olivier 1989; Winslow and Insel 1990; Olivier et al 1998). In mouse pups, non-selective 5-HT_{1A} and 5-HT_{1B} agonists have been reported to reduce USVs, whereas 5-HT_2 receptor agonists increase USVs (Nastiti et al 1991b).

The objective of the current experiment was to demonstrate that mouse pups could be used as experimental animals to investigate the potentially anxiolytic effect of drugs that selectively act on 5-HT_{1A} and 5-HT_{1B} receptors or the $GABA_A$ receptor complex. Distress-like USVs were produced by separation of mouse pups from their dam and littermates. Pups treated with the 5-HT_{1A} or 5-HT_{1B} receptor agonists, the neurosteroid allopregnanolone, or the benzodiazepine midazolam dose dependently emitted fewer UVSs than vehicle-treated pups. The dose-effect curves for the 5-HT_1 agonists were shifted to the right in the presence of the receptor specific antagonist. These results confirm that 5-HT_1 agonists reduce USVs and moreover extend these observations to mice.

Materials and methods

Animals

CFW mouse pups (n=746), weighing between 3.5 and 5.5 g, were bred on site from parents obtained from Charles River Breeding Labs (Wilmington, Mass., USA) and housed with both parents in clear polycarbonate cages (28 cm×17 cm×14 cm). The cages were covered by a stainless steel wire lid and lined with pine shavings that were changed once a week. Purina rodent chow and tap water were freely available through the lid. The vivarium was maintained at a constant temperature of 21±1°C, humidity 30–40% and a 12-h light/dark photocycle (lights on at 0800 hours). The pups were counted at birth (day 0) and not disturbed until the day of the test. Only litters between 8 and 12 pups were tested. The mice were cared for according to the guidelines of the "Guide for the Care and Use of Laboratory Animals" (1996). All procedures were approved by the Institutional Care and Use Committee (IACUC) of Tufts University.

Apparatus and measurements

The testing apparatus was located in a procedure room that was separate from the animal housing colony. USVs were recorded in a sound-attenuated chamber (49.5 cm×38 cm×34 cm) that was illuminated by a 10 W red light and was fitted with a one-way mir-

ror (19 cm×16.5 cm) to allow observation. A square aluminum pan (23 cm×23 cm) was suspended in a water bath to maintain a surface temperature of ca. 20°C. The surface was divided into 2 cm squares that served as grids for the measurement of locomotor behavior. Ultrasounds were detected by a high-frequency Bruel & Kjaer (Naerum, Denmark) condenser microphone (Model No. 4135) placed in the center of the testing chamber, ca. 2 cm from the aluminum surface. The condenser microphone formed a unit with a Bruel & Kjaer preamplifier (Model No. 2633). It detected sounds that were filtered by a Krohn-Hite (Cambridge, Mass., USA) filter (Model No. 3550R), and amplified by a Bruel & Kjaer amplifier (Model No. 2610) to produce a flat frequency response between 30 and 100 kHz. The output was monitored on a 20 MHz oscilloscope (Goldstar 059020 A, Cerritos, Calif., USA) and connected to a computer (Macintosh II) running customized software for signal detection. The analog signals were converted to digital representations using a GW Instruments (Somerville, Mass., USA) GWI-AMP analog-to-digital converter that also enabled additional amplification of the signal. Audible sounds produced by the mouse such as "squeals" or scratches upon the surface were eliminated using rejection parameters that accepted sounds between 30 and 100 kHz, longer than 0.01 s in duration, and that had an inter-sound interval of longer than 0.02 s. The program recorded the total number of USV, the total duration of USV (s), and the average duration of USV (s) for each pup tested. A sonogram was recorded using a Pettersson Elektronik ultrasound detector (model D 940).

Procedure

Each session began by removing an entire litter of pups and a handful of bedding from the home cage and placing them in an incubator that maintained nest temperature (35°C). Around 20 min later, the pups were weighed, marked, and screened for the emission of USVs. Only pups that reached the criterion of six USVs during a 30-s separation were used as experimental animals (ca. 75%). The pups were injected with the appropriate drug or vehicle, and rectal temperatures were taken using a thermo-probe (YSI 555 N034, Yellow Springs Instruments, Yellow Springs, Ohio, USA) attached to a YSI-2100-Tele Thermometer (Yellow Springs Instruments). The probe was lubricated with mineral oil and inserted ca. 10 mm and remained in place until the temperature measurement was stable for at least 3 s. After recording rectal temperature, the pups were returned to the incubator until the time of the test. After a specific injection interval (see Drugs), a second rectal temperature was taken immediately before the separation test. The pup was placed in the center of the cool surface and USVs and the number of grid crossings were recorded for 4 min. An experimenter counted a grid crossing when half of the pup's body crossed into the next grid. The experimental sessions were conducted between 0800 and 2300 hours. No differences in baseline rates of vocalization were observed according to time of day. At the conclusion of the experimental session, the pups were killed by CO_2 inhalation.

Drugs

(+)8-Hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT; Research Biochemicals Inc., Natick, Mass., USA), *R*(+)-*N*-(2[4-(2,3-dihydroxymethyl-1,4-benzodioxin-5-yl)-1-piperazinyl]-4)-fluorobenzoamide (flesinoxan; Solvay Pharmaceutica, Weesp, The Netherlands), *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexancarboxamine trihydrochloride (WAY 100635; American Home Products, Ayerst Wyeth), 1-(m-trifluoromethylphenyl)piperazine (TFMPP; Research Biochemicals), and 8-chloro-6-(2-fluorophenyl)-2-methyl-4H-imidazol(1,5-a)-(1,4) benzodiazepine (midazolam; Sigma, St Louis, Mo, USA) were dissolved in 0.9% saline. 3-(1,2,5,6-Tetrahydro-4-pyridyl)-5-propoxypirolo[3,2-b]pyridine (CP-94,253; Charles Pfizer, Groton, Conn., USA) was dissolved with the aid of sonication in a vehicle that was 5% Tween 80, 5% DMSO (dimethyl sulfoxide) and 90% distilled water. *N*-(4-Methoxy-3-[4-methyl-1-piperazinyl]phenyl)-2'-methyl-4'-(5-methyl-1,2,4-oxadiozol-3-yl)(1,1-biphenyl)-4-carboxamide (GR 127935; Glaxo Research and Development) and 5α-3α-pregnan-ol-20-one (allopregnanolone; Steraloids, Inc., Newport, R.I., USA) were suspended with the aid of sonication in a 20% hydroxypropyl-betacyclodextrin (Research Biochemicals) in distilled H_2O solution. All drugs were administered via the subcutaneous (SC) route using a 30 ga. needle in a volume of 1 ml/100 g body weight. To prevent leakage, a small amount of glue was applied to the injection site.

8-OH-DPAT, flesinoxan, and allopregnanolone were injected 15 min before the separation test. For antagonism, WAY 100635 was given 15 min prior to injection of the agonist. Midazolam was injected 10 min before the separation test. These injection intervals were derived based on intervals previously used in studies with rat pups (Vivian et al. 1997; Olivier et al. 1998). CP-94,253 and TFMPP were injected 30 min before the separation test and GR 127935 was given 30 min prior to the injection of the agonist, based on injection intervals effective in adult mice and rat pups (Olivier et al. 1998; Fish et al. 1999).

Statistical analysis

The rate of USVs, frequency of grid crossings, and change in body temperature were analyzed by one-way between-subjects analysis of variance (ANOVA) with post-hoc Dunnett's test to compare each treatment with the appropriate vehicle control. α was defined at 0.05 for all comparisons. To calculate the ED_{50}s, the data on frequency of USVs and grid crossing were transformed into percent of vehicle control. The ED_{50} was defined as the dose of a drug that produced a 50% change in behavior as compared to the vehicle control. ED_{50}s were calculated from first order regression equations. Non-overlapping 95% confidence intervals were considered to be significantly different.

Results

Postnatal development

The rates of ultrasonic vocalization and grid crossing by mouse pups differed daily during the first 2 weeks of life [$F(6,121)=10.66$, $P<0.05$ and $F(6,121)=28.62$, $P<0.05$ (Fig. 1). The highest rates of USVs were emitted by 7-day-old pups ($P<0.05$) and grid crossing increased from day 9 to day 13 ($P<0.05$). A spectragraph of ultrasonic vocalizations emitted by a 7-day-old mouse pup is shown as an inset to Fig. 1. These calls are in the 60 kHZ range and show downsweeps characteristic of whistles.

Ultrasonic vocalizations

5-HT$_{1A}$ receptors

8-OH-DPAT and flesinoxan dose-dependently reduced the emission of ultrasonic vocalizations [$F(3,58)=13.75$, $P<0.05$ and $F(3,55)=13.48$, $P<0.05$, respectively (Fig. 2)]. Post-hoc Dunnett's comparisons revealed that pups treated with 0.03 or 0.1 mg/kg 8-OH-DPAT and 0.1 or 0.3 mg/kg flesinoxan emitted fewer USVs than did the pups treated with saline ($P<0.05$). Pretreatment with WAY 100,635 produced a significant rightward shift in

Table 1 5-HT_1 and $GABA_A$ effects on ultrasonic vocalization and grid crossing by 7-day-old mouse pups

Treatment	USV		Grid crossing	
	ED_{50} (mg/kg)	95%CI	ED_{50} (mg/kg)	95%CI
(+)8-OH-DPAT	0.03	0.02, 0.05	0.04	0.01, 0.10
°+ WAY 100,635	4.82*	1.72, 10.64	1.00**	090, 1.34
Flesinoxan	0.09	0.03, 0.15	0.17	0.08, 0.29
°+WAY 100,635	2.05*	0.91, 4.22	>3.0	
CP-94,253	1.15	0,71, 1.55	>0.6**	
°+GR 127935	16.44*	4.24, 31.78	>3.0**	
TFMPP	1.43	0.63, 2.12	0.40**	0.36, 0.51
°+GR 127935	4.23*	2.35, 5.49	1.16*	0.71, 1.84
Allopregnanolone	5.40	3.45, 7.41	10.90	6.24, 13.62
Midazolam	1.41	0.78, 2.20	>0.3**	

* Indicates significant shift in dose effect curve of agonist
** Indicates increase in the number of grid crossings

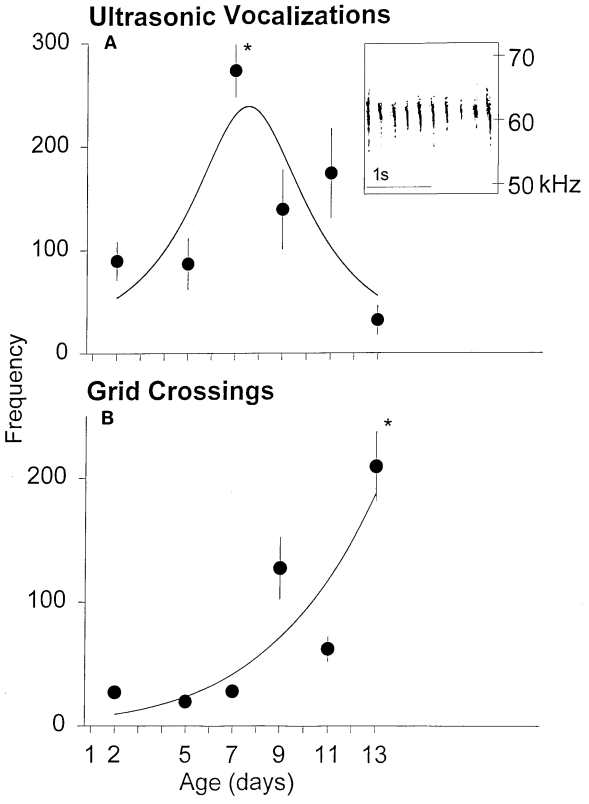

Fig. 1 **A** The developmental time course for ultrasonic vocalizations. *Filled circles* represent data for ultrasonic vocalizations. Data are presented as mean frequency±1 SE (*vertical lines*). The data are fitted with a regression line to illustrate the peak in this behavior. *Asterisks* denote significance as compared to day 1 (P<0.05). *Inset*: a spectragraph of ultrasonic vocalizations emitted by a 7-day-old mouse pup. The frequency of each vocalization (kHz) is plotted against time in seconds. **B** The developmental time course for locomotor behavior. *Filled circles* represent the mean frequency of grid crossings. *Vertical lines* indicate±1 SE. The data are fitted with a regression line to illustrate the peak in this behavior. *Asterisks* denote significance as compared to day 1 (P<0.05)

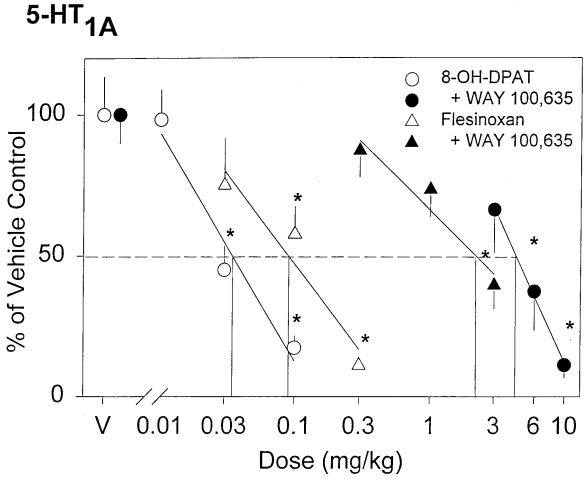

Fig. 2 The effects of 5-HT_{1A} agonists on the ultrasonic vocalizations of mouse pups, expressed as percent change from vehicle. The data are presented as mean frequency±1 SE (*vertical lines*) and are fit with regression lines to determine the ED_{50}. (≥12 mice/group). *Open symbols* represent data for the agonists. *Filled symbols* depict data for the agonists following pretreatment with WAY 100,635 (0.1 mg/kg) 15 min before administration of the agonist. *Circles* represent 8-OH-DPAT. *Triangles* represent flesinoxan. *Asterisks* indicate significance from vehicle control, P<0.05

the ED_{50} for the agonists (Table 1 and Fig. 2). In the presence of the antagonist, doses of 6.0 and 10.0 mg/kg 8-OH-DPAT [F(3,59)=9.64, P<0.05] and 3.0 mg/kg flesinoxan [F(3, 68)=5.22, P<0.05] were required to reduce the emission of USVs (P<0.05).

5-HT_{1B} receptors

Administration of CP-94,253 also reduced the emission of USVs [F(3,58)=13.29, P<0.05 (Fig. 3)]. Pups treated with CP-94,253 at the 1.0 and 3.0 mg/kg doses vocalized less than did the pups treated with the vehicle control (P<0.05). The mixed $5HT_{1B/2C}$ agonist, TFMPP exerted a biphasic effect on mouse pup USVs [F(5,85)=28.26, P<0.05 (Fig. 4)]. USVs were increased above control values by 0.3 mg/kg TFMPP (P<0.05) and decreased by 1.0, 3.0 and 5.6 mg/kg (P<0.05). In the presence of GR 127935, the dose-effect curve for CP-94,253 was significantly shifted to the right (Table 1 and Fig. 4). Doses of 10.0 and 30.0 mg/kg were necessary to reduce the rate of vocalization from the level of the vehicle-treated controls, [F(3,40)=12.97, P<0.05 (P<0.05 for post-hoc)]. GR 127935 shifted the descending portion of the dose-effect curve for TFMPP to the right (Table 1 and Fig. 4).

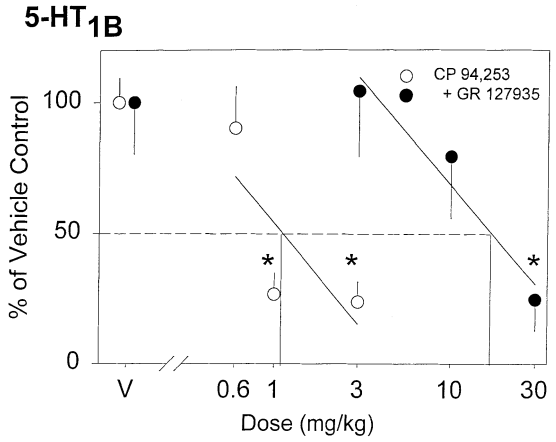

Fig. 3 The effects of 5-HT_{1B} agonist CP-94,253 on the ultrasonic vocalizations of mouse pups, expressed as percent change from vehicle. The data are presented as mean frequency±1 SE (*vertical lines*) and are fit with regression lines to determine the ED_{50}. (≥12 mice/group.) *Open circles* represent data after administration of CP-94,253. *Filled circles* represent data for the agonist following pretreatment with GR 127935 (0.1 mg/kg) 30 min before administration of the agonist. *Asterisks* indicate significance from vehicle control, $P<0.05$

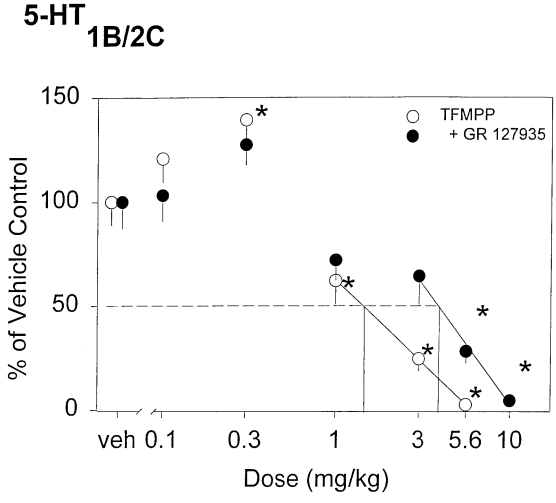

Fig. 4 The effects of 5-$HT_{1B/2C}$ agonist TFMPP on the ultrasonic vocalizations of mouse pups, expressed as percent change from vehicle. The data are presented as mean frequency±1 SE (*vertical lines*). The descending portion of the dose effect curves were fit with regression lines to determine the ED_{50} (≥8 mice/group). *Open circles* represent data after administration of TFMPP. *Filled circles* represent data for the agonist following pretreatment with GR 127935 (0.1 mg/kg) 30 min before administration of the agonist. *Asterisks* indicate significance from vehicle control, $P<0.05$

The doses of 5.6 and 10.0 mg/kg TFMPP reduced the emission of USVs by pups pretreated with GR 127935 [$F(6,91)=17.13$, $P<0.05$ (post-hoc $P<0.05$)]. A separate two-way between subjects ANOVA comparing the 0.1 and 0.3 mg/kg and 1.0 mg/kg doses alone or in the presence of GR 127935 revealed that GR 127935 did not antagonize the effects of these doses [$F(2,76)=0.67$, $P>0.05$].

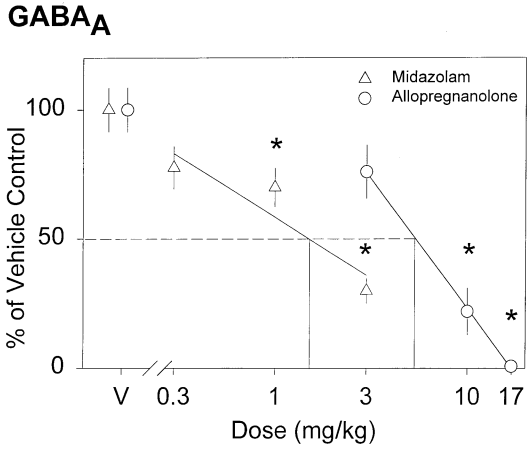

Fig. 5 The effects of the $GABA_A$ positive modulators allopregnanolone and midazolam on the ultrasonic vocalizations of mouse pups, expressed as percent change from vehicle. The data are presented as mean frequency±1 SE (*vertical lines*) and fit with a regression line to calculate the ED_{50}. *Asterisks* indicate significance from vehicle control, $P<0.05$

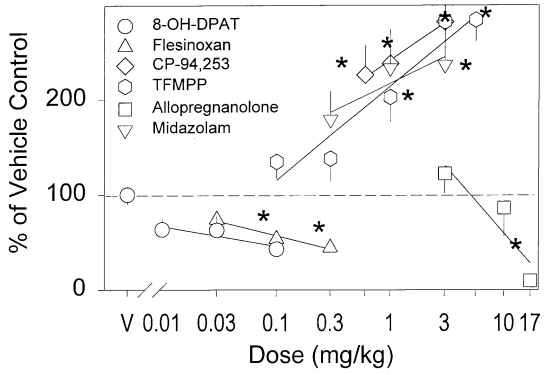

Fig. 6 The effects of 5-HT_{1A}, 5-HT_{1B} agonists and $GABA_A$ positive modulators on the frequency of grid crossings by mouse pups, expressed as percent change from vehicle. The data are presented as mean frequency±1 SE (*vertical lines*) and fit with a regression line to calculate the ED_{50}. *Circles* represent 8-OH-DPAT. *Triangles* represent flesinoxan. *Hexagons* represent TFMPP. *Diamonds* represent CP-94,253. *Squares* represent allopregnanolone. *Upside-down triangles* represent midazolam. *Asterisks* indicate significance from vehicle control, $P<0.05$

$GABA_A$

Midazolam and allopregnanolone reduced the number of vocalizations emitted by pups separated from their dams, [$F(3,50)=15.34$, $P<0.05$; $F(3,61)= 28.73$, $P<0.05$, respectively) (Fig. 5, Table 1). Post-hoc analysis indicated that the 1.0 and 3.0 mg/kg doses of midazolam and the 10.0 and 17.0 mg/kg doses of allopregnanolone significantly decreased these calls ($P<0.05$).

Locomotor behavior

8-OH-DPAT had no significant effect on, but tended to decrease, the number of grid crossings [$F(3,58)=2.23$,

p=0.08 (Fig. 6, Table 1)]. After pretreatment with WAY 100,635, 8-OH-DPAT dose-dependently increased grid crossings, [$F(3,59)=9.87$, $P<0.05$]. Pups treated with all doses of 8-OH-DPAT (3.0, 6.0, and 10.0 mg/kg) crossed significantly more grids than did pups treated with the antagonist alone ($P<0.05$). Flesinoxan reduced the number of grid crossings [$F(3,55)= 13.87$, $P<0.05$ (see Fig. 6, Table 1)], significantly at the 0.1 and 0.3 mg/kg doses ($P<0.05$). When WAY 100,635 was administered prior to flesinoxan, this suppressive effect was blocked [$F(3,68)=2.24$, $P=0.09$ (Table 1)].

In contrast to the $5HT_{1A}$ agonists, CP-94,253 dose-dependently increased the number of grid crossings [$F(3,58)=9.90$, $P<0.05$] with significant effects at the 0.6, 1.0, and 3.0 mg/kg doses, ($P<0.05$) (Fig. 6 and Table 1). TFMPP also increased the number of grid crossings [$F(5,85)=9.67$, $P<0.05$] (see Fig. 6 and Table 1) at the 1.0, 3.0, and 5.6 mg/kg doses ($P<0.05$). The locomotor stimulating effects of CP-94,253 and TFMPP were shifted to the right by pretreatment with GR 127935 (Table 1). In the presence of GR 127935, only the 30.0 mg/kg dose of CP-94,253 [$F(3,40)=12.37$, $P<0.05$; $P<0.05$ for post-hoc test] and 10.0 mg/kg dose of TFMPP [$F(3,57)=8.29$, $P<0.05$; $P<0.05$ for post-hoc test] increased the number of grid crossings above vehicle control values ($P<0.05$).

Allopregnanolone dose-dependently reduced the number of grid crossings, [$F(3,61)=6.94$, $P<0.05$] (Fig. 6, Table 1). Only the highest dose of allopregnanolone (17.0 mg/kg) significantly decreased locomotor behavior ($P<0.05$). Midazolam dose-dependently increased the number of grid crossings, [$F(3,50)=3.44$, $P<0.05$] (Fig. 6, Table 1). The 1.0 and 3.0 mg/kg doses of midazolam significantly increased the number of grid crossings.

Body temperature

None of the drugs exerted significant effects on body temperature (data not shown).

Discussion

Mouse pups, like other myomorph rodents, when separated from their dam and littermates, emit ultrasonic vocalizations. Developmentally, the emission of maternal separation-induced USVs peaks around day 7 of life and declines as the pups grow older and locomotor behavior increases. Treatment of mouse pups with compounds acting on $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, or $GABA_A$ reduces the rate of vocalization. These effects are consistent with previous demonstrations in rat pups (Vivian et al. 1997; Olivier et al. 1998). Action at $5\text{-}HT_{1A}$ or $5\text{-}HT_{1B}$ receptors is indicated by rightward shifts in the dose effect curves of the agonist by pretreatment with receptor-specific antagonist WAY 100,635 or GR 127935, respectively. Midazolam and allopregnanolone were effective at reducing the emission of USVs, confirming the sensitivity of these calls to positive modulators of the $GABA_A$ receptor complex. The current experimental procedure enabled evaluation of potential behavioral and physiological confounds by concurrent measurement of an index of locomotor behavior, grid crossing, as well as effects of these compounds on body temperature.

Maternal separation-induced USVs as a screen for drugs with anti-anxiety potential demands the use of a between subjects experimental design because this measure is highly sensitive to the age of the pups. It is also possible that the pharmacological manipulations may be influencing a developmental process (Whitaker-Azmitia 1991; Whitaker-Azmitia et al. 1995), although this is less likely because the $5\text{-}HT_1$ agonists suppress USVs similarly as they suppress behaviors in other screens for anxiolytic action (Miczek et al. 1995). Hypothermia may represent a confounding variable in the interpretation of USVs (Allin and Banks 1971; Blumberg and Alberts 1990; Sokoloff and Blumberg 1997). However, rodents primarily use ultrasonic vocalizations during thermoregulation in extreme hypothermia (Hofer and Shair 1991, 1993; Hofer et al. 1993). Agents like 8-OH-DPAT typically lower body temperature, and suppress rather than increase USVs (Hard and Engel 1988; Mos and Olivier 1989; Olivier et al. 1998).

There are few reports of pharmacological modulation of maternal separation-induced USVs by mouse pups (Benton and Nastiti 1988; Nastiti et al. 1991a; 1991b). Most experiments have relied on rat pups as experimental subjects. Nastiti et al. (1991b) reported that drugs with non-selective actions at the $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, or $5\text{-}HT_{2A}$ reduced USVs without altering body temperature or locomotor behavior but that the prototypic $5\text{-}HT_{1A}$ agonist 8-OH-DPAT increased vocalization. In the present study, two $5\text{-}HT_{1A}$ agonists, 8-OH-DPAT and flesinoxan, decreased USVs in a dose-dependent, antagonist-reversible manner, which is consistent with findings from rat pups (Mos and Olivier 1989). The discrepancy between the current experiment and Nastiti et al. (1991b) may be due to the use of higher doses of 8-OH-DPAT (0.25 and 0.5 mg/kg) and the lower baseline rates of USVs in the Nastiti et al. (1991b) study. 8-OH-DPAT and flesinoxan were similarly efficacious at reducing USVs and did not significantly differ in their potency. Interestingly, the ED_{50}s for 8-OH-DPAT (0.03 mg/kg) and flesinoxan (0.09 mg/kg) in mouse pups were similar to those reported in rats, 0.04 mg/kg and 0.07 mg/kg (Olivier et al. 1998). The two agonists differed slightly in behavioral specificity. Flesinoxan produced a significant reduction in locomotor behavior, whereas there was only a trend towards significance for pups treated with 8-OH-DPAT. Treatment with the $5\text{-}HT_{1A}$ agonists in the lower dose range did not produce the expected hypothermia, an effect predicted by studies in rats. $5\text{-}HT_{1A}$-induced hypothermia is an effect attributed primarily to presynaptic receptors in both rats and mice (Goodwin et al. 1986; Hillegaart 1991). In the currently used dose range, 8-OH-DPAT might preferentially activate post-

synaptic sites. The lack of hypothermia could also be due to the maintenance of the pups at nest temperature preceding the temperature measurement.

CP-94,253 is currently the most selective ligand to the 5-HT_{1B} receptor in rat cerebral cortex (K_i=2.0±0.4 for inhibition of [^{3}H]5-HT binding and 4.4±0.9 nM for inhibition of [^{125}I]ICP binding versus 89±15 nM for the inhibition of [^{3}H]8-OH-DPAT binding) (Koe et al. 1992). Administration of CP-94,253 reduced USVs and is consistent with findings from other studies using less selective 1B agonists (Mos and Olivier 1989; Olivier et al. 1998). Interestingly, the active dose range for CP-94,253 was remarkably steep, between the doses of 0.6 and 1.0 mg/kg. Studies using this drug to modulate feeding (Lee and Simansky 1997) or aggression (Fish et al. 1999; Sekinda et al. 1999) in adult rodents have yielded a more gradual dose-effect curve for this agonist and may indicate particular sensitivity of anxiety-like behavior to modulation by the 5-HT_{1B} receptors. Surprisingly, the reduction in USVs by CP-94,253 contrasts with the data from mouse pups lacking the 5-HT_{1B} receptor (Brunner et al. 1999). 5-HT_{1B} receptor "knockout" adult mice have been reported to be less anxious, more aggressive, acquire cocaine self-administration faster, and hyperactive when compared to wild type mice (Saudou et al. 1994; Rocha et al. 1998; Brunner et al. 1999).

The mixed 5$HT_{1B/2C}$ receptor agonist TFMPP produced a biphasic effect on mouse pup USVs, confirming similar observations in rat pups (Olivier et al. 1998). Using GR 127935, the 5-HT_{1B} receptor was implicated as the relevant site of action for TFMPP's reduction in vocalizations. GR 127935 did not alter the increase in vocalizations observed at the lower doses of TFMPP, suggesting that the increased rate of calling may be due to action at the 2C receptor. DOI, an agonist with action at the 5-HT_{2C} receptor, has also been shown to increase the rate of vocalization (Nastiti et al. 1991b).

In contrast to the 5-HT_{1A} agonists, CP-94,253 and TFMPP increased the number of grid crossings. Hyperactivity following treatment with 5-HT_{1B} agonists has been repeatedly demonstrated following treatment with CP-94,253 or RU 24969 (Kennett et al. 1987; Callaway and Geyer 1992; Rempel et al. 1993; O'Neill et al. 1997). The striking difference between the 5-HT_{1A} and 5-HT_{1B} receptor agonists on locomotor behavior indicates the functional dissociation of these receptors; while the 5-HT_{1A} and 5-HT_{1B} receptors appear to modulate similarly USVs they differentially affect locomotor behavior.

GR 127935 was originally developed as an antagonist to the 5-$HT_{1B/D}$ receptors (Skingle et al. 1993) but recent evidence suggests that it may be working as a partial agonist (Watson et al. 1996; Pauwels 1997; Parsons et al. 1998). In this study, GR 127935 produced a parallel rightward shift in the 5-HT_{1B} agonist dose-effect curves indicating effective antagonism as has been seen in other behavioral studies (Bell et al. 1995; O'Neill et al. 1996; Fletcher and Korth 1999). Despite the contradictory evidence on the action of GR 127935 at the 5$HT_{1B/D}$ receptors, this apparent antagonism indicates that the USV reducing effects of CP-94,253 and TFMPP are due to their actions at the 5-HT_{1B} receptors.

The primary clinical application of benzodiazepines is in the treatment of anxiety (e.g., Noyes et al 1984). The benzodiazepine midazolam dose-dependently reduced the emission of USVs as has been shown for other benzodiazepines (Nastiti et al 1991a). Interestingly, midazolam increased the number of grid crossings, rather than producing the sedation, as expected in adults. The locomotor behavior of the pups was ataxic and occurred in a burst-like fashion. We are currently observing this same pattern of locomotor behavior with other benzodiazepines (unpublished observations).

Allopregnanolone, a neurosteroid that positively modulates the $GABA_A$ receptor complex, dose-dependently reduced the emission of USVs by mouse pups as has been shown previously in rat pups (Zimmerberg et al. 1994; Vivian et al. 1997). The ED_{50} for allopregnanolone's USV reducing effect was similar in mice and rats, 5.40 mg/kg and 4.55 mg/kg (Vivian et al. 1997), respectively. The absence of adequate antagonists to the neurosteroid recognition site on the $GABA_A$ receptor complex precluded antagonism of the dose-effect curve for allopregnanolone. The benzodiazepine receptor antagonist flumazenil, the GABA antagonist bicuculline, and the convulsant picrotoxin were not capable of significantly altering the dose-effect curve of allopregnanolone in rat pups (Vivian et al. 1997). Flumazenil and bicuculline do not antagonize the effects of compounds active at the neurosteroid site in other experiments performed in vivo or in vitro (Cottrell et al. 1987; Morrow et al. 1990; Britton et al. 1991; Wieland et al. 1991; Reddy and Kulkarni 1998). In addition to its effects on USVs, allopregnanolone at higher doses reduced the number of grid crossings (ED_{50}=10.90 mg/kg), a side effect typical of other positive modulators of the $GABA_A$ receptor (Vanover et al. 1999). Allopregnanolone did not reduce body temperature, an effect consistent with Zimmerberg et al. (1994) using ICV administration but different from Vivian et al. (1997) using SC administration. The similarity between the behavioral effects of $GABA_A$ and 5-HT_1 agonists raises the possibility of mechanistic interactions between these two neurotransmitter and receptor systems (File and Andrews 1991; Andrews and File 1993; Gonzalez et al. 1996; Solderpalm et al. 1997). Investigating the effects of combined manipulations of 5-HT_1 and $GABA_A$ receptors could elucidate whether these systems modulate USVs independently or in tandem.

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