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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 (Kuryatov et al., 1994; Legendre, 2001). 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