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Rat pup ultrasonic vocalization: effects of benzodiazepine receptor ligands

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Abstract

The involvement of the $GABA_A$-benzodiazepine receptor complex in rat pup ultrasonic vocalisations was studied by testing benzodiazepine receptor ligands with varying intrinsic activity and selectivity for benzodiazepine subtype receptors. Ultrasonic vocalisations were recorded under two temperature conditions (37°C and 18°C), presumably reflecting a low and high stress state. The latency to the negative geotaxis response, a measure of motor coordination and the rectal temperature were determined to assess putative side effects of drugs. The full, non-selective benzodiazepine receptor agonists diazepam, chlordiazepoxide, alprazolam and oxazepam suppressed ultrasonic vocalisations both at 37°C and 18°C conditions, although more efficaciously at 37°C. The partial, non-selective benzodiazepine receptor agonist bretazenil and the partial benzodiazepine$_1$ selective receptor agonist alpidem significantly reduced ultrasonic vocalisations at 37°C, but not at 18°C. The full benzodiazepine$_1$ selective receptor agonist zolpidem behaved like other full, non-selective benzodiazepine receptor agonists by reducing ultrasonic vocalisations under both high and low temperature. The effects of zolpidem indicate that activation of benzodiazepine$_1$ receptors alone already suffices to suppress ultrasonic vocalisations. The non-selective, benzodiazepine receptor antagonist flumazenil and the partial, non-selective benzodiazepine receptor inverse agonist FG 7142 (*N′*-methyl-β-carboline-3-carboxamide) and the full, non-selective benzodiazepine receptor inverse agonist DMCM (6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) had no significant effect on ultrasonic vocalisations under both temperature conditions. The involvement of benzodiazepine receptors in rat pup ultrasonic vocalisations (37°C-condition) was confirmed by antagonism of the ultrasonic vocalisations reducing effects of chlordiazepoxide by flumazenil (1 or 3 mg/kg). Using the rat pup ultrasonic vocalisations paradigm under 18°C and 37°C conditions combined with measurements of negative geotaxis-latencies and rectal temperatures it is possible to (1) distinguish benzodiazepine receptor agonists from other anxiolytics because of dissimilar dose response curves at 37°C and 18°C, (2) differentiate partial from full receptor agonists by absence of effects at the 18°C condition, (3) suggest a key role for benzodiazepine$_1$ receptors in the modulation of ultrasonic vocalisations. These data contribute to the predictive validity of pup vocalizations as an animal model of anxiety.

Keywords: Benzodiazepine receptor; Receptor occupancy; Allosteric modulation; Ultrasonic vocalisation, rat pup; Anxiety; Animal model; Predictive validity

1. Introduction

Rat pups emit ultrasonic vocalisations in the 35–40-kHz range when separated from their mother and littermates. Calling gradually develops a few days after birth, is maintained at high levels until eye-opening at day 14 (Insel et al., 1988) and subsequently disappears around day 18 (Allin and Banks, 1971). Ultrasonic vocalisations evoke searching and retrieving of the pup by its mother (Allin and Banks, 1972; Smotherman et al., 1974) and stimulate prolactin release in the mother (Terkel et al., 1979). Since rat pups this age are blind, deaf, have no fur, are poikilothermic and depend on their mother for excretion processes (Brouette-Lahlou et al., 1992), they are not able to survive if left unattended outside the nest. Ultrasonic vocalisations may therefore serve a vital communicative

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function between the mother and her progeny. Besides by mere separation, calling is enhanced by stressful stimulation like exposure to low temperatures (Allin and Banks, 1971; Okon, 1972), tactile stimulation (Noirot, 1972; Oswalt and Meier, 1975) or unfamiliar olfactory stimuli (Oswalt and Meier, 1975). Stimuli emanating from the mother and/or littermates have a quieting effect (Hofer and Shair, 1980, 1987). On these grounds infant rat ultrasonic vocalizations were hypothesized to reflect a state of distress (see also Insel and Winslow, 1991; Miczek et al., 1991).

Established anxiolytic drugs belonging to different pharmacological classes, in particular the benzodiazepine receptor agonists (Gardner, 1985a,b; Insel et al., 1986; Gardner and Budhram, 1987; Gardner et al., 1987; Vivian et al., 1997), reduced ultrasonic vocalisations. The neurosteroid allopregnanolone, also acting on the $GABA_A$-benzodiazepine receptor complex, was also capable to reduce ultrasonic vocalisations (Vivian et al., 1997). The partial 5-HT_{1A} receptor agonists buspirone, ipsapirone and gepirone (Benton and Nastiti, 1988; Mos and Olivier, 1989; Olivier and Mos, 1989; Kehne et al., 1991; Winslow and Insel, 1991b; Olivier et al., 1998) and the full 5-HT_{1A} receptor agonist 8-OH-DPAT (8-hydroxy-2-(di-*N*-propylamino)tetralin) and flesinoxan (Hård and Engel, 1988; Olivier et al., 1998) also reduced rat pup ultrasonic vocalisations. Furthermore, drugs with a mixed antidepressant/anxiolytic profile like the 5-HT uptake inhibitors clomipramine, fluoxetine, fluvoxamine, citalopram and paroxetine (Mos and Olivier, 1989; Winslow and Insel, 1990; Olivier et al., 1998) as well as the opioid receptor agonist morphine (Gardner, 1985a) and the putative anxiolytic kynurenic acid derivatives (Winslow et al., 1990; Kehne et al., 1991) are active in this paradigm. Some putative anxiogenic drugs, like pentylenetetrazol, lindane or $cholecystokinin_4$ have been shown to enhance calling (Insel et al., 1986; Gardner and Budhram, 1987; Rivera et al., 1992; Rex et al., 1994), whereas a number of other psychotropic agents devoid of anxiolytic properties, such as the neuroleptic haloperidol, the psychostimulant amphetamine or the muscle relaxant mephenesin, were inactive (Gardner, 1985a). Based on these pharmacological arguments isolation-induced ultrasonic vocalizations of rat pups were proposed as a sensitive test for anxiolytic drugs by Gardner (1985a) and Winslow and Insel (1991a).

To extend studies on the role of the $GABA_A$-benzodiazepine receptor complex on rat pup ultrasonic vocalisations, the effects of selected benzodiazepine receptor ligands with varying intrinsic activity at, or selectivity for putative benzodiazepine receptors were studied in this paradigm. The $GABA_A$-benzodiazepine receptor complex constitutes a very remarkable system in the brain, in which GABA-neurotransmission is modulated via diverse mechanisms. The $GABA_A$ receptor is a pentameric ligand-gated ion channel admitting Cl^- into the cell upon binding of GABA. Benzodiazepines, barbiturates, steroids, polyvalent anions, picrotoxin and ethanol act as positive or negative modulators of GABA-receptor function (Rabow et al., 1995; Doble and Martin, 1996). The benzodiazepine receptor displays an intriguing pharmacology. The efficacy spectrum for benzodiazepine-receptor ligands stretches from full receptor agonists (e.g., diazepam) to full inverse receptor agonists (e.g., DMCM) including ligands with all possible degrees of intermediate intrinsic activities (Doble and Martin, 1996). Moreover, receptor multiplicity has been found, presumably reflecting heterogeneity in the subunit composition of the $GABA_A$ receptor. At present, at least two benzodiazepine receptor subtypes have been proposed, $benzodiazepine_1$ (ω_1) and $benzodiazepine_2$ (ω_2) receptors (Sanger et al., 1994).

The present study tries to get insight into the putative role of these different factors by testing full non-selective benzodiazepine receptor agonists (diazepam, chlordiazepoxide, alprazolam and oxazepam), a full $benzodiazepine_1$ receptor agonist (zolpidem), the partial $benzodiazepine_1$ receptor agonist alpidem (Langer et al., 1990; Morton and Lader, 1992), the partial non-selective benzodiazepine receptor agonist bretazenil (Haefely et al., 1990), the non-selective benzodiazepine receptor antagonist flumazenil (Brogden and Goa, 1991), the partial non-selective benzodiazepine receptor inverse agonist FG 7142 (*N′*-methyl-β-carboline-3-carboxamide) (File and Pellow, 1984) and the full non-selective inverse receptor agonist DMCM (6,7-dimethyl-4-ethyl-β-carboline-3-carboxylate) (Petersen and Jensen, 1984) in the ultrasonic vocalization test in rat pups. Two levels of distress were applied: a low-stress situation where the ambient temperature was kept at 37°C, representing isolation distress only, and a more stressful situation with a temperature of 18°C, where additional cold stress was imposed. Moreover, we tried to antagonize the effects of chlordiazepoxide with flumazenil.

In an attempt to assess the possible specificity of drug effects on ultrasonic vocalisations, the latency to the negative geotaxis response, a measure for motor coordination, as well as the rectal temperature were also determined. Although simultaneous effects of drugs on negative geotaxis and body temperature not necessarily means that such effects are responsible for effects on ultrasonic vocalisations, absence of such effects may be supportive for selective anxiolytic effects.

2. Materials and methods

2.1. Animals

Subjects were infant Wistar rats (Harlan/CPB, Zeist, Netherlands) derived from litters bred in our own laboratory. Within 24 h after birth (Day 0) litters were culled to nine regardless of sex. Each litter was housed in a MAK3 cage (RUCA, Valkenswaard, The Netherlands) under normal daylight lighting conditions (lights on at 7:00 a.m.,

lights off at 7:00 p.m.) in the presence of the mother. Food and water were ad lib available. The pups were tested at 9–11 days of age. One group of pups was used for ultrasonic vocalisations testing, another group was used for negative geotaxis and body temperature measurements.

2.2. Apparatus

Ultrasounds were recorded with a Bruel and Kjaer 4135 microphone, connected to a Bruel and Kjaer 2619 preamplifier and a Bruel and Kjaer 2608 amplifier (all Bruel and Kjaer Nederland, Nieuwegein, Netherlands). The signal was filtered using two Interelectronic AF 173 filters (settings: 30 kHz high pass, 60 kHz low pass) before being fed into a custom-made detector (Solvay Pharmaceuticals, Netherlands) with variable high and low trigger levels which transformed the analogue signals into digital block pulses. The block pulses were further processed by a CTM-05 counter-timer card and a custom-made computer program (Solvay Pharmaceuticals, Netherlands).

The low trigger level was set just above the ambient noise level and the high trigger level was set at a fixed distance above the low level. The start of a call was designated when the intensity of a signal surpassed the high level. The end of a call was designated when the signal level subsequently dropped below the low trigger level. The end of a call and the beginning of a new call were registered only if they were separated by a gap ('dead time') of at least 20 ms. In this way frequent short-lasting fluctuations in sound intensity did not generate spurious calls. Onset and end of each call were timed to the nearest ms. Eventually the number and the total duration of ultrasounds produced by each individual animal were obtained.

2.3. Procedure

2.3.1. Ultrasounds

On the experimental day the pups were separated from their mother and transported in their home cage to the experimental room. The cage was placed on a warm plate (37°C) and left undisturbed for at least 15 min. Pups were then weighed, marked and injected intraperitoneally (needle: ϕ 0.5 mm, 16 mm long; Terumo Europe 'orange', Leuven, Belgium) with vehicle or a test dose and put back in the home cage.

Thirty minutes later each pup was placed singly on one of two aluminium plates (ϕ 19 cm), which were kept at a temperature of either 18°C (cold plate) or 37°C (warm plate) by circulating water through a reservoir below the plate, similar to the set-up described by Gardner (1985a,b). A clear plexiglass cylinder (∅ 19 cm, 25 cm high) was placed on the plate and covered by a plexiglass top on which the microphone was mounted. Animals remained on the plate for 5 min, while their vocalizations were registered. At the end of the test pups were returned to their home cage.

2.3.2. Negative geotaxis and body temperature

Similar to the former procedure pups were transported to the experimental room, placed on a warm plate (37°C), left undisturbed for at least 15 min, weighed, marked and injected intraperitoneally with vehicle or a test dose, and put back in the home cage. Thirty minutes later each pup was placed singly head-down on the inclined plane (20° angle, covered with cloth) in order to measure its negative geotaxis response. The median latency to turn around in a head-up position obtained from five consecutive trials, with a cut-off time of 30 s, was taken as an adequate measure of this response. Immediately following the negative geotaxis trials the rectal temperature was measured using a rectal probe and a Keithley 871A digital thermometer. These measurements were performed under room temperature (22°C).

2.4. Drugs

Diazepam base, oxazepam base, chlordiazepoxide base (O.P.G., Netherlands), alprazolam base (Upjohn, USA), alpidem base (Synthélabo Recherche, France), bretazenil base, flumazenil base (Hoffmann La Roche, Basel), *N′*-methyl-β-carbolin-3-carboxamide base (FG 7142), 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate base (DMCM) (RBI, USA) were suspended in a mixture of gelatine (0.5%) and mannitol (5%) in water. Zolpidem 1/2 tartrate (Synthé Labo Recherche, France) was dissolved in water. All solutions/suspensions were adjusted (if necessary) to pH between 5 and 8. Control groups received the appropriate vehicle treatment. All injections were administered intraperitoneally in a volume of 10 ml/kg.

2.5. Allocation of subjects and data analysis

2.5.1. Ultrasounds

Eight pups from one litter were randomly allocated to vehicle and three doses of a drug, according to a 2×4 balanced block design. In alternating order the first or the second block of four pups was tested on the warm plate; the other half of the same nest was tested on the cold plate. This allowed direct comparisons of drug effects in both test conditions. As such, eight litters were used to obtain eight individuals per treatment group for each test condition ($n = 8$). For the interaction study, 2×4 treatments (agonist in four doses, including vehicle, in the absence and presence of single-dose antagonist) were tested on the test condition. Pups within a litter received one of these treatments at random.

Effects of pharmacological treatments on ultrasonic vocalisations were statistically analyzed using on Analysis of Variance (ANOVA) with drug treatment and nest as the explanatory factors, for each test condition. Comparisons between drug-treated groups and control groups used a *t*-test. Drug effects were considered significant when both the ANOVA and the *t*-test revealed a *P*-value less than

0.05. The factor nest usually appeared significant, which is a well-known observation, and which has led us to develop the balanced block allocation of experimental subjects as described above.

To establish whether dose response curves for a given drug were different under warm and cold test-conditions, the interaction drug treatment × test condition was considered. Dose–response curves were normalized by expressing them as percentage of their control response. An ANOVA using multiplicative (instead of additive) factors was used. A significant interaction indicates that the dose–response curves for the two test conditions are disproportional. Furthermore, ED_{50}s for the suppression of the total number of ultrasounds were estimated using the best fitting sigmoidal curves on data sets in which indeed a decrease of ultrasonic vocalisations was observed. The parameter for the slope was kept constant for both test conditions now. Confidence intervals (90%) of the ED_{50} estimates were revealed within the same procedure. The ratio of the ED_{50} value in the warm vs. the cold test condition was calculated and it was tested with a *t*-test whether or not this ratio was equal to 1. Ratios not equal to 1 would indicate a shift of the dose–response curve to the left or the right. With the interaction experiment an additional ANOVA for the interaction agonist × antagonist was performed. In the case of oxazepam and the higher dose range of alprazolam, non-parametric statistics were applied, using the Friedman ANOVA test.

2.5.2. Negative geotaxis and body temperature

For the negative geotaxis and rectal temperature experiments eight pups from one litter were randomly designated to vehicle or three doses of drug, using a 2×4 balanced block design. Thus, four litters were required to form a group size of eight per treatment. Data were analyzed by an ANOVA, with drug treatment and nest as explanatory factors, followed by a *t*-test. $P < 0.05$ (two-tailed) was accepted as statistically significant.

A square root transformation on the ultrasound data was performed to ascertain a normal distribution. Multiple comparisons between drug groups were made by Fisher's protected LSF method.

3. Results

3.1. Baseline responding

During 5 min of isolation, rat pups emitted on average 117 calls (range of group means: 71–320) in the warm (37°C) and 426 (range of group means: 222–784) calls in the cold (18°C) environment. The average rectal temperature of vehicle treated rat pups was 36.8°C (range of group means: 36.1–37.7°C) and the average latency to the negative geotaxis response 7.0 s (range of group means: 5.0–11.3 s).

3.2. Effect of benzodiazepine receptor ligands

Diazepam efficaciously suppressed ultrasonic vocalisations in both test conditions ($F(3,21) = 6.72$, $P < 0.05$ and $F(3,21) = 13.02$, $P < 0.001$ for warm and cold plate re-

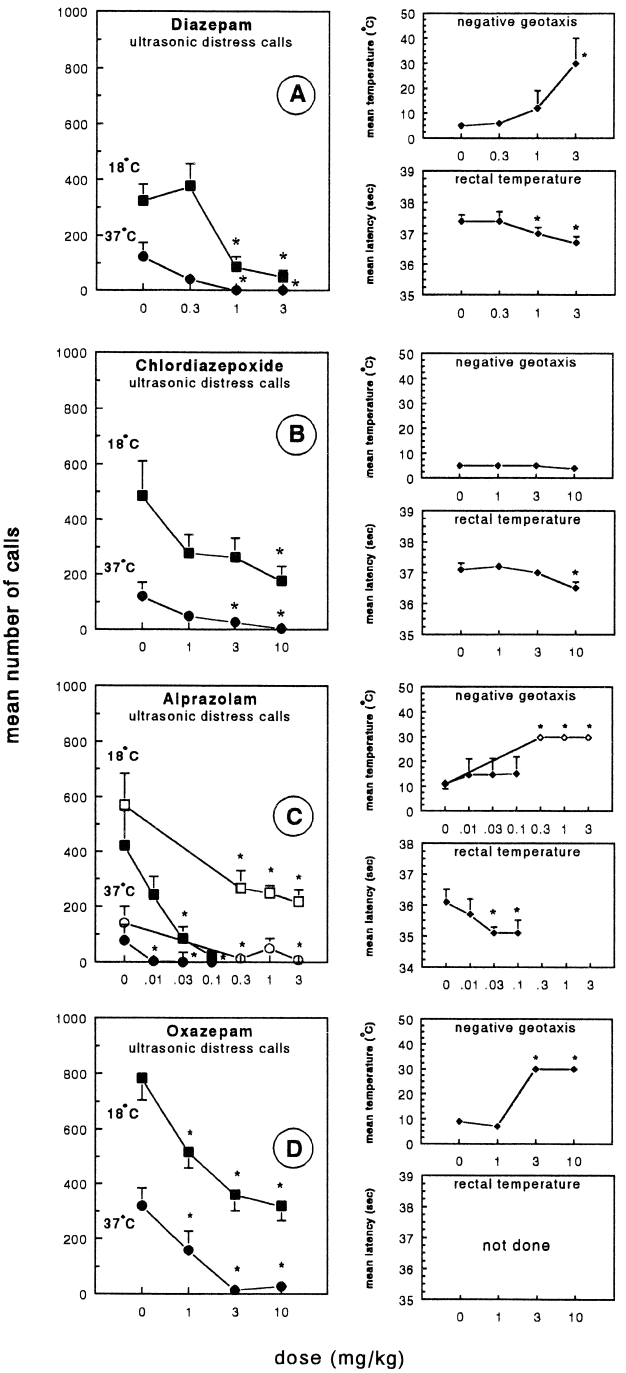

Fig. 1. Effects of the full and non-selective benzodiazepine receptor agonists diazepam (A), chlordiazepoxide (B), alprazolam (C) and oxazepam (D) on rat pup ultrasonic vocalisations in the warm (circles) and cold (squares) test situation (left panel) and on the latency to the negative geotaxis (right panel) and rectal temperature (right, bottom panel). Asterisks indicate significant ($P < 0.05$) effects compared to vehicle treatment. Alprazolam was tested in two dose ranges, 0.01–0.1 mg/kg (filled symbols) and 0.3–3 mg/kg (open symbols).

spectively) (Fig. 1A). On the warm plate ultrasounds were completely abolished. The ED_{50} for ultrasonic vocalisations suppression on the warm plate was 0.2 mg/kg i.p. and on the cold plate 0.6 mg/kg i.p., indicating that diazepam is more potent in the warm test condition (Table 1). The dose response curve on the cold plate is significantly more to the right as compared to the warm plate, but both curves were not significantly disproportional (Table 1). Both ultrasonic vocalisations-active doses (1 and 3 mg/kg i.p.) reduced rectal temperature ($F(3,25) = 5.48$, $P < 0.01$), whereas the latency to the negative geotaxis was only increased at the highest dose ($F(3,25) = 5.58$, $P < 0.01$) (Fig. 1A; right panel).

Chlordiazepoxide reduced ultrasonic vocalisations at 3 and completely suppressed calling at 10 mg/kg i.p. in the warm test situation ($F(3,21) = 5.45$, $P < 0.01$) (Fig. 1B). In the cold situation, ultrasonic vocalisations were reduced after 10 mg/kg i.p. only ($F(3,21) = 4.38$, $P < 0.05$). The ED_{50} values for ultrasonic vocalisations suppression were 0.3 and 3.0 mg/kg i.p. for the warm and the cold plate respectively, revealing a 11-fold potency difference (Table 1). Although this ratio vs. warm plate is higher than with diazepam statistical significant difference between these curves was not obtained due to large confidence intervals. Hypothermia was found at 10 mg/kg i.p. ($F(3,25) = 4.50$, $P < 0.05$), whereas the negative geotaxis response was not affected (Fig. 1B; right panel). Alprazolam was tested in two dose ranges. The first experiment used 0.3–1 mg/kg, the second one 0.01–0.1 mg/kg. Base lines were quite different in both experiments. In the lower dose-range studied alprazolam very potently and efficaciously, reduced ultrasonic vocalisations in the warm and the cold environment ($F(3,21) = 5.04$, $P < 0.01$ and $F(3,21) = 6.11$, $P < 0.001$ for warm and cold plate respectively) (Fig. 1C; closed symbols). The ED_{50}-values were 0.001 and 0.01 mg/kg i.p. for the warm and the cold test condition, respectively, showing alprazolam's 9-fold higher potency on the warm than on the cold plate. This shift in the horizontal direction was not statistically significant, but a significant drug treatment × test condition effect indicated that the dose–response curves on warm and cold plate were not proportional (Table 1). Despite the complete suppression of ultrasonic vocalisations the negative geotaxis response was not disturbed after any of these doses. Hypothermia, however, was found after 0.03 as well as 0.1 mg/kg i.p. ($F(3,25) = 3.81$, $P < 0.01$) (Fig. 1C; right panel). In a second experiment (0.3, 1 and 3 mg/kg) alprazolam also decreased ultrasonic vocalisations on the warm ($X^2 = 6.08$, $df = 3$, $P < 0.05$) and the cold plate ($X^2 = 6.95$, $df = 3$, $P < 0.05$). The effect on the cold plate however, was far less efficacious than in the lower dose experiment again confirming inequal effects under both conditions. The negative geotaxis in the high-dose range was severely affected ($X^2 = 19.43$; $df = 3$, $P < 0.0002$). In this experiment no temperature measurements were done.

Oxazepam reduced ultrasonic vocalisations on the warm and cold plate ($X^2 = 17.55$; $df = 3$, $P = 0.0005$ and $X^2 = 17.70$, $df = 3$; $P = 0.0005$ for warm and cold plate respectively (Fig. 1D; left panel). The negative geotaxis (Fig. 1D; right panel) was significantly affected ($X^2 = 11.70$; $df = 3$, $P < 0.009$). In this experiment temperature was not measured.

Bretazenil reduced rat pup ultrasounds in the warm environment almost completely ($F(3,21) = 5.12$, $P < 0.01$) (Fig. 2A; $ED_{50} = 0.2$ mg/kg i.p., Table 1), whereas no effects were found in ultrasonic vocalisations on the cold plate, the negative geotaxis or the rectal temperature (Fig. 2B; right panel). As such bretazenil was at least 50-fold more potent on the 37°C than on the 18°C plate. Because this difference was so extreme, the dose range tested was not wide enough to produce estimate curves with acceptable confidence intervals.

Alpidem produced a significant reduction of ultrasonic vocalisations at 10 mg/kg i.p. on the warm plate only ($F(3,21) = 4.09$, $P < 0.05$) (Fig. 2B; left panel), with an

Table 1
ED_{50} values and 90% confidence intervals (mg/kg i.p.) for the suppression of ultrasonic vocalisations in the warm and cold test condition

Drug	Warm test condition		Cold test condition		Ratio ED_{50} cold/warm
	ED_{50}	90% confidence interval	ED_{50}	90% confidence interval	
Diazepam	0.2	0.006–0.5	0.6	0.4–1.1	3.8[a]
Chlordiazepoxide	0.3	0.01–6.0	3.0	0.7–13	11
Alprazolam	0.001	0–0.016	0.01	0.001–0.02	8.7[b]
Oxazepam	1.8	0.3–10	2.6	0.5–10	1.5
Bretazenil	0.2	0.005–7.5	> 10		> 50
Alpidem	2.5	1.0–6.6	> 10		> 4
Zolpidem	0.3	0.1–0.5	0.7	0.4–1.1	2.6[a]
Flumazenil	> 10		> 10		
FG 7142	> 10		> 10		
DMCM	> 0.3		> 0.3		

[a] Significant ($P < 0.05$) difference between ED_{50}'s on warm and cold plate.
[b] Significant ($P < 0.05$) interaction 'drug treatment × test condition'.
Ratio ED_{50}-cold/ED_{50}-warm based on non-rounded data.

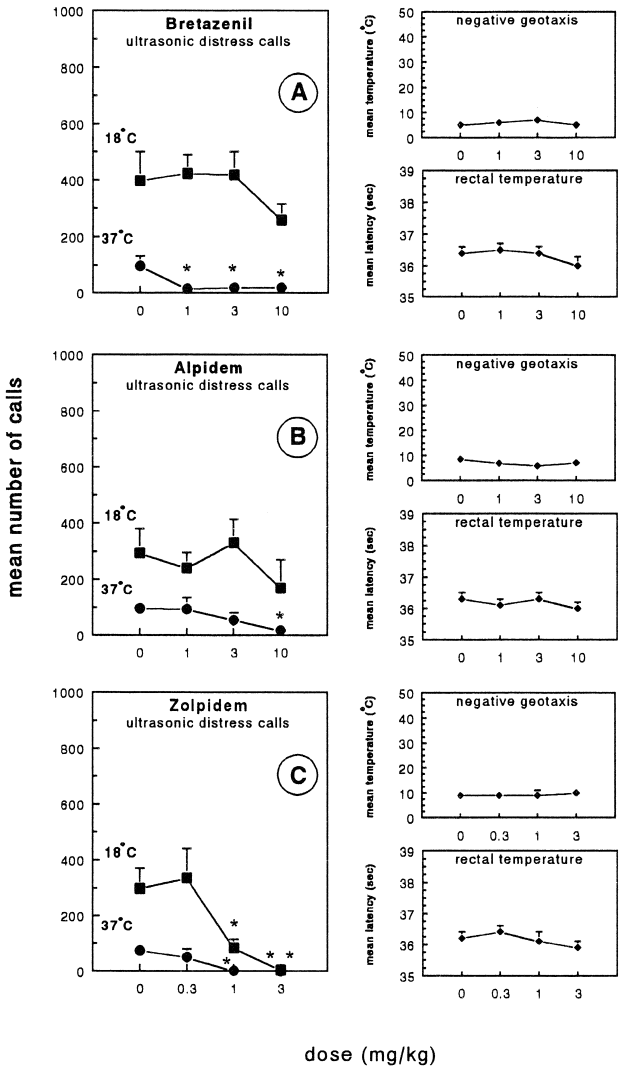

Fig. 2. Effects of partial and subtype selective benzodiazepine receptor agonists bretazenil (A), alpidem (B) and zolpidem (C) on rat pup ultrasonic vocalisations in the warm (circles) and cold (squares) test situation (left panel) and on the latency to the negative geotaxis (right top panel) and rectal temperature (right bottom panel). Asterisks indicate significant ($P < 0.05$) effects compared to vehicle treatment.

ED_{50} of 2.5 mg/kg i.p.. Therefore, alpidem is at least a factor 4 more potent on the warm plate than on the cold plate. Alpidem did neither affect negative geotaxis nor rectal temperature (Fig. 2B; right panel).

Zolpidem reduced ultrasonic vocalisations under both temperature conditions ($F(3,21) = 12.37$, $P < 0.001$ and $F(3,21) = 15.89$, $P < 0.01$ for warm and cold plate, respectively), without affecting the negative geotaxis latency or the rectal temperature (Fig. 2C). The ED_{50}-values for suppression of ultrasonic vocalisations were 0.3 and 0.7 mg/kg i.p. on the warm and the cold plate respectively. Zolpidem was about 2.5 times more potent on the warm than on the cold plate and this difference was significant (Table 1).

Flumazenil and FG 7142, both given at 1, 3 and 10 mg/kg i.p. to rat pups, had no significant effects on any of the variables measured (Fig. 3A and B, and Table 1).

DMCM had no significant effect on ultrasounds when applied at 0.03, 0.1 and 0.3 mg/kg i.p. (Fig. 3C). Despite the lack of effects on ultrasonic vocalisations, the negative geotaxis response was completely disrupted at 0.3 mg/kg i.p. ($F(3,25) = 28.66$, $P < 0.001$), as is shown by the maximal latencies (Fig. 3C; right panel). Also a strong hypothermia was measured at this dose ($F(3,25) = 4.19$, $P < 0.05$) (Fig. 3C; right panel).

In Fig. 4 the effects of a selected dose of flumazenil (1 or 3 mg/kg i.p.) on the suppressive effects of a dose-range

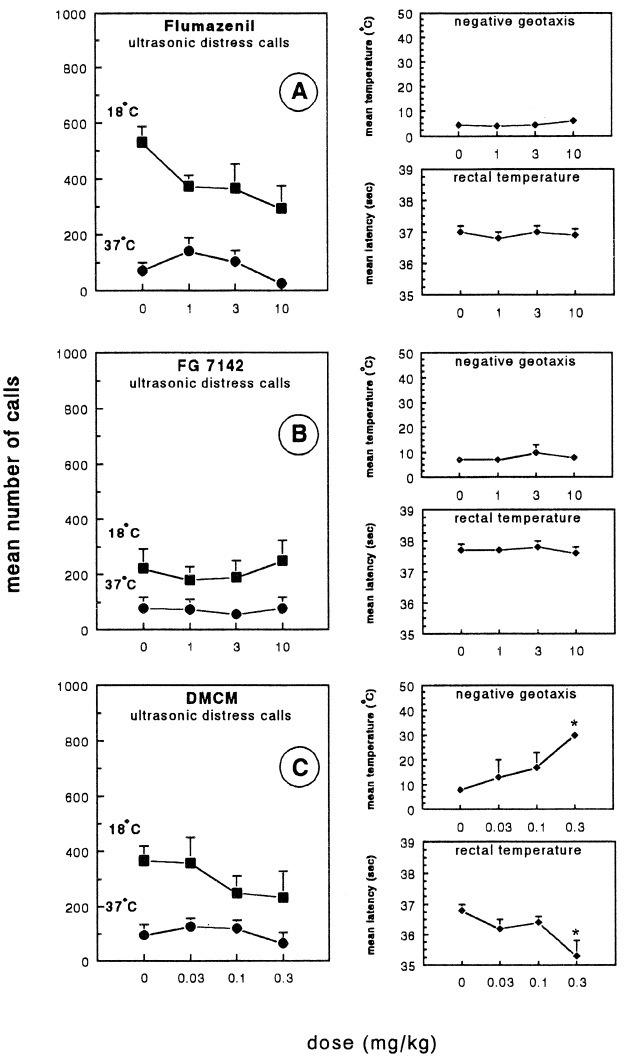

Fig. 3. Effect of the benzodiazepine receptor antagonist flumazenil (A) and two inverse benzodiazepine receptor agonists FG7142 (B) and DMCM (C) on rat pup ultrasonic vocalisations in the warm (circles) and cold (squares) test situation (left panel) and on the latency to the negative geotaxis (right top panel) and rectal temperature (right bottom panel). Asterisks indicate significant ($P < 0.05$) effects compared to vehicle treatment.

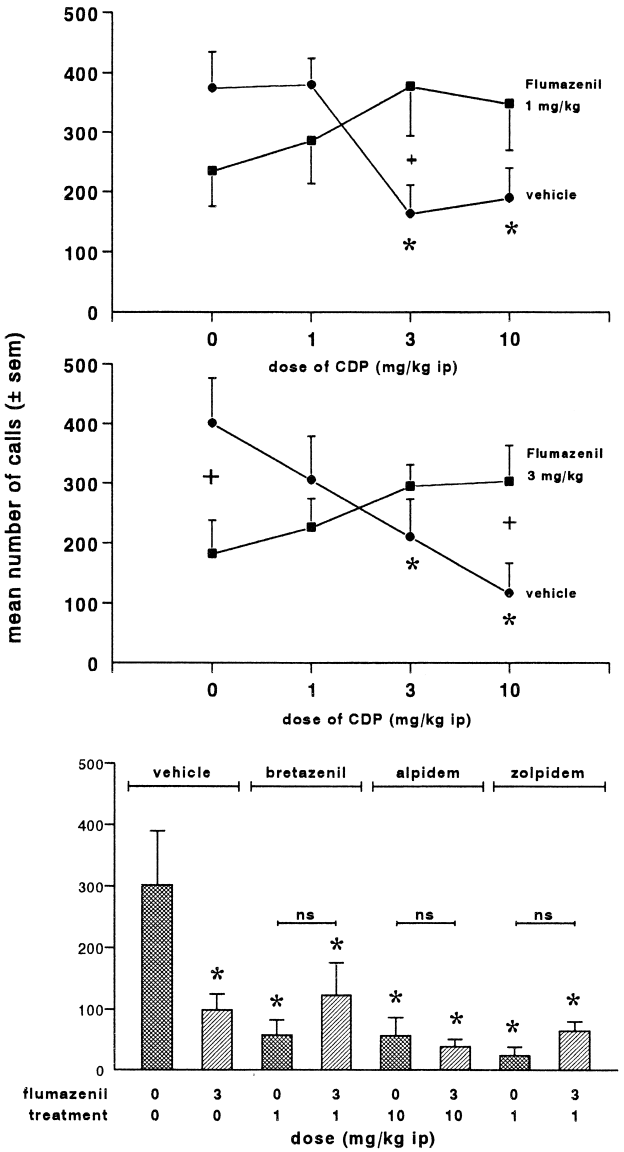

Fig. 4. Effect of chlordiazepoxide (0, 1, 3 and 10 mg/kg i.p.) with vehicle pretreatment (solid line with circles) or with (dotted line with triangles) the benzodiazepine receptor antagonist flumazenil (1 mg/kg i.p., top panel) or with 3 mg/kg, i.p. (bottom panel) on rat pup ultrasonic vocalisations in the warm environment. Asterisks indicate significant ($P < 0.05$) effects compared to the vehicle–vehicle (0+0) treatment. (+) reflects a significant difference ($P < 0.05$) between vehicle and flumazenil pretreated groups at similar chlordiazepoxide doses.

of chlordiazepoxide (1, 3 and 10 mg/kg i.p.) at 37°C is shown. Flumazenil (1 mg/kg i.p.) alone not significantly influenced ultrasonic vocalisations, but significantly antagonized the effects of chlordiazepoxide at 3 and 10 mg/kg i.p. (CDP × flumazenil: $F(3,49) = 3.64$; $P < 0.02$). Flumazenil 3 mg/kg i.p. (Fig. 4; bottom panel) had a significant effect itself on ultrasonic vocalisations ($P = 0.02$), but was also able to antagonize the CDP-induced suppression of ultrasonic vocalisations ($F(3,49) = 3.68$; $P < 0.02$).

4. Discussion

Ultrasonic vocalizations were elicited in rat pups between the postnatal age of 9 and 11 days in response to separation from mother and littermates. Additional cold stress (18°C) further increased the number of ultrasounds, suggesting that the 18°C condition represents a higher level of distress and confirming the results of Allin and Banks (1971) and Okon (1972).

The clinically effective anxiolytics and full, non-selective benzodiazepine receptor agonists diazepam, chlordiazepoxide, alprazolam (Chouinard et al., 1982) and oxazepam reduced rat pup ultrasonic vocalisations both on the warm and the cold plate, in line with earlier studies using full benzodiazepine receptor agonists, including, e.g., diazepam, chlordiazepoxide, clobazepam, alprazolam, premazepam and quazepam (Gardner, 1985a,b; Engel and Hård, 1987; Gardner and Budhram, 1987; Insel et al., 1986, 1988, 1989; Benton and Nastiti, 1988; Mos and Olivier, 1988; Hård and Engel, 1991; Insel and Winslow, 1991; Kehne et al., 1991; Vivian et al., 1997). In contrast to these studies, which measured ultrasonic vocalization under more limited, less well controlled or defined conditions, our experiments were specifically designed to measure ultrasonic vocalisations under two stress conditions. In our experiments on the warm plate, a total suppression of calling was induced by diazepam, chlordiazepoxide, alprazolam and oxazepam. Alprazolam was by far the most potent drug, followed by diazepam, oxazepam and chlordiazepoxide confirming similar findings by Vivian et al. (1997). These drugs were more potent to suppress ultrasonic vocalisations on the warm than on the cold plate. Moreover, the receptor agonists used were able to completely suppress ultrasonic vocalisations at 37°C, but not completely at 18°C. Alprazolam appears somewhat contradictory, in that it almost completely reduces ultrasonic vocalisations at the cold plate at the 0.1 mg/kg dose, whereas in higher doses a limited reduction is obtained, more similar to the other benzodiazepine-receptor agonists. Whether this has to do with different baseline levels is uncertain, although lower starting baseline levels seem to be more sensitive to decreasing effects than initial higher ones (cf. diazepam vs. oxazepam). Moreover, rate-dependency could play a role in which a greater response is more difficult to antagonise, irrespective of whether this greater response is associated with a state of greater stress. Although higher doses would be needed to prove this, it may be possible that full, non-selective benzodiazepine receptor agonists are simply not able to fully suppress ultrasonic vocalisations under high stress conditions. This is in contrast to the effects of 5-HT_{1A} anxiolytics that are able to completely suppress ultrasonic vocalisations at the cold plate. Alternatively, higher doses of full benzodiazepine receptor agonists may be necessary to reach anxiolytic effects under high stress conditions. This may parallel clinical observations that higher doses of full and high

efficacy benzodiazepine receptor agonists are necessary to reach a therapeutic effect in panic disorder, whereas in a less intense form, generalised anxiety disorder, lower doses and less efficacious benzodiazepine are also active (McNair and Kahn, 1981; Ballenger, 1993). Except chlordiazepoxide, the other benzodiazepine receptor agonists impaired the negative geotaxis response at higher doses. Benzodiazepine use is commonly associated with sedation, rather than stimulation, although stimulation of aggressive behaviour has been observed at lower doses (Mos and Olivier, 1987). It could be suggested that the ultrasonic vocalisations-suppressing effects of diazepam, chlordiazepoxide, alprazolam and oxazepam are relatively specific, in that they are not accompanied by severe side effects like sedation or motor impairment as measured by the negative geotaxis. The hypothermic effects of benzodiazepine are a well-known phenomenon which does not interfere, at least at a clinical level, with their anxiolytic profile. The similarity in hypothermia induced by benzodiazepine in pups and adults points to the functional integrity of the benzodiazepine receptor system in pups.

The partial, non-selective benzodiazepine receptor agonist bretazenil (Haefely et al., 1990) produced a distinct anxiolytic profile in that it quite potently and efficaciously reduced ultrasonic vocalisations on the warm plate, while having no effect at all at the doses chosen on the cold plate. The difference in dose response curve between the warm and cold plate is much larger here than observed after full, non-selective benzodiazepine receptor agonists. This may be attributed to the partial agonistic character of bretazenil at benzodiazepine receptors. Partial benzodiazepine receptor agonists have limited intrinsic activity at benzodiazepine receptors and thus produce pharmacological effects associated with low doses of full benzodiazepine receptor agonists without producing effects associated with high doses of full benzodiazepine receptor agonists (Haefely et al., 1990; Gardner et al., 1993). The relatively low intrinsic activity of bretazenil at benzodiazepine receptors may therefore not be sufficient to exert anxiolytic activity under more stressful conditions. In line with this, bretazenil did not suppress conditioned ultrasonic vocalizations in adult rats, a putative model for panic disorder, an intense form of anxiety (Molewijk et al., 1995), whereas bretazenil is active in animal models of anxiety related to milder forms of anxiety, like conflict procedures (Martin et al., 1988). This hypothesis should be further explored using different partial non-selective benzodiazepine receptor agonists. Bretazenil had no effect on the negative geotaxis latency or rectal temperature, in line with the partial agonistic character of the compound.

The relatively selective partial benzodiazepine$_1$ receptor agonist alpidem, which shows anxiolytic activity in some animal models, such as marble burying, punished drinking (Vogel test), feeding under stress (Zivkovic et al., 1990), and the elevated plus maze test (Sanger et al., 1994), produced a significant although not complete suppression of ultrasonic vocalisations only on the warm plate. Similar to the inactivity of alpidem in shock-induced fighting, shock-induced suppression of exploration (four-plates test) or punished operant responding for food (Geller–Seifter) (Zivkovic et al., 1990), ultrasonic vocalisations were not reduced in the more stressful condition (18°C) up to 10 mg/kg i.p.. Whether higher doses would have produced ultrasonic vocalisations-inhibition also on the cold plate remains to be established. Like bretazenil, the relatively low intrinsic activity of alpidem may be responsible for its present inactivity in high stress procedures. Since alpidem is a relatively selective benzodiazepine$_1$ (ω_1) receptor agonist (compared to benzodiazepine$_2$) it seems feasible that the anxiolytic profile is due to the selective activation of benzodiazepine$_1$ receptors. The anxiolytic effect of alpidem has a relatively high specificity, in that no disruption was found in the negative geotaxis response and body temperature.

The full benzodiazepine$_1$ receptor agonist and hypnotic zolpidem (Sanger et al., 1987; Langtry and Benfield, 1990) reduced ultrasonic vocalisations significantly more potent (about 2.5-fold) in the warm than in the cold test. Remarkably, zolpidem was able to fully (100%) inhibit ultrasonic vocalisations under both stress conditions. Zolpidem had neither any effect on the negative geotaxis response nor on body temperature. The anxiolytic effect of zolpidem therefore seems quite specific, which does not seem in agreement with its reported hypnotic properties (Langtry and Benfield, 1990).

Since zolpidem is selective for the benzodiazepine$_1$ (or ω_1) receptor subtype and is able to fully suppress ultrasonic vocalisations under both conditions, it could be suggested that this particular subtype may play a key role in the regulation of ultrasonic vocalisations by benzodiazepine receptor ligands. The hypothesis that in particular the benzodiazepine$_1$ receptor is modulating anxiety has been proposed before (see Sanger et al., 1994), but is not undisputed and still has to be established more definitively.

In our dose–response study, the non-selective benzodiazepine receptor antagonist flumazenil (1, 3 and 10 mg/kg i.p.) had no effects in the ultrasonic vocalisations paradigm. Moreover, in antagonism studies flumazenil (1 or 3 mg/kg) was able to antagonize the effects of chlordiazepoxide on ultrasonic vocalisations, thereby showing the involvement of benzodiazepine receptors. However, in our present studies, the dose of 1 mg/kg, but certainly the 3 mg/kg dose of flumazenil exerted intrinsic activity, reducing ultrasonic vocalisations by approx. 40–60%. This suggests that flumazenil exerts partial benzodiazepine receptor agonistic properties. A lack of effects of flumazenil on rat pup ultrasonic vocalisations was found earlier by Gardner and Budhram (1987), Benton and Nastiti (1988) and Vivian et al. (1997). However, in other studies, flumazenil reduced rat pup ultrasonic vocalisations (Insel et al., 1986; Engel and Hård, 1987; Cagiano et al., 1993), again suggesting partial benzodiazepine receptor agonistic properties. Alter-

natively, the release of an endogenous benzodiazepine receptor ligand with anxiogenic properties by isolation distress, which would in turn be displaced by flumazenil (Insel et al., 1986), has been suggested. This hypothesis was supported by a reduction of in vivo [^{3}H]flumazenil binding in the brain of rat pups that were isolated for 25 min (Insel et al., 1989). If this hypothesis is correct, one could predict that this endogenous anxiogenic ligand is released more under the higher stress conditions and consequently that flumazenil would show higher intrinsic activity at the 18°C than at the 37°C condition. This cannot really be proven, but the antagonist experiments, performed under the less stressful conditions are not supportive. Further explicit experiments to unravel this hypothesis are needed.

In humans, flumazenil has been characterized as anticonvulsive (Scollo-Lavizzari, 1988), inactive (Darragh et al., 1983) or as panic provocative (Nutt et al., 1990). It has been hypothesized that the effect of flumazenil depends on basal conditions, test, dosage (Brogden and Goa, 1991) or on the status ('set point') of the benzodiazepine receptor system (Nutt et al., 1990). Alternative to the anxiogenic endogenous benzodiazepine receptor ligand-hypothesis, a modifiable set-point of the receptor efficacy spectrum has been proposed (Nutt et al., 1992). Under this hypothesis, it is suggested that environmental factors, previous drug treatment and stress may produce shifts in the receptor set-point such that acute challenge with flumazenil might reveal partial agonistic (or partial inverse agonistic) properties.

The inverse and non-selective benzodiazepine receptor agonists and putative anxiogenic (weak partial inverse receptor agonist) drugs FG 7142 (File and Pellow, 1984) and (full inverse receptor agonist) DMCM (Petersen and Jensen, 1984) did not increase the number of ultrasounds, not even in the warm environment where there is relatively low baseline ultrasonic vocalisations responding. FG 7142 was also devoid of effects on other variables in this set-up. Higher doses of FG 7142 should probably be tried in subsequent experiments. DMCM on the other hand, had profound side effects in absence of effects on ultrasounds. The negative geotaxis totally disappeared at the highest dose concomitant with strong hypothermia. Apparently, the full inverse receptor agonistic effect of DMCM may lead to side effects, which are not induced by the partial inverse receptor agonistic effects of FG 7142.

This paradigm apparently is not suited to detect putative anxiogenic or anxiolytic qualities of inverse benzodiazepine receptor agonists. Also in other studies, FG 7142 (Insel et al., 1986; Gardner and Budhram, 1987) and DMCM (Gardner and Budhram, 1987) only produced inconsistent and often non-significant increases in rat pup ultrasonic vocalisations. Benton and Nastiti (1988) however, showed anxiogenic activity of various benzodiazepine inverse receptor agonists, including CGS 8216, FG 7142, DMCM and β-CCM (methyl-β-carboline-3-carboxylate), using a warm test condition (37°C) to obtain low levels of calling in mice pups. The maximal increase in calling (DMCM > β-CCM > FG7142 > CGS8216) correlates with the intrinsic activity of these ligands at the benzodiazepine receptor (Doble and Martin, 1996). The lack of stimulation by FG7142 or DMCM in our experiments can most probably not be attributed to ceiling-ultrasonic vocalisations levels, not even on the cold plate, since stimulations have been recorded for instance after administration of clonidine (Hård et al., 1988; Kehoe and Harris, 1989; Van der Poel et al., 1991), ketanserine (Olivier et al., 1998), ritanserine (Winslow and Insel, 1991b; Van der Poel et al., 1992) or noradrenaline uptake inhibitors (Winslow and Insel, 1990; Van der Poel et al., 1992). According to the set-point shift theory of the benzodiazepine-receptor it may be speculated that in rat pups, compared to mice, the set-point has shifted to an agonistic state, so that weak partial inverse receptor agonists behave like 'silent' receptor antagonists, whereas full inverse agonists would behave like partial inverse receptor agonists. The lack of effect of DMCM on ultrasonic vocalisations calling could then be due to the inability to activate the benzodiazepine-receptor sufficiently, due to the severe side effects at higher doses (convulsions, death).

This study tried to assess the specificity of anxiolytic effects, by measuring some additional motoric and physiologic variables. Full receptor agonists which activate all subtypes of benzodiazepine receptors (diazepam, chlordiazepoxide, alprazolam, oxazepam) also affected body temperature and negative geotaxis (with the exception of chlordiazepoxide). Only the non-specific full benzodiazepine receptor agonists decreased body temperature, but not the partial receptor agonists, neither the full benzodiazepine$_1$ selective receptor agonist zolpidem. This suggests that the hypothermic effects of the non-selective full benzodiazepine receptor agonists are caused by activation of non-benzodiazepine$_1$ benzodiazepine receptors. This hypothesis needs verification. The negative geotaxis was thought of as reflecting motoric/sensoric capabilities of rat pups, ideally suited to measure side effects of drugs. However, the data obtained, especially on the hypnotic zolpidem cast doubt on the usefulness of negative geotaxis to measure ultrasonic vocalisations-interfering side effects. Far more studies are needed to evaluate the further practical meaning of negative geotaxis.

Whether or not benzodiazepine-induced anxiolysis, both in animals and in humans, depends on global dampening of behaviour is not clear. As for the rat pup ultrasonic vocalisations paradigm, the results with DMCM show that impairment of motor responses (negative geotaxis) per sé does not lead to changes in ultrasounds. Apparently, pups can vocalize despite severe motor incoordination. Therefore, a disruption of the negative geotaxis does not necessarily imply ultrasonic vocalisations reduction. Furthermore, it may be questioned whether hypothermia induced ultrasonic vocalisations suppression by itself, although sev-

eral ultrasonic vocalisations-reductions have been found after drugs without hypothermia (e.g., bretazenil, alpidem, zolpidem). In literature however, cold stress is associated with increases in ultrasonic vocalisations rather than decreases (Allin and Banks, 1971; Okon, 1972; Blumberg and Alberts, 1990). Isolation-induced ultrasonic vocalisations in rat pups may therefore vary relatively independent of the additional behavioural and physiological variables, and may probably represent a sensitive measure for anxiety.

Ethological and pharmacological arguments also suggest that rat pup ultrasonic vocalisations can be used as model to identify anxiolytic drugs. Moreover, it can be argued that separation-induced ultrasonic vocalisations has face validity towards separation-induced anxiety in children. Interestingly, in panic disorder patients there is evidence of reduced benzodiazepine receptor binding as measured by iomazenil SPECT (Kaschka et al., 1995), which may be congruous to the reduction in flumazenil binding found after social isolation of rat pups (Insel et al., 1989).

However, pharmacological experiments have also indicated that there are distinct exceptions to rat pup ultrasonic vocalisations representing an anxiety model. Whereas benzodiazepine- and 5-HT_{1A} receptor agonists and 5-HT uptake inhibitors reduce ultrasonic vocalisations and have clinical anxiolytic/antipanic effects, some compounds decrease ultrasonic vocalisations (e.g., m-CPP (metachlorophenyl-piperazine): Winslow and Insel, 1991b) while having anxiogenic properties in humans (Kahn et al., 1988), and reversely, some compounds enhance ultrasonic vocalisations (e.g., clonidine: Kehoe and Harris, 1989; Van der Poel et al., 1991) while having clinical anxiolytic properties (Hoehn-Saric et al., 1981). It thus seems that although benzodiazepine and 5-HT_{1A} receptors and 5-HT uptake sites in these immature rats are already operational (Mallorga et al., 1980), this may not be true for other receptors, like α_2-adrenoceptors (Kehoe and Harris, 1989), 5-HT_{1B} and 5-$HT_{2A/2C}$ (Winslow and Insel, 1991b; Olivier et al., 1998) receptors. Regarding the α_2-adrenoceptor, the work of Kehoe and Harris (1989) has demonstrated that these increases in ultrasonic vocalisations are only found until postnatal day 18, thereafter clonidine reduced ultrasonic vocalisations. Moreover, the anxiogenic benzodiazepine inverse receptor agonists do not increase ultrasonic vocalisations or do so inconsistently (Insel et al., 1986; Gardner and Budhram, 1987; this study). When using rat pup ultrasonic vocalisations as a screening method for anxiolytic drugs, these limitations should be taken into account.

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