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Involvement of the strychnine-sensitive glycine receptor in the anxiolytic effects of GlyT1 inhibitors on maternal separation-induced ultrasonic vocalization in rat pups

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ABSTRACT

Several studies have shown that glycine transporter 1 (GlyT1) inhibitors have anxiolytic actions. There are two types of glycine receptor: the strychnine-sensitive glycine receptor (GlyA) and the strychnine-insensitive glycine receptor (GlyB); however, which receptor is the main contributor to the anxiolytic actions of GlyT1 inhibitors is yet to be determined. Here, we clarified which glycine receptor is the main contributor to the anxiolytic effects of GlyT1 inhibitors by using maternal separation-induced ultrasonic vocalization (USV) by rat pups as an index of anxiety. We confirmed that administration of the benzodiazepine diazepam or the selective serotonin reuptake inhibitor escitaloplam, which are both clinically proven anxiolytics, or the GlyT1 inhibitor SSR504734 (2-chloro-N-[(S)-phenyl[(2S)-piperidin-2-yl] methyl]-3-trifluoromethyl benzamide), decreases USV in rat pups. In addition, we showed that another GlyT1 inhibitor, ALX5407 ((R)-N-[3-(4′-fluorophenyl)-3(4′-phenylphenoxy)propyl]sarcosine) also decreases USV in rat pups. SSR504734- or ALX5407-induced decreases in USV were dose-dependently reversed by administration of the GlyA antagonist strychnine, whereas the diazepam- or escitalopram-induced decreases in USV were not. Furthermore, GlyT1-induced decreases in USV were not reversed by administration of the GlyB antagonist L-687,414. Together, these results suggest that GlyA activation is the main contributor to the anxiolytic actions of GlyT1 inhibitors and that the anxiolytic actions of diazepam and escitalopram cannot be attributed to GlyA activation. Our findings provide new insights into the importance of the activation of GlyA in the anxiolytic effects of GlyT1 inhibitors.

1. Introduction

The neurotransmitter glycine acts through two receptors: a strychnine-sensitive glycine receptor (GlyA) and a strychnine-insensitive glycine receptor (GlyB). GlyA is localized to the neuronal membrane post-synaptic to inhibitory glycinergic neurons, whereas GlyB is associated with the NR1 subunit of the excitatory N-methyl-D-aspartate (NMDA) receptor (Legendre, 2001; Kuryatov et al., 1994). Glycine therefore has bidirectional actions on neuronal excitability.

The extracellular concentration of glycine is regulated by glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2) (Aragón and López-Corcuera, 2005). GlyT1 is expressed on glial cells and glutamatergic neurons (Cubelos et al., 2005; Raiteri and Raiteri, 2010), whereas GlyT2 is predominantly expressed at glycinergic nerve terminals (Jursky and Nelson, 1995). NMDA receptor function is enhanced in the hippocampus of *GlyT1* heterozygous-knockout mice, suggesting that GlyT1 regulates the concentration of glycine at NMDA receptor-containing excitatory synapses (Gabernet et al., 2005). Thus, GlyT1 inhibitors likely promote NMDA receptor function.

GlyT1 inhibitors may have anxiogenic actions, because NMDA receptor activation induces anxiety-like behavior in mice (Miguel and Nunes-de-Souza, 2008). However, GlyT1 inhibitors may also have anxiolytic actions, because SSR504734, a GlyT1 inhibitor, both attenuates the acquisition and expression of contextual conditioned fear in rats (Nishikawa et al., 2006) and decreases maternal separation-induced ultrasonic vocalization (USV) in rat pups (Depoortère et al., 2005). Furthermore, the NMDA receptor antagonists MK-801 and DL-amino-5-phosphonovaleric acid (AP5) have been shown to have anxiolytic actions in rats (Kehne et al.,

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1991), and 7-chlorokynurenic acid (7-Cl-KYN), a GlyB antagonist, has been shown to have anxiolytic actions in mice (Trullas et al., 1989). Together, these reports suggest that the anxiolytic action of GlyT1 inhibitors is not induced by activation of GlyB. However, which glycine receptor subtype is the main contributor to the anxiolytic actions of GlyT1 inhibitors is yet to be fully elucidated. Recently, it was reported that the hyperlocomotion induced by a GlyT1 inhibitor in mice was antagonized by the GlyA antagonist strychnine (Kopec et al., 2010), suggesting that not only GlyB but also GlyA plays a significant role in the behavioral changes induced by GlyT1 inhibitors.

Rodent pups emit USVs (peak frequency around 40 kHz) when they are separated from their mother and littermates (Brudzynski et al., 1999; Zippelius and Schleidt, 1956). Because clinically proven anxiolytics such as the benzodiazepines and selective serotonin reuptake inhibitors reduce the number of maternal separation-induced USVs in rat pups, USV is thought to be a predictive animal model of the anxiolytic effect (Insel et al., 1986; Winslow and Insel, 1991). Therefore, in the present study, maternal separation-induced USV in rat pups was used as an index of anxiety.

Here, we examined which glycine receptor is the major contributor to GlyT1 inhibitor-induced decreases in rat pup USV. We first examined the effect of GlyT1 inhibitors and anxiolytics on USV and then examined whether GlyT1 inhibitor-induced decreases in USV were reversed by administration of a GlyA or GlyB antagonist.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats, each with 10 pups at postnatal day 4, were purchased from Charles River Laboratories (Tokyo, Japan). Animals were housed at room temperature and maintained under a 12-h/12-h light/dark cycle with ad libitum access to food and tap water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Eisai Co., Ltd. (Ibaraki, Japan).

2.2. Drugs

SSR504734 (2-chloro-N-[(S)-phenyl[(2S)-piperidin-2-yl] methyl]-3-trifluoromethyl benzamide), ALX5407 ((R)-N-[3-(4′-fluorophenyl)-3(4′-phenylphenoxy)propyl]sarcosine; (+)-NFPS), and L-687,414 ((3R,4R)-3-amino-1-hydroxy-4-methylpyrrolidin-2-one) were synthesized at the medicinal chemistry department of Eisai Co., Ltd. Diazepam, escitalopram, and strychnine were purchased from Wako Pure Chemical Industries (Osaka, Japan), AK Scientific (Union City, CA), and Sigma-Aldrich (Tokyo, Japan), respectively. SSR504734 was dissolved in distilled water, and the pH was adjusted to 6 to 7 using 1 N HCl. ALX5407 was dissolved in distilled water, and the pH was adjusted to 6 to 7 using 1 N NaOH. Escitalopram was dissolved in saline. Diazepam was suspended in 0.5% methyl cellulose (Wako Pure Chemical Industries, Osaka, Japan). Strychnine was dissolved in saline, and the pH was adjusted to 6 to 7 using 1 N HCl. L-687,414 was dissolved in saline with 0.3% Tween 80 (Kanto Chemical Co., Inc., Tokyo, Japan). Several doses of each drug were used and are indicated in the figures. We chose the doses of drugs used in this study by referencing the results of previous studies (Depoortère et al., 2005; Kopec et al., 2010; Olivier et al., 1998a; Sánchez et al., 2003). After determining the dose–response relationship of each compound, in the subsequent antagonism study we used the dose at which the number of USVs was suppressed to less than 35% of that in vehicle-treated control rat pups. All solutions and suspensions were prepared daily and administered orally or subcutaneously in a volume of 10 ml/kg body weight.

2.3. Ultrasonic vocalization test

The procedure was modified from that described by Olivier et al. (1998a, 1998b). Briefly, pre-weaning Sprague–Dawley rat pups were used at postnatal day 10. Each pup was separated from its mother and littermates and immediately placed in a plastic cylinder kept at room temperature. The number of USVs was recorded for 3 min by using a Sonotrack™ measurement system (Metris, Netherland). USVs picked up by the microphones were digitally recorded. The band-pass filter was adjusted to 30–70 kHz. Within this range, the Sonotrack™ software automatically counted the number of USVs produced by each rat pup.

SSR504734, diazepam, or escitalopram was administered orally 1 h prior to the USV test. ALX5407 was administered orally 3 h prior to the USV test. A 3-h pretreatment time was selected because of the irreversible nature of ALX5407 binding (Atkinson et al., 2001; Kopec et al., 2010). For the antagonism test, strychnine (GlyA antagonist) or L-687,414 (GlyB antagonist) was administered subcutaneously 30 min before the USV test. To avoid direct interactions between the compounds, different routes of administration were used for the two compounds. After administration of the test compound, the pups were returned to their home cage until use.

2.4. Measurement of rectal temperature

To evaluate whether or not any decrease in the number of USVs was secondary to a decrease in body temperature, the influence of each drug on rectal temperature, when administered at the maximum ineffective and minimum effective doses as determined in the USV test, was examined by using a rectal probe (Physitemp Instruments, Inc., Clifton, NJ) and a TX1002 digital thermometer (Yokogawa Meters & Instruments Corporation, Japan). Pretreatment times were the same as those used in the USV test.

2.5. Statistical analysis

All statistical analyses were carried out by using GraphPad Prism software version 6.0 for Windows (GraphPad Software, San Diego, CA). Data were analyzed by using Kruskal–Wallis followed by Dunn's multiple comparison test or the Mann–Whitney *U* test.

3. Results

3.1. Effects of GlyT1 inhibitors or anxiolytics on USV and rectal temperature in Sprague–Dawley rat pups

The effects of administration of the test compounds on the number of USVs recorded in 3 min are shown in Fig. 1. The SSR504734 (Fig. 1A) doses were 3, 10, or 30 mg/kg; administration at 30 mg/kg significantly decreased the number of USVs recorded (H [4, 32] = 14.90, $P < 0.01$). Rectal temperature did not change compared with that in vehicle-treated control rats after administration of SSR504734 at 10 or 30 mg/kg (Table 1).

Similarly, ALX5407 doses were 0.1, 0.3, or 1 mg/kg (Fig. 1B); administration at 1 mg/kg significantly decreased the number of USVs (H [4, 35] = 20.26, $P < 0.01$]) without affecting rectal temperature at 0.3 or 1 mg/kg (Table 1).

Both diazepam (Fig. 1C) and escitalopram (Fig. 1D) significantly decreased the number of USVs when administered at 1 or 3 mg/kg (diazepam; H [4, 32] = 18.91, $P < 0.05$ and $P < 0.01$, escitalopram; H

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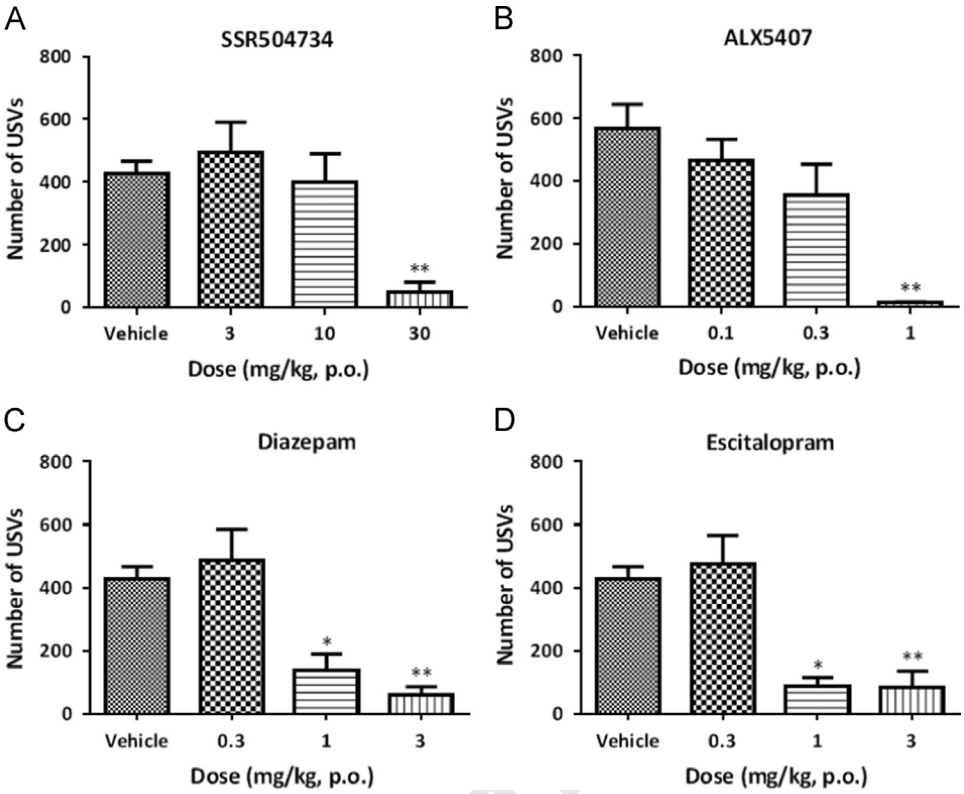

Fig. 1. Effects of SSR504734, ALX5407, diazepam, or escitalopram on the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. SSR504734 (A), diazepam (C), or escitalopram (D) was administered orally 1 h prior to the test. ALX5407 (B) was administered orally 3 h prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mother and littermates. Data are presented as mean ± S.E.M. $N=8$–9 per group. $^{*}P<0.05$, $^{**}P<0.01$, compared with the vehicle-treated control group (Kruskal–Wallis followed by Dunn's test).

Table 1
Effects of SSR504734, ALX5407, diazepam, or escitalopram on rectal temperature. SSR504734, diazepam, or escitalopram was orally administered 1 h prior to the test. ALX5407 was orally administered 3 h prior to the test. Data are presented as mean ± S.E.M. $N=4$ per group. Each treatment group was compared with its vehicle-treated control group.

Compound	Dose (mg/kg)	Mean ± S.E.M.
Vehicle	–	36.9 ± 0.17
SSR504734	10	36.6 ± 0.20
SSR504734	30	36.7 ± 0.21
Vehicle	–	36.5 ± 0.07
ALX5407	0.3	36.7 ± 0.29
ALX5407	1	36.1 ± 0.14
Vehicle	–	36.7 ± 0.18
Diazepam	0.3	36.7 ± 0.18
Diazepam	1	1.0 ± 0.29
Vehicle	–	36.9 ± 0.13
Escitalopram	0.3	36.3 ± 0.32
Escitalopram	1	36.1 ± 0.30

[4, 32] = 16.71, $P<0.05$ and $P<0.01$), but did not affect rectal temperature at 0.3 or 1 mg/kg (Table 1).

3.2. Effects of strychnine, a GlyA antagonist, on GlyT1 inhibitor-induced or anxiolytic-induced decreases in the number of USVs produced by Sprague–Dawley rat pups

The number of USVs was not changed by administration of strychnine alone at 0.1 or 0.2 mg/kg, but was significantly increased compared with control when strychnine alone was administered at 0.4 mg/kg (H [4, 39] = 7.06, $P<0.05$) (Fig. 2). The SSR504734-induced decrease in the number of USVs (30 mg/kg; $U=7.0$, $P<0.01$) was significantly and dose-dependently reversed by administration of strychnine at 0.2 or 0.4 mg/kg (H [4, 40] = 19.15, $P<0.05$ and $P<0.01$) (Fig. 3A). The ALX5407-induced decrease in the number of USVs (1 mg/kg; $U=0.0$, $P<0.01$) was also significantly and dose-dependently reversed by administration of strychnine at 0.2 or 0.4 mg/kg (H [4, 38] = 29.40, both $P<0.01$) (Fig. 3B). However, the diazepam-induced (1 mg/kg; $U=8.0$, $P<0.01$) or escitalopram-induced (1 mg/kg; $U=4.0$, $P<0.01$) decrease in the number of USVs was not reversed by the administration of strychnine at any of the doses examined (H [4, 40] = 2.84 and H [4, 40] = 4.50) (Fig. 4A and B).

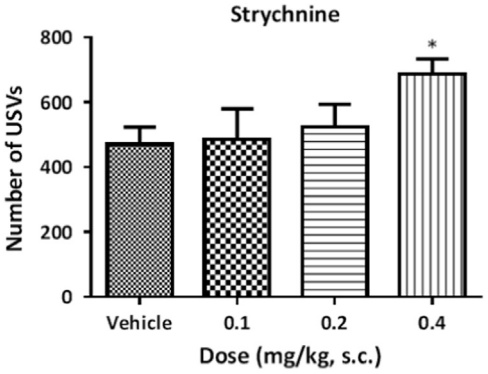

Fig. 2. Effects of strychnine on the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. Strychnine was subcutaneously administered 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mothers and littermates. Data are presented as mean ± S.E.M. $N=9$–10 per group. $^{*}P<0.05$, compared with the vehicle-treated control group (Kruskal–Wallis followed by Dunn's test).

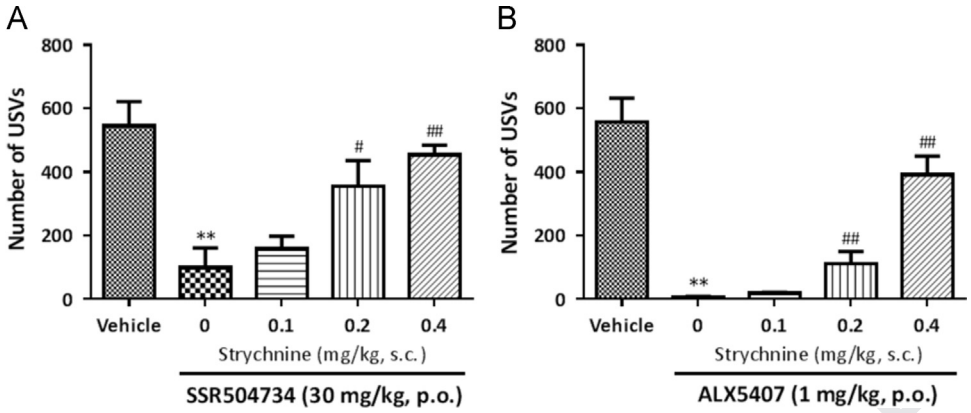

Fig. 3. Effects of strychnine on SSR504734- or ALX5407-induced decreases in the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. SSR504734 (A) was orally administered 1 h and ALX5407 (B) was orally administered 3 h prior to the test. In both tests, strychnine was administered subcutaneously 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mothers and littermates. Data are presented as mean ± S.E.M. $N=8$–10 per group. $^{**}P<0.01$, compared with the vehicle-treated control group (Mann–Whitney U test). $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the group treated with SSR504734 alone or ALX5407 alone (Kruskal–Wallis followed by Dunn's test).

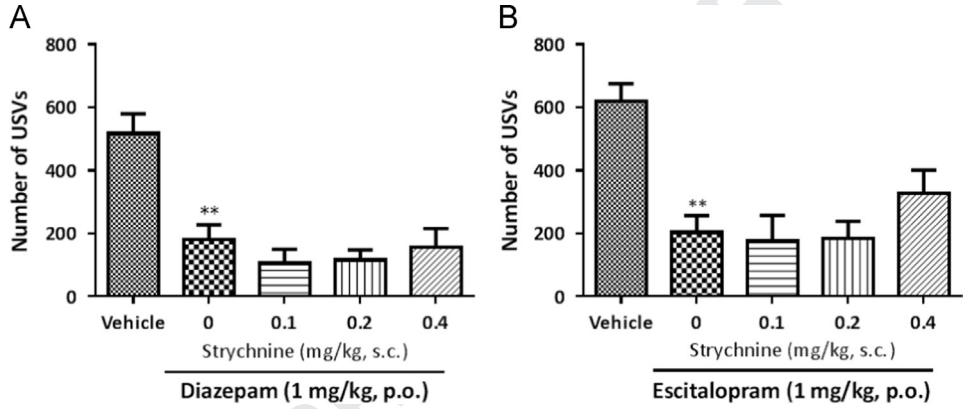

Fig. 4. Effects of strychnine on diazepam- or escitalopram-induced decreases in the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. Diazepam (A) or escitalopram (B) was orally administered 1 h prior to the test. In both tests, strychnine was administered subcutaneously 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mothers and littermates. The number of USVs was measured for 3 min immediately after separation of the pups from their mother and littermates. Data are presented as mean ± S.E.M. $N=10$ per group. $^{**}P<0.01$, compared with the vehicle-treated control group (Mann–Whitney U test).

3.3. Effects of L-687,414, a GlyB antagonist, on SSR504734-induced decreases in the number of USVs produced by Sprague–Dawley rat pups

The number of USVs was significantly decreased by the administration of L-687,414 alone at 30 mg/kg (H [3, 30] = 9.45, $P<0.01$), but not at 10 mg/kg (Fig. 5). Furthermore, the SSR504734-induced decrease in the number of USVs (30 mg/kg; $U=13.0$, $P<0.01$) was not reversed by the administration of L-687,414 at 3, 10, or 30 mg/kg (Fig. 6). L-687,414 appeared to strengthen the SSR504734-induced decrease in the number of USVs, but this was not statistically significant (H [4, 39] = 4.01).

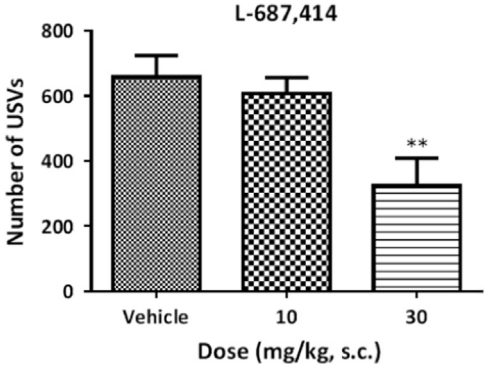

Fig. 5. Effects of L-687,414 on the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. L-687,414 was subcutaneously administered 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mothers and littermates. Data are presented as mean ± S.E.M. $N=10$ per group. $^{**}P<0.01$, compared with the vehicle-treated control group (Kruskal–Wallis followed by Dunn's test).

4. Discussion

The results of the present study suggest that GlyT1 inhibitor-induced decreases in USV are mediated through the activation of inhibitory GlyA rather than excitatory GlyB, which is localized to the NMDA receptor. Furthermore, we found that diazepam- or escitalopram-induced decreases in USV are not related to GlyA. Thus, GlyA plays an important role in GlyT1 inhibitor-induced decreases in USV.

Maternal separation-induced USV is a well-established index of anxiety in rodents (Olivier et al., 1998a, 1998b; Winslow et al., 1990). In agreement with previous reports, we showed that diazepam and escitalopram, which are clinically proven anxiolytics, significantly decreased USV, which suggests that the USV test may be predictive of anxiolytic effect in humans. We also

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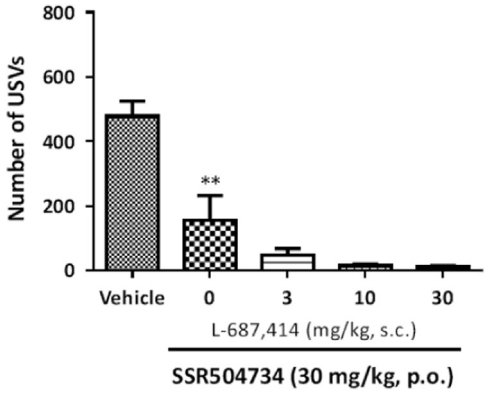

Fig. 6. Effects of L-687,414 on SSR504734-induced decreases in the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. SSR504734 was orally administered 1 h prior to the test and L-687,414 was subcutaneously administered 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mother and littermates. Data are presented as mean ± S.E.M. $N = 9–10$ per group. $^{**}P < 0.01$, compared with the vehicle-treated control group (Mann–Whitney U test).

confirmed previously published data showing that SSR504734 significantly decreases USV (Depoortère et al., 2005).

We also evaluated another GlyT1 inhibitor, ALX5407, which has a different chemical structure from that of SSR504734. ALX5407 decreased USV when administered at a dose of 1 mg/kg; however, it has been reported that ALX5407 does not have anxiolytic actions at a dose of 3 or 10 mg/kg, as assessed by behavioral tests in adult rats (Harsing et al., 2003). These inconsistent results may be explained by the different pretreatment times and routes of administration used in the studies. Harsing et al. (2003) administered ALX5407 intraperitoneally 30 min prior to conducting the tests, whereas we orally administered it 3 h prior to conducting the tests. Because ALX5407 has an amino acid moiety within its structure (Atkinson et al., 2001), we predicted that its penetration into the brain would be slow. Indeed, in a microdialysis study, extracellular glycine levels gradually increased, reaching peak levels in the prefrontal cortex at 3 h and in the cerebellum at 6 h after oral administration of 10 mg/kg (±)-NFPS, a racemic form of ALX5407 (Perry et al., 2008). Kinney et al. (2003) demonstrated that intraperitoneal administration of ALX5407 (1 mg/kg) 2 h prior to the test increased the degree of prepulse inhibition in mice. Thus, it may be possible to detect the anxiolytic actions of ALX5407 by taking the pharmacokinetics of ALX5407 and pharmacodynamics of glycine in the brain into account.

It is important to clarify whether the decrease in USV after administration was secondary to the central depressant actions of the test compounds. Several reports have addressed the possibility that central depressant actions such as motor incoordination or changes in body temperature might affect USV (Olivier et al., 1998a, 1998b). However, while diazepam is known to induce muscle relaxation at higher doses, it has only been shown to suppress USV at lower doses (Olivier et al., 1998a). And although diazepam reduces rectal temperature at effective doses, overall the hypothermic actions of the benzodiazepines are not thought to contribute to their anxiolytic actions, at least in the clinical setting (Olivier et al., 1998a). Furthermore, in the present study, diazepam did not decrease rectal temperature at the effective doses used, which provides support for the idea that diazepam decreases USV via its anxiolytic actions. Our results also show that escitalopram did not affect rectal temperature at the effective doses used, which is consistent with the results of previous studies showing that selective serotonin reuptake inhibitors have no effect on motor coordination, body temperature, or the righting reflex (Hodgson et al., 2008; Olivier et al., 1998b). We also showed that SSR504734 did not affect rectal temperature at the effective doses used, which supports previous work by Depoortère et al. (2005) that did not report any abnormal behavior in rats and mice caused by SSR504734, and that administration of ALX5407 at effective doses also did not cause a decrease in rectal temperature. Several studies have shown that high-dose ALX5407 induces motor incoordination. For example, Harsing et al. (2006) have reported that intraperitoneal administration of (±)-NFPS at 30 mg/kg produces motor dysfunction in mice, and Perry et al. (2008) have reported that orally administered (±)-NFPS at 30 mg/kg, but not at 10 mg/kg, produces motor dysfunction, impaired gait in rats. Kopec et al. (2010) have shown that intraperitoneal administration of ALX5407 at 6 mg/kg, but not at 3 mg/kg, exhibited hyperlocomotion. Here, we observed the decrease in USV at the lower dose of 1 mg/kg without affecting rectal temperature and we did not observe any abnormal behaviors in rat pups at the same dose. Therefore, we consider the ALX5407-induced decrease in USV to not be secondary to the central depressant actions of the compound. Together, the above results suggest that GlyT1 inhibitors have an anxiolytic action.

Next, we examined whether GlyA or GlyB is the main contributor to the anxiolytic action of GlyT1 inhibitors. Inhibitory GlyA and excitatory GlyB mediate opposing actions on neuronal excitability. GlyT1 inhibitors efficiently activate NMDA receptors because GlyT1 is distributed closely to the NMDA receptor (Smith et al., 1992). However, a recent autoradiograph study (Herdon et al., 2010) confirmed the results of a previous study that showed that GlyT1 is enriched in the hindbrain (Jursky et al., 1994), where the distribution is consistent with that of GlyA (Zarbin et al., 1981). Therefore, it might be possible that GlyT1 inhibitors contribute to the activation of GlyA. Consistent with this hypothesis, our results indicate that the anxiolytic action of GlyT1 inhibitors is reversed by the GlyA antagonist strychnine, but not by the GlyB antagonist L-687,414, and therefore that the anxiolytic action of GlyT1 inhibitors is mediated by GlyA. Strychnine administration (0.4 mg/kg) slightly increased USV; however, we can exclude the possibility that this apparent reversal of the action of SSR504734 and ALX5407 by strychnine was actually due simply to an independent increase in USV, because strychnine also showed significant reversal when administered at 0.2 mg/kg, a dose at which USV remained unchanged when strychnine was administered alone. Administration of L-687,414 alone at 30 mg/kg decreased USV, which agrees with the results of previous studies in which GlyB antagonists were reported to have anxiolytic actions (Trullas et al., 1989; Winslow et al., 1990). These data support the notion that activation of GlyB does not induce anxiolytic actions. Although L-687,414 has been reported to be a partial agonist of GlyB (Priestley et al., 1998), it acts as a substantial NMDA receptor antagonist in vivo because of its weak intrinsic agonist activity. Tricklebank et al. (1994) have shown that L-687,414 has dose-dependent anticonvulsant effects in a variety of animal models, and that the anticonvulsant effects of L-687,414 are completely reversed by administration of the GlyB agonist D-serine. In contrast to GlyT1 inhibitors, neither the diazepam- nor escitalopram-induced reductions in USV were reversed by strychnine. These results suggest that GlyA is not associated with $GABA_A$ receptor- or serotonin transporter-mediated anxiolytic actions.

It is possible that maternal separation-induced USV in neonatal rodents is not a good model of adult anxiety for examining GlyT1 inhibitors, if the expression levels of GlyT1, GlyA, and GlyB markedly differ between pups and adults. However, several reports provide evidence that this is not the case. Lall et al. (2012) have reported that the expression levels of both GlyT1 and GlyA differ by 20% in the brain stems of 10-day-old mouse pups and adult mice. Suen et al. (1998) have reported that the protein levels of NR1 subunits in the rat cortical postsynaptic

density is 1.6-fold greater in adult rats than in rats at postnatal day 10. Although these differences are unlikely to change the conclusions of the present study, other paradigms such as the elevated plus maze test should be examined in adult rodents to provide further confirmatory evidence of our conclusions.

In conclusion, the present data suggest that the anxiolytic effects of GlyT1 inhibitors are mediated through GlyA but not through GlyB. Several GlyT1 inhibitors are currently in clinical development for the treatment of schizophrenia and obsessive–compulsive disorder (Umbricht et al., 2014; ClinicalTrials.gov Identifier: NCT01674361), and a recent clinical study has demonstrated that sarcosine, a GlyT1 inhibitor, improves psychic anxiety, as assessed by means of the 17-item Hamilton Depression Rating Scale, in patients with major depression (Huang et al., 2013). Our findings further demonstrate the anxiolytic effects of GlyT1 inhibitors and provide new insights into the mechanism of these anxiolytic effects.

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