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ORIGINAL INVESTIGATION

Glutamatergic and GABAergic modulations of ultrasonic vocalizations during maternal separation distress in mouse pups

Aki Takahashi · Jasmine J. Yap · Dawnya Zitzman Bohager · Sara Faccidomo · Terry Clayton · James M. Cook · Klaus A. Miczek

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Abstract
Introduction Dysregulation of GABAergic inhibition and glutamatergic excitation has been implicated in exaggerated anxiety. Mouse pups emit distress-like ultrasonic vocalizations (USVs) when they are separated from their dam/siblings, and this behavior is reduced by benzodiazepines (BZs) which modulate GABAergic inhibition. The roles of glutamate receptors on USVs remain to be investigated.
Materials and methods We examined the roles of glutamate receptor subtypes on mouse pup USVs using *N*-methyl-D-aspartate (NMDA) receptor antagonists with different affinities [dizocilpine (MK-801), memantine, and neramexane] and group II metabotropic glutamate receptor agonist (LY-379268) and antagonist (LY-341495). These effects were compared with classic BZs: flunitrazepam, bromazepam, and chlordiazepoxide. To assess the role of $GABA_A$ receptor subunits on USVs, drugs that have preferential actions at different $GABA_A$-α subunits (L-838417 and QH-ii-066) were tested. Seven-day-old CFW mouse pups were separated from their dam and littermates and placed individually on a 19°C test platform for 4 min. Grid crossings and body rolls were measured in addition to USVs.
Results Dizocilpine dose-dependently reduced USVs, whereas memantine and neramexane showed biphasic effects and enhanced USVs at low to moderate doses. The NMDA receptor antagonists increased locomotion. LY-379268 reduced USVs but also suppressed locomotion. All BZs reduced USVs and increased motor incoordination. Neither L-838417 nor QH-ii-066 changed USVs, but both induced motor incoordination.
Conclusion Low-affinity NMDA receptor antagonists, but not the high-affinity antagonist, enhanced mouse pup distress calls, which may be reflective of an anxiety-like state. BZs reduced USVs but also induced motor incoordination, possibly mediated by the α5 subunit containing $GABA_A$ receptors.

Keywords Anxiety · Ultrasonic vocalizations · Mouse pups · NMDA receptor · mGluR2/3 · $GABA_A$ receptor subtypes · Motor activity · Motor incoordination

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A. Takahashi · J. J. Yap · D. Z. Bohager · S. Faccidomo · K. A. Miczek (✉)
Department of Psychology, Tufts University,
530 Boston Ave. (Bacon Hall),
Medford, MA 02155, USA
e-mail: klaus.miczek@tufts.edu

K. A. Miczek
Departments of Psychiatry, Pharmacology and Neuroscience,
Tufts University,
Medford, MA, USA

T. Clayton · J. M. Cook
Department of Chemistry and Biochemistry,
University of Wisconsin–Milwaukee,
Milwaukee, WI, USA

Present address:
D. Z. Bohager · S. Faccidomo
Bowles Center for Alcohol Studies,
University of North Carolina at Chapel Hill,
Chapel Hill, NC, USA

Introduction

Rodent pups that are separated from their dam and/or siblings emit distress calls (ultrasonic vocalizations, USVs) that prompt retrieval by their dam (Noirot 1972; Brunelli et al. 1994). This behavior may represent an expression of affect by the pup, and separation-induced USVs have been

used as one of the ethologically validated measures for preclinical characterization of anxiolytic drugs (Brunelli et al. 1994; Gardner 1985; Miczek et al. 1995, 2008; Noirot 1972; Sales and Pye 1974). One of the proposed mechanisms of exaggerated anxiety may be attributed to an imbalance between GABAergic inhibition and glutamatergic excitation in limbic areas (Herman et al. 2004; Rainnie et al. 1991; Sajdyk and Shekhar 1997; Smith and Dudek 1996). It has been shown that drugs which target those pathways modulate pup USVs.

Benzodiazepines (BZs) allosterically modulate gamma-aminobutyric acid A ($GABA_A$) receptors, and they have been widely used for the treatment of general anxiety disorders (Shader and Greenblatt 1993). Reduced benzodiazepine receptor binding in some forebrain areas has been observed in patients of panic disorder, posttraumatic stress disorder, and general anxiety disorder (Bremner et al. 2000a, b; Malizia et al. 1998; Roy-Byrne et al. 1996; Tiihonen et al. 1997), and gene-targeted mice that had disruption of the GABAergic pathway changed their expression of anxiety-like behavior (Crestani et al. 1999; Stork et al. 2000; Liu et al. 2007). BZs and other positive modulators of $GABA_A$ receptors inhibit distress-like calls in several animal species, and these calls are enhanced by inverse agonists (Fish et al. 2000; Insel et al. 1986; Miczek et al. 1995; Nastiti et al. 1991; Olivier et al. 1994, 1998a; Rowlett et al. 2001; Vivian et al. 1997; Watson et al. 1999).

The anxiolytic effects of BZs depend on the compositions of $GABA_A$ receptor subunits. Zolpidem, an agonist with preferential action at $GABA_A$ receptors containing the $\alpha 1$ receptor subunits, suppressed USVs in mouse and rat pups (Olivier et al. 1998a; Rowlett et al. 2001). In contrast, genetic and pharmacological studies showed that anxiolytic effects of BZs in the other behavioral models were primarily mediated by $\alpha 2$- and $\alpha 3$-containing $GABA_A$ receptors (Löw et al. 2000; Dias et al. 2005; Atack et al. 2006; Collins et al. 2002; Griebel et al. 2001), and $\alpha 1$- and $\alpha 5$-containing receptors were more responsible for the sedative effects (Rudolph et al. 1999; McKernan et al. 2000; van Rijnsoever et al. 2004; Savić et al. 2008). Therefore, further characterization of the pharmacological profile of $GABA_A$ α receptor subtypes on USVs is needed.

Antagonists of specific glutamate receptor subtypes, the major excitatory neurotransmitter in the vertebrate brain, have emerged as potential anxiolytic compounds (Swanson et al. 2005). Systematic and local administration of drugs that act at *N*-methyl-D-aspartate (NMDA), AMPA/kainite, or metabotropic glutamate receptors showed anxiolytic-like effects in several animal models (Bergink et al. 2004; Clineschmidt et al 1982; Guimarães et al. 1991; Nordquist et al. 2008; Pérez de la Mora et al. 2006; Tatarczyńska et al. 2001), but it is often difficult to distinguish the anxiolytic effects from those on motor activity and other physiological effects since most models depend on bodily movement (Wiley 1997; Criswell et al. 1994). The measurement of USVs in maternally separated pups is advantageous because it can distinguish between anxiolytic effects on vocalizations from those on locomotor activation or sedation (Gardner 1985; Benton and Nastiti 1988; Miczek et al. 1995). Ligands that antagonize ionotropic glutamate receptors, NMDA and AMPA, have been shown to reduce USVs in rat pups (Kehne et al. 1991, 1995; Winslow and Insel 1991), and it is important to extend the role of these receptors towards mouse models. Furthermore, it has been shown that drugs that have different affinities on NMDA receptors sometimes have varied effects on behavior (Maldonado et al. 2003; Zajaczkowski et al. 1996; Zoladz et al. 2006), and thus, comparison of several drugs targeting the same receptor gives us more insights of the role of this receptor on USVs.

In the present study, we investigated the effects of glutamatergic compounds including high-affinity noncompetitive NMDA receptor antagonist dizocilpine (MK-801), low-affinity noncompetitive antagonists memantine and neramexane, and group II metabotropic glutamate receptor agonist LY-379268 (Monn et al. 1999) and antagonist LY-341495 (Kingston et al. 1998) on USVs in mouse pups. Locomotor activity, body roll, and body temperature were measured in addition to USVs in order to assess potential side effects. Those effects were compared with BZs of different potency and pharmacokinetic characteristics: flunitrazepam, bromazepam, and chlordiazepoxide. In addition, to examine the role of GABA α subunits in USVs, we tested here the drugs that have different preferential actions at the other $GABA_A$-α subunits: L-838417, a partial agonist with preferential action at $\alpha 2$, $\alpha 3$, $\alpha 5$ subunits and antagonist for $\alpha 1$ (McKernan et al. 2000), and QH-ii-066, an agonist with preferential action at $\alpha 5$ subunit (Skolnick et al. 1997, Huang et al. 2000).

Materials and methods

Animals

Subjects were Carworth Farms Webster (CFW) mouse pups (n=746) from litters ranging from six to 13 pups. We used 7-day-old pups because the highest rate of USV emission was observed around this age (Noirot 1966; Fish et al. 2000). The number of animals for each treatment group was summarized in Electronic supplementary material, Table 1. CFW mice were purchased from Charles River Laboratories International (Wilmington, MA, USA), pair-housed, and were bred to obtain pups in our vivarium with controlled humidity and temperature (35–40%, 21±1°C) on a 12-h light/dark cycle (lights on at 8:00 A.M.) with food

(Purina, St. Louis, MO, USA) and water freely available. Mice were housed in a clear polycarbonate cage (28×17×14 cm) with pine shavings as bedding material. All procedures were approved by the Institutional Animal Care and Use Committee of Tufts University. The animals were cared for according to the 'Guide for the Care and Use of Laboratory Animals' (National Academy Press, Washington, DC, 1996).

Apparatus and measurements

Behavioral tests were performed in a procedure room that was separated from the vivarium. USVs were recorded in a sound-attenuating chamber (49.5×38×34 cm) that held a water bath that maintained the temperature of the test platform (23×23 cm; 19±0.5°C). The platform was divided by a 2×2-cm grid to measure motor activity. The chamber was illuminated by a red light (10 W) and had a one-way vision window (19×16.5 cm) for observation. As described previously (Fish et al. 2000, 2004), USVs were detected with a high frequency condenser microphone (Bruel & Kjaer model 4135; Naerum, Denmark) and preamplifier (Bruel & Kjaer Model 2633) placed 2.5 cm above the testing platform. The sounds were amplified (Bruel & Kjaer Model 2610) and filtered (Krohn-Hite Model 3550R, Avon, MA, USA) to produce a flat frequency response between 30- and 80-kHz range, which was then monitored by an oscilloscope and detected by a Mac computer after digitizing the frequency-filtered signal with an analog-to-digital converter (GW Instruments; Somerville, MA, USA). The system counted the sound as an USV when the sound was between 30 and 80 kHz, lasted longer than 0.01 s, and the interval between sounds was longer than 0.02 s. For Bromazepam, LY379268 and LY341495, we used a different recording system, Sonotrack (Metris B.V., The Netherlands) to detect USVs. Gold foil electrostatic transducer microphone (Metris B.V.) was placed 45 cm above the testing platform and connected to a PC computer through a Sonotrack interface. Sonotrack software was used to count USVs with 30- to 90-kHz range bandpass filter, and sounds which had more than 0.07-s peak width were counted as USVs. These criteria were developed in order to exclude the artificial sources for high frequency signals but still remained sensitive for the detection of USVs.

Procedure

An entire litter of pups was separated from its parents into a small transport cage with some home cage shavings, transferred to the procedure room, and placed on an incubator that maintained at 34°C, mimicking the nest temperature. Twenty-five minutes later, each pup was individually weighed, marked for identification, and placed on the test platform for 30 s to screen for the emission of USVs (screening). Pups that vocalized more than six times during the screening and weighed between 3.5 and 5.5 g were accepted as subjects and were injected subcutaneously either with one dose of the drug or the vehicle. Assignment to drug treatments was random, with the restriction that no more than three pups per dose stemmed from the same litter. Each drug had a corresponding vehicle treatment group, and pups in each litter were assigned to drug doses and the corresponding vehicle treatments. After the injection, the pup was returned to the incubator and stayed with its littermates for 30 to 45 min until the separation test. Immediately before the test, the thermo-probe, lubricated with mineral oil, was inserted 2 mm into the rectum and kept in place until stable body temperature was observed for at least 3 s. The pup was placed in the center of test platform (19±0.5°C), and its USVs were automatically was counted for 4 min. In addition, an experimenter manually recorded the number of grid crossings and body rolls. A grid crossing was measured as an index of 'motor activity' and was counted when the hind limb of the pup crossed into the next grid. A body roll was counted when the back of the pup made contact with the testing floor as an index of 'motor incoordination'. After the test session, the litter was euthanized by CO_2 inhalation in accordance with the recommendation by the American Veterinary Medical Association.

For the further analysis of the enhancement of USV by a moderate dose (5.6 mg/kg) of memantine, we tested pup USVs in the cold (9±0.5°C), cool (19±0.5°C), moderate (26±0.5°C), and nest temperatures (34±0.5°C). The procedure was closely similar to the procedure described above, except 25 min after the separation, each pup was weighed and injected with saline or memantine 5.6 mg/kg without the 30-s screening.

Drugs

Dizocilpine (MK-801; Sigma-Aldrich, St. Louis, MO, USA), memantine, and neramexane (provided by Forest Laboratories, Jersey City, NJ, USA), (1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268; provided by Lilly Research Laboratories, Indianapolis, IN, USA), 2S-2-amino-2-(1S,2S-2-carboxycycloprop-1-yl)-3-(xanth-9-yl)propanoic acid (LY341495; Lilly Research Laboratories), and chlordiazepoxide (Hoffmann-La Roche, Nutley, NJ, USA) were dissolved in 0.9% saline. Flunitrazepam and bromazepam (Sigma-Aldrich) were dissolved in a vehicle that was 17% propylene glycol, 1% Tween 80, and 82% saline. 7-*tert*-butyl-3-(2,5-difluoro-phenyl)-6-(2-methyl-2*H*-[1,2,4]triazol-3-ylmethoxy)-[1,2,4]triazolo[4,3-b]pyridazine (L-838417; gift from Merck Sharp & Dohme Research Laboratories, Harlow, UK) was suspended in a saline

solution containing 0.5% methyl cellulose. 1-Methyl-7-acetyleno-5-phenyl-1,3-dihydro-benzo[*e*]-1,4-diazepin-2-one (QH-ii-066; synthesized by the laboratory of James M. Cook, University of Wisconsin-Milwaukee) was dissolved in a vehicle that consisted of 2% Tween 80, 13% emulphor, and 85% saline. All drugs were injected subcutaneously in a volume of 0.1 ml/10 g body weight. To prevent leakage, a small amount of tissue-glue (3M™ Vetbond™ Tissue Adhesive; 3M, St. Paul, MN, USA) was applied to the injection site.

Data analysis

The data for USVs and grid crossings were converted to percentage of corresponding vehicle control in each drug. That is, each individual value of drug treatment was divided by the mean value of control with the vehicle for that drug. One-way between-groups analysis of variance (ANOVA) was performed to examine the effect of the drug treatments on the number of USVs, grid crossings, and rolls. In case of significant F values, Dunnett's t tests were conducted as a post hoc analysis to determine which doses of the drug had significant effect compared to vehicle (α=0.05). For further examination of ambient temperature on memantine, we used unconverted raw data for all measurements. Two-way ANOVA was performed to examine the effect of ambient temperature and drug treatment. If significant interaction of temperature and drug treatment was observed, Tukey's post hoc tests were conducted for each temperature. Because we did not perform 30-s screening for this test, a few animals showed extremely different number of USVs. These outliers (n=4, exceed more than ±2 SD from mean) were excluded from the analysis.

Results

Figure 1 shows an example of a sonogram of USVs in a 7-day-old CFW mouse pup. With vehicle treatment, pups emitted on average 338.96±11.95 (SEM) USVs during the 4 min separation on the 19°C test platform. Their baseline locomotor activity was on average 29.21±1.85 grid crossings and the presence of rolls was infrequent (on average 1.98±0.29). Mean body temperature was 33.27±0.11.

GABAergic drugs

Ultrasonic vocalizations

Flunitrazepam, bromazepam, and chlordiazepoxide dose-dependently reduced USVs [$F(4,46)=10.091$, $F(4,52)=3.170$, and $F(4,74)=16.141$, respectively, all $p<0.05$] (Fig. 2). Dunnett's t test showed that flunitrazepam (0.056–0.1 mg/kg), bromazepam (1–3 mg/kg), and chlordiazepoxide (10–30 mg/kg) significantly suppressed USVs ($p<0.05$). QH-ii-066 also exhibited a significant effect of drug [$F(5,71)=6.432$, $p<0.001$] (Table 1) but showed a reduction of USVs only at the highest dose tested (3.0 mg/kg). No significant change was observed with L-838417 [$F(6,75)=1.143$, $p=0.347$] (Table 1).

Motor activity

Flunitrazepam, bromazepam, and chlordiazepoxide did not significantly alter the number of grid crossings (Fig. 2), although these drugs did tend to enhance activity with low doses and suppress locomotor activity with high doses. Similarly, L-838417 also did not significantly change locomotion (Table 1). In contrast, QH-ii-066 showed a biphasic effect [$F(5,71)=4.485$, $p<0.001$] (Table 1) as moderate doses (1.0 and 17 mg/kg) significantly increased grid crossings.

Motor incoordination

Flunitrazepam, bromazepam, and chlordiazepoxide dose-dependently increased body rolls [$F(4,46)=8.803$, $F(4,52)=7.172$, and $F(4,74)=10.546$, respectively, all $p<0.001$] (Fig. 2). Dunnett's t test showed that flunitrazepam

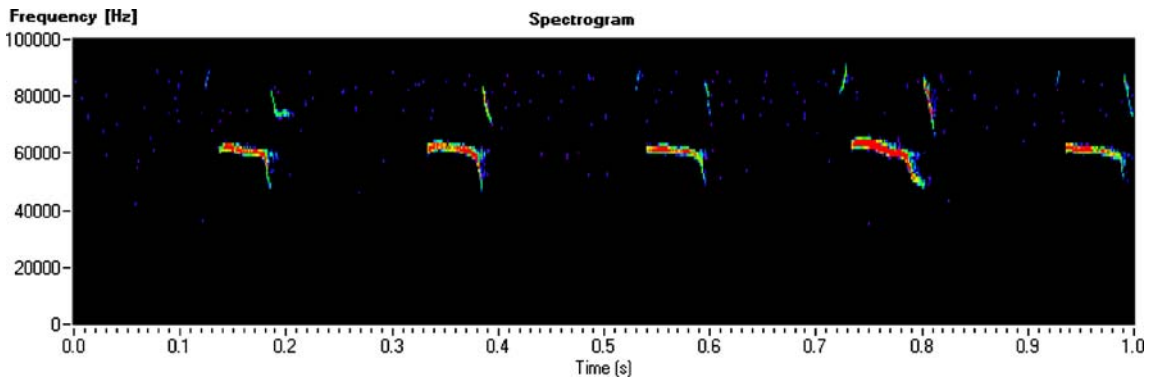

Fig. 1 An example of a sonogram of separation-induced USVs in 7-day-old CFW mouse pups

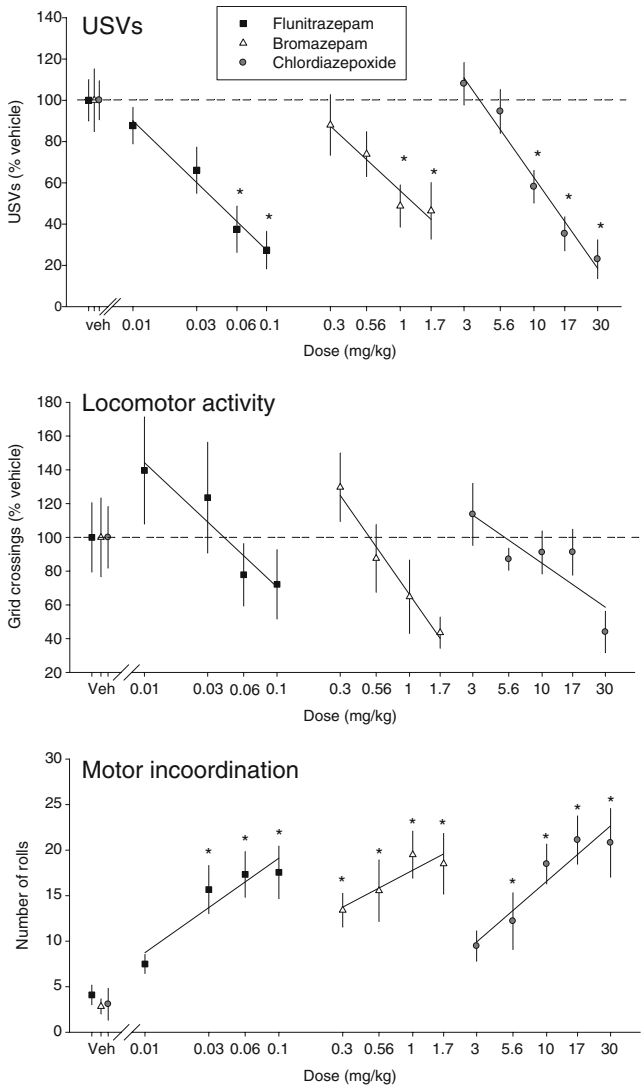

Fig. 2 The effects of flunitrazepam (*square*), bromazepam (*circle*), and chlordiazepoxide (*triangle*) on mouse pup ultrasonic vocalizations (*USVs*), motor activity, and incoordination. Total numbers of USVs and grid crossings during 4 min session are expressed as percent of each vehicle group, whereas total number of rolls is exhibited as non-transformed value. First-order regression line calculated by each drug treatment is indicated. *Asterisks* denote values that are significantly different from vehicle (Dunett's t *t*est, $p<0.05$). *Error bar* indicates SEM

(0.03–0.1 mg/kg), all doses of bromazepam, and chlordiazepoxide (10–30 mg/kg) significantly increased rolls. QH-ii-066 also exhibited a dose-dependent increase [$F(5,71)=15.712$, $p<.001$] (Table 1) since 1.0 mg/kg and higher doses significantly increased rolls. All doses of L-838417 increased rolls [$F(6,75)=6.018$, $p<0.001$] (Table 1) in a dose-independent manner.

Body temperature

One-way ANOVA indicated that there were no significant effects of drug on body temperature with any of these GABAergic positive modulators (Electronic supplementary material, Table 2).

Glutamatergic drugs

Ultrasonic vocalization

Dizocilpine dose-dependently reduced USVs [$F(5,66)=19.888$, $p<0.001$] (Fig. 3), and significant reductions were observed after administration of 0.18–0.56 mg/kg dizocilpine. On the other hand, memantine and neramexane showed a biphasic change in USVs [$F(5,71)=10.922$ and $F(5,86)=14.416$, respectively, for both $p<0.001$] (Fig. 3). Moderate doses of those drugs (5.6 mg/kg for memantine, 10 mg/kg for neramexane) significantly increased USVs, whereas the highest dose (30 mg/kg for both memantine and neramexane) strongly reduced USVs. The mGlu2/3 agonist LY379268 also dose-dependently reduced USVs [$F(3,38)=21.109$, $p<0.001$] (Table 2) at the two highest doses (0.3 and 1.0 mg/kg). The antagonist LY341495 did not significantly modulate USVs [$F(4,48)=2.066$, $p=0.102$] (Table 2).

Motor activity

Memantine and neramexane dose-dependently increased grid crossings [$F(5,71)=13.722$ and $F(5,86)=11.757$, respectively, for all $p<0.001$] (Fig. 3) as 5.6–30 mg/kg of memantine and 10–30 mg/kg of neramexane significantly increased the number of grid crossings. However, it is likely that motor activity after administration of the highest dose (30 mg/kg) of both compounds was slightly reduced from the maximal level of activity due to significant motor incoordination. Dizocilpine had a biphasic effect on grid crossings [$F(5,66)=7.885$, $p<0.001$], and only moderate doses of dizocilpine (0.18 and 0.3 mg/kg) significantly increased motor activity. In contrast, a significant reduction in motor activity was observed with all doses of LY379268 [$F(3,38)=12.454$, $p<0.001$] (Table 2). LY341495 did not have any significant effect [$F(4,48)=0.175$, $p=0.95$] (Table 2).

Motor incoordination

Dizocilpine, memantine, and neramexane dose-dependently increased rolls [$F(5,66)=22.729$, $F(5.71)=21.041$, and $F(5,86)=10.298$, respectively, for all $p<0.001$] (Fig. 3). Dunnett's *t* test showed that rolls were significantly increased after administration of dizocilpine (0.1–0.56 mg/kg), memantine (17–30 mg/kg), and neramexane (23–30 mg/kg). On the other hand, LY379268 and LY341495 did not significantly affect rolls [$F(3,38)=0.659$ and $F(3,42)=2.825$, respectively, for both $p>0.05$] (Table 2).

Table 1 The effect of L-838417 and QH-ii-066 on USVs and motor activity

Dose (mg/kg)	Veh	0.3	0.56	1	1.7	3	5.6	10	17	30
USVs (% from Veh)										
L-838417	100±8			118±11		101±11	84±11	94±8	95±6	107±10
QH-ii-066	100±9	98±9	107±13	99±14	83±13	20±7				
Grid crossings (% from Veh)										
L-838417	100±20			217±33		168±25	126±24	139±21	145±38	138±36
QH-ii-066	100±16	192±28	178±31	306±65	259±45	117±32				
Rolls										
L-838417	0.8±0.2			4.5±0.9		6.4±0.9	5.2±0.9	5.0±0.7	6.8±1.1	5.0±1.5
QH-ii-066	2.4±0.7	7.0±1.3	8.3±0.8	10.5±1.7	18.8±2.5	22.8±4.0				

Body temperature

There were no significant effects of dizocilpine, LY379268, and LY341495 on body temperature (BT). Although a significant effect of drug on BT was detected for memantine [$F(5,71)=3.527$, $p=0.007$], the change was very subtle and Dunnett's t test showed that there were no significant differences at any dose (Electronic supplementary material, Table 2). The BT data of neramexane were not available.

Ambient temperature on the effect of memantine

In order to examine the enhancement of USV by a moderate dose (5.6 mg/kg) of memantine in more detail, the pups were tested under four different temperature conditions (9°C, 19°C, 26°C, and 34°C). Each group consists of ten to 14 pups. As indicated previously, we replicated the result that 5.6 mg/kg of memantine enhances USVs and locomotor activity compared to vehicle in the 19°C condition. Two-way ANOVA indicated that there is a significant interaction of testing temperature and drug treatment on USVs [$F(3,78)=3.491$, $p=0.02$] and on grid crossings [$F(3,82)=2.867$, $p=0.042$]. Tukey's post hoc test indicated that memantine enhanced USVs at 9°C and 19°C, but not at 26°C and 34°C, compared to each of the vehicle groups (Fig. 4). This drug also enhanced grid crossings at 9°C, 19°C, and 26°C, but not at 34°C, compared to vehicle (Fig. 4). We did not analyze the rolls because there was neither significant interaction [$F(3,82)=2.175$, $p=0.097$] nor main effect of drug [$F(1,82)=2.380$, $p=0.127$].

Discussion

In this study, our first goal was to examine the effect of inhibition of glutamatergic excitation through NMDA receptors on USVs using antagonists that possess different affinities for this receptor. Our results showed that the low-affinity noncompetitive NMDA receptor antagonists, memantine and neramexane, had bidirectional effect on separation-induced USVs: enhanced USVs after moderate doses and reduced USVs after higher doses. This result is surprising because it is expected that NMDA receptor antagonists exert anxiolytic effects (Swanson et al. 2005), and it has been shown previously that noncompetitive NMDA receptor antagonists dizocilpine, the competitive antagonist AP-5 and AP-7, and the partial agonist ACPC reduced USVs in rats (Kehne et al. 1991, 1995; Winslow and Insel 1991). We also found that dizocilpine dose-dependently reduced mouse pup USVs, and these effects are consistent with those in the rat studies. Since lower doses of dizocilpine (0.01, 0.03 mg/kg) did not enhance USVs (data not shown), the observed increase in

Table 2 Effect of mGluR2/3 agonist and antagonist on USVs and motor activity

Dose (mg/kg)	Veh	0.1	0.3	1	3	10	20	40
USVs (% from Veh)								
LY379268	100±19	96±8	44±5	3.4±1.7				
LY341495	100±13				51±15	69±15	64±12	101±20
Grid crossings (% from Veh)								
LY379268	100±24	26±10	12±3	0.8±0.5				
LY341495	100±22				78±15	88±24	87±18	100±30
Rolls								
LY379268	1.3±0.6	1.0±0.6	1.7±0.6	0.7±0.3				
LY341495	2.3±0.8				0.8±0.3	0.6±0.2	0.7±0.3	0.0

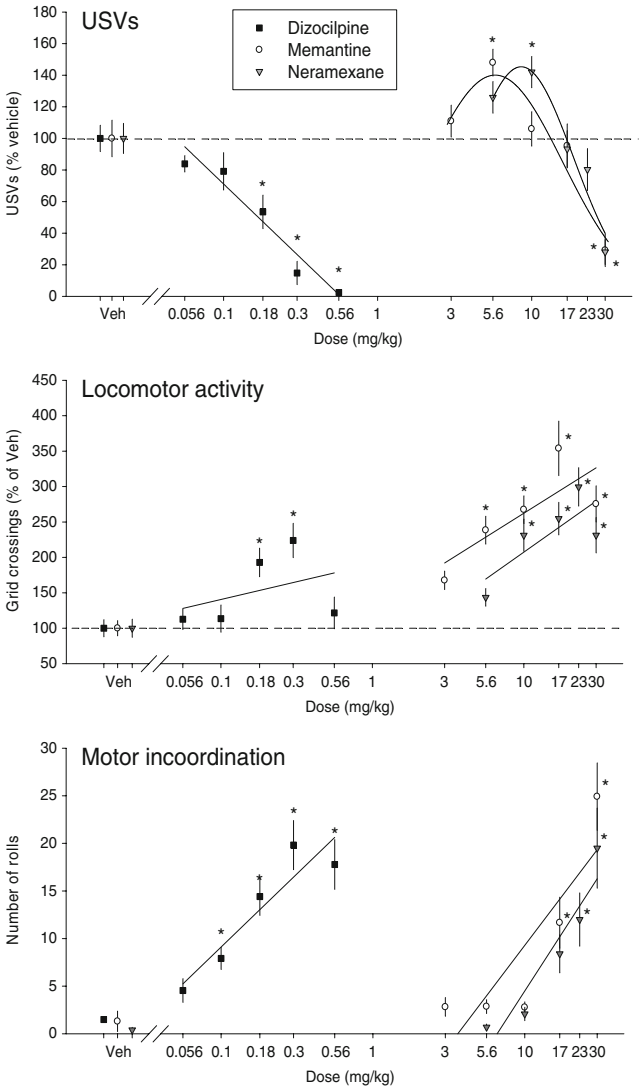

Fig. 3 The effects of dizocilpine (MK-801; *square*), memantine (*circle*), and neramexane (*triangle*) on mouse pup ultrasonic vocalizations, motor activity, and incoordination. Total numbers of USVs and grid crossings during 4-min session are expressed as percent of each vehicle group, whereas total number of rolls is exhibited as non-transformed value. Linear regression is fitted for grid crossings and rolls, and regression line of Gaussian peak is indicated for USVs. *Asterisks* denote values that are significantly different from vehicle (Dunett's *t* test, $p<0.05$). *Error bar* indicates SEM

USVs was specific for low-affinity NMDA receptor antagonists. It has been shown that low-affinity antagonists have a behavioral profile that differs from that of dizocilpine. Dizocilpine is known to have psychotomimetic side effects, whereas low-affinity antagonists possess much less psychotomimetic activity but also improve cognitive functions and inhibit morphine dependence (Maldonado et al. 2003; Zajaczkowski et al. 1996; Zoladz et al. 2006). All of dizocilpine, memantine, and neramexane bind to the PCP-binding site inside the NMDA receptor and block channel activity (Kornhuber and Weller 1997). However, the receptor binding kinetics of these drugs differ. High-affinity dizocilpine has very slow kinetics, whereas low-affinity memantine is strongly voltage-dependent and has quick blocking and unblocking kinetics (Parsons et al. 1995, 2007), which may be due to the diverse binding affinity to different subtypes of NMDA receptors of these drugs (Bresink et al. 1995; Parsons et al. 1999). In addition to the differences of binding affinity and kinetics of these drugs at NMDA receptors, dizocilpine and memantine bind at higher doses to other receptors such as acetylcholine receptors (Buisson and Bertrand 1998; Drever et al. 2007; Plazas et al. 2007). Further studies to elucidate how dizocilpine and memantine/neramexane differently modulate USVs are required.

The problem of studying glutamate receptor drugs on anxiety-like behavior is that it is often difficult to distinguish the anxiolytic effects from those on motor activity (Wiley 1997; Criswell et al. 1994). In this study, both memantine and neramexane had strong dose-dependent, locomotor-

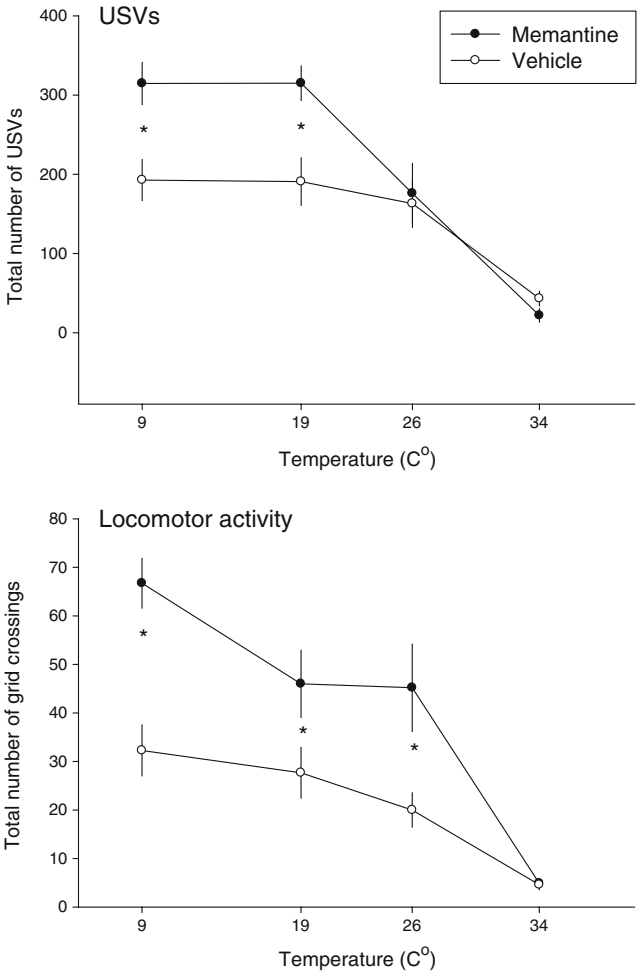

Fig. 4 The effects of test temperature on the enhancement of USVs and locomotor activity by moderate dose (5.6 mg/kg) of memantine. *Asterisks* indicates a significant difference between memantine and vehicle for each temperature (Tukey's *t* test, $p<0.05$). *Error bar* indicates SEM

activating effects. These dose-dependent patterns of locomotion did not correspond to the bidirectional patterns of the USVs. Thus, the enhancement of USVs by these drugs is independent of locomotor activation. In contrast, motor incoordination was observed with the doses that reduced USVs; the decreases in calling may be linked to motor coordination by NMDA receptor antagonists. Our data indicate that the enhancement of USVs with moderate doses of low-affinity NMDA receptor antagonists is dissociated from locomotor activation and motor incoordination.

Then, does this enhancement of USVs by low-affinity NMDA receptor antagonists reflect an anxiogenic effect of these drugs? Or do moderate doses of these drugs enhance certain responses that produce sounds as a by-product (Blumberg et al. 2000) in a stress-independent manner? To examine the effect of stress intensity on this behavior, we tested the pups under different ambient temperatures. Consistent with previous reports, the cold stress enhances mouse pup USVs compared to its nest temperature (Olivier et al. 1998a, b; Dirks et al. 2002). Memantine (5.6 mg/kg) strongly heightened USVs under cold temperature conditions (9°C and 19°C), but not under moderate (26°C) or nest temperature conditions (34°C). Therefore, a moderate dose of memantine enhances "distress" calls in a stress-intensity-dependent manner. Previously, it has been shown that inverse agonists of BZs, which is considered to have anxiogenic effects in several behavioral models, can enhance USVs (Insel et al. 1986; Nastiti et al. 1991). Our results indicate that low-affinity NMDA antagonists have similar effect and also showed anxiogenic-like effect on USVs. Interestingly, enhanced locomotor activity by memantine also depends on the stress intensity. Strong enhancement of locomotor activity was observed in the coldest temperature, while there was no difference in activity at the nest temperature.

We then examined the role of group II metabotropic glutamate receptors (mGlu2 and mGlu3) on USVs. These receptors are coupled with inhibitory G proteins and inhibit pre- and postsynaptic neuronal activity (Swanson et al. 2005). Therefore, in contrast to the NMDA receptor antagonist, group II mGlu receptors agonist inhibit glutamatergic excitations. It has been found that group II mGlu agonists showed anxiolytic effects in the elevated plus maze, fear-potentiated startle, and conflict tests in adults (Helton et al. 1998; Kłodzińska et al. 1999; Satow et al. 2008) and also reduced separation-induced USVs in the rat pups (Satow et al. 2008). Consistently, our results showed that LY-379268 dose-dependently reduces USVs in the mouse. However, this drug had strong sedative effects even at the lowest dose, which was ineffective in altering USVs. We could not differentiate the anxiolytic inhibition, as indicated by USVs, from the sedative effect of this drug. LY-341495, an antagonist of mGlu2/3, did not have any effect on the USVs or locomotor activity. Therefore, intrinsic activation of mGlu2/3 may not be necessary for separation-induced USVs in mouse.

Classic BZs chlordiazepoxide, flunitrazepam, and bromazepam that have different affinities for the modulatory site on the $GABA_A$ receptor dose-dependently reduced USVs of mouse pups, consistent with previous studies using other BZs or other positive modulators of $GABA_A$ receptor (Benton and Nastiti 1988; Fish et al. 2000; Rowlett et al. 2001; Vivian et al. 1997). We did not observe an affinity-dependent difference of effects on USVs with BZs like with NMDA antagonists. In addition, in contrast to glutamatergic drugs, BZs did not have a significant effect on locomotor activity. However, similar to glutamatergic compounds, BZs dose-dependently increased body rolls, and the inhibition of USVs cannot be clearly dissociated from the motor incoordination. Thus, we can not reject the possibility that the reduction of USVs occurred due to the behavioral disruption by drugs used in this experiment. However, it has previously been shown that the selective serotonin reuptake inhibitors can reduce USVs without motor incoordination using the same procedure as in this experiment in our laboratory (Fish et al. 2004) and with similar procedures (Olivier et al. 1998b). Also, muscle-relaxing drugs which can induce motor incoordination did not affect USVs (Gardner 1985). Therefore, it is possible that the reduction of USVs by BZs may reflect anxiolytic effects that are dissociable from behavioral disruption.

The anxiolytic effect and the sedative/incoordination effect of BZs may be mediated by different compositions of $GABA_A$ receptor subunits. Genetic and pharmacological studies indicated that anxiolytic effects of BZs were mediated by α2- and, possibly, α3-containing $GABA_A$ receptors (Löw et al. 2000; Dias et al. 2005; Atack et al. 2006; Collins et al. 2002; Griebel et al. 2001), whereas α1- and α5-containing receptors may mediate the sedative effects (Rudolph et al. 1999; McKernan et al. 2000; van Rijnsoever et al. 2004; Savić et al. 2008). However, some studies have indicated that the α1 and α5 subunits also are relevant to anxiolytic-like properties (Belzung et al. 2000; Lippa et al. 2005; Olivier et al. 1998a; Brunelli et al. 1994). A previous study showed that zolpidem, preferentially acting on $GABA_A$ receptors containing the α1 subunit, suppressed USVs in the mice (Rowlett et al. 2001). In the present study, we examined different $GABA_A$-α subunit preferring agonists L-838417 and QH-ii-066 on mouse pup USVs. L-838417 binds preferentially to α2-, α3-, and α5-containing $GABA_A$ receptors as agonist, but also is an antagonist at α1 subunit (McKernan et al. 2000). Behaviorally, this drug has anxiolytic-like effects in the elevated plus maze, fear-potentiated startle, and Vogel conflict test in mice (Mathiasen and Mirza 2005; McKernan et al. 2000) and also in primates (Rowlett et al. 2005). However, we

found that L-838417 did not demonstrate an anxiolytic-like effect on pup USVs with doses that had anxiolytic effects in other models, although modest significant motor incoordination was observed. In addition, the α5-containing $GABA_A$ receptor agonist QH-ii-066 reduced USVs only at the highest dose, which was associated with increased body rolls. Our results suggest that α5-containing $GABA_A$ receptors are associated more with sedative effects rather than anxiolytic effects. From our results with L-838417, activation of α2 and α3 subunits was insufficient to reduce pup USVs. Therefore, other α subunit containing $GABA_A$ receptors, possibly α1 subunit (Rowlett et al. 2001), may have an important role on maternal separation-induced mouse pup USVs. Further examination using knock-in mice that have a point mutation in the BZ binding site of either α subunits will give us further understanding of the role of α subunit on USVs (Takahashi, Rudolph and Miczek, in preparation).

In this study, we characterized the effects of glutamate receptors and $GABA_A$ receptors on separation-induced USVs, locomotor activity, and motor incoordination in mouse pups. However, it is important to consider how the results of a pup study can be related to the neuronal mechanisms underlying anxiety in the adult, since it has been shown that the expression of some receptor subunits changes across development. Specifically, the expression of most $GABA_A$ receptor subunits is different in the adult compared to the presently studied 7-day-old pups, and those patterns of change appear to be different across brain regions (Davis et al. 2000; Fritschy et al. 1994; Laurie et al. 1992). Similarly, developmental changes in NMDA receptor subunit composition have also been observed (Sheng et al. 1994; Monyer et al. 1994). Because of these caveats, in order to fully understand the contribution of each receptor subtype in the expression of anxiety-like behavior, additional complimentary studies using other behavioral models in the adult will be required.

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