Glycine Transporter Blockade Ameliorates Motor Ataxia in a Mouse Model of Spinocerebellar Atrophy

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Abstract. Ataxic movement, the common major symptom of spinocerebellar atrophy, has been considered to involve impaired glutamatergic excitatory neurotransmission in the cerebellum. Considering the therapeutic importance of ataxia control, we assessed the effectiveness of increasing the extracellular concentration of glycine by administering it exogenously or via blockade of glycine transporter 1, using its selective inhibitors sarcosine and N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS), for amelioration of motor ataxia in a mouse model of spinocerebellar atrophy developing after neonatal treatment with cytosine β-D-arabinofuranoside. Intracerebroventricular (i.c.v.) injection of sarcosine (3, 10, and 30 μg) and NFPS (0.01 and 0.03 μg) reduced the number of falls without affecting spontaneous motor activity, and therefore the falling index [(number of falls / spontaneous motor activity) × 100], and dose-dependently ameliorated ataxic movements. Similar effects were observed upon i.c.v. injection of D-serine (1 and 10 μg), an agonist of the glycine-recognition site of the N-methyl-D-aspartate (NMDA) receptor. However, exogenously injected glycine (1, 3, and 10 μg, i.c.v.) only weakly ameliorated the ataxic movements at 3 μg. These results suggest the therapeutic relevance of GlyT1 inhibitors for amelioration of motor ataxia in spinocerebellar atrophy by increasing the endogenous concentration of glycine near the glycine-recognition site of the NMDA receptor.

Keywords: motor ataxia, spinocerebellar atrophy, glycine, D-serine, glycine transporter

Introduction

Ataxic movement is the common major symptom of spinocerebellar atrophy; and it has been considered to involve impaired glutamate signaling in the cerebellum, as supported by several lines of evidence including a decrease in the glutamate content of the cerebellar tissue taken postmortem from patients with spinocerebellar atrophy (1, 2), generation of ataxic movements by blockade of ataxic movements by blockade of N-methyl-D-aspartate (NMDA) receptors in humans and animals (3, 4), and their improvement by the NMDA receptor allosteric agonists D-serine and D-cycloserine in animals (5, 6) and also humans (7).

D-Serine and D-cycloserine bind to the glycine-recognition site of the NMDA receptor where glycine acts as a co-agonist of glutamate to facilitate excitatory synaptic transmission mediated by the NMDA receptor. Therefore, an increase in the extracellular glycine concentration may have a similar therapeutic impact on motor dysfunction associated with spinocerebellar atrophy. The extracellular concentration of glycine is regulated by its reuptake via sodium/chloride-dependent glycine transporters (GlyTs) into presynaptic terminals of glycinergic inhibitory neurons and glial cells adjacent to inhibitory and excitatory synapses (8, 9). Two GlyT subtypes, GlyT1 and GlyT2, have been identified so far, and both participate in high-affinity uptake of glycine in axons and synapses (10). It is likely that GlyT2, which is present in axons and presynaptic terminals of inhibitory glycinergic neurons, has an essential role in the refilloming of synaptic vesicles with glycine and therefore in the maintenance of glycinergic inhibitory synaptic transmission (11). By contrast, GlyT1, localized mostly in glial cells, reduces glycine concentration near NMDA receptors. Since GlyT1 also eliminates glycine from the synaptic cleft to terminate glycinergic neurotransmission

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Materials and Methods

All of the experimental protocols were approved by the Animal Care and Use Committee of Nagoya City University and were carried out according to the guidelines of the National Institutes of Health and the Japanese Pharmacological Society.

Preparation of the animal model

Male ICR-strain mice were used for preparation of the ataxic model. In brief, ataxia was chemically induced by repeated subcutaneous administration of Ara-C (60 mg/kg per day) on the second, third, and fourth postnatal days, following a modification of the method of Saigoh et al. (6, 15). Animals were allowed free access to food and water and maintained under a 12-h light / 12-h dark cycle in temperature- and humidity-controlled rooms.

Assessment of motor dysfunction and evaluation of the effects of drugs

At 5 weeks of age, the spontaneous motor activity of each mouse during exploratory behavior in an open arena (18 cm × 28 cm floor with 13-cm-high walls) was measured for 30 min by using an automated behavioral experimental apparatus (Animex IIIA; Shimadzu, Kyoto). In this equipment, the movement detector operates by counting the number of times an animal elicits a capacitance change. The number of falls was measured for 30 min by using an automated behavioral experimental apparatus (Animex IIIA; Shimadzu, Kyoto). In this equipment, the movement detector operates by counting the number of times an animal elicits a capacitance change. The number of falls was also counted by behavioral observation at the same time. Falling index was estimated as: falling index = (number of falls / spontaneous motor activity) × 100.

Measurements of spontaneous motor activity and the number of falls were conducted once a day for two consecutive days. Mice showing more than 10 falls at the first measurement were used for further evaluation of the effects of drugs on the second day.

Drugs

Glycine was purchased from Nacalai Tesque (Kyoto), and sarcosine, D-serine, and Ara-C were from Sigma Chemical Co. (St. Louis, MO, USA). All were dissolved in 0.9% physiological saline. NFPS was obtained from Tocris Cookson (Bristol, United Kingdom; in vivo experiments conducted with the permission of NPS Pharmaceuticals, Inc., Parsippany, NJ, USA) and dissolved in 20% 2-hydroxypropyl-β-cyclodextrine (2-HP-β-CD). All drugs and their vehicle except for Ara-C were administered intracerebroventricularly (i.c.v.) immediately before assessment of motor dysfunction on the second day in a volume of 5 μL via a disposable 27-gauge needle, which was inserted into the lateral ventricle (16).

Statistics

All data were expressed as the mean ± S.E.M. To evaluate the effect of drugs on the falling index, the paired t-test was used. Dose-dependent effects of drugs were assessed using the ratio of the falling index after drug administration divided by that on the first day (falling index ratio), and two-tailed multiple t-test with Bonferroni correction following one-way analysis of variance (ANOVA) was employed (17). Differences at P<0.05 were considered significant.

Results

Mice generally exhibited lower spontaneous motor activity when tested after i.c.v. administration of drugs (D-serine, sarcosine, NFPS, and glycine) or their vehicles (saline for D-serine, sarcosine and glycine and 20% 2-HP-β-CD for NFPS) compared with that assessed one day before. However, there were no significant differences in spontaneous motor activity between the vehicle- and drug-treated animal groups (Fig. 1). In this study, therefore, the changes in the falling index closely reflected the changes in the number of falls during unaffected spontaneous motor activity.

We first assessed the effectiveness of D-serine in ameliorating motor ataxia. As shown in Fig. 2, D-serine (1 and 10 μg, i.c.v.) reduced the falling index (significant at 1 and 10 μg), and dose-dependently decreased the falling index ratio (1.04 ± 0.17 for saline; 0.75 ± 0.10 and 0.26 ± 0.04 for 1 and 10 μg, respectively; P<0.01 at 10 μg, n = 8−10) and therefore dose-dependently ameliorated the motor dysfunction, thus supporting the study by Saigoh et al. (6). A similar reduction of the falling index (data not shown) and a dose-dependent
decrease in the falling index ratio were obtained after i.c.v. injection of the GlyT1 inhibitors sarcosine (3, 10, and 30 μg; Fig. 3A) and NFPS (0.01 and 0.03 μg, Fig. 3B). Sarcosine decreased the falling index ratio from 1.00 ± 0.13 (saline) to 0.73 ± 0.11, 0.54 ± 0.09, and 0.40 ± 0.05 at 3, 10, and 30 μg, respectively (P<0.01 at 10 and 30 μg, n = 9 – 10). NFPS decreased the falling index ratio from 1.04 ± 0.08 (2-HP-β-CD) to 0.46 ± 0.10 and 0.35 ± 0.06 at 0.01 and 0.03 μg, respectively (P<0.01 at 0.01 and 0.03 μg, n = 6 – 7).

In contrast, exogenously applied glycine (1, 3, and 10 μg, i.c.v.) produced a weak reduction of the falling index only at 3 μg (data not shown). As was revealed in the falling index ratio, glycine in this dose range did not cause any dose-dependent or significant decrease in this ratio (Fig. 3C; 0.85 ± 0.08 for saline; 0.89 ± 0.14, 0.58 ± 0.07, and 0.83 ± 0.13 for 1, 3, and 10 μg, respectively, n = 7 – 10).

**Discussion**

In this study, we employed the DNA polymerase inhibitor Ara-C to produce spinocerebellar ataxia involving impaired glutamate signaling in the cerebellum in mice. Early postnatal administration of Ara-C results in hypoplasia of the cerebellum and prominent loss of granule cells (15) and therefore insufficient excitatory synaptic inputs to Purkinje cells via parallel fibers from the granule cells. Restoration of excitatory transmission by facilitating NMDA-receptor function in the cerebellum has been considered to be of crucial importance in the treatment of spinocerebellar ataxia. Consistently, i.c.v. injection of D-serine, the agonist of the glycine-recognition site of the NMDA receptor, resulted in amelioration of motor dysfunction. More importantly, we demonstrated for the first time that stimulation of the glycine-recognition site of the NMDA receptor by an endogenous increase in the extracellular glycine concentration via blockade of GlyT1 is strikingly effective in reducing ataxic movement and therefore has therapeutic potential for patients with spinocerebellar ataxia.

In addition to the previously proposed role of GlyT1 in regulating the concentration of glycine at excitatory
synapses containing NMDA receptors, which require glycine as a co-agonist, recent evidence employing GlyT1-deficient mice has demonstrated that GlyT1 has a role in terminating glycinergic neurotransmission by uptake of glycine into neighboring glial cells (12). Hence, blockade of GlyT1 potentially elicits both excitatory and inhibitory effects. It is likely, however, that the net effect of endogenously increased glycine via blockade of GlyT1 on neuronal excitatory and inhibitory balance depends on the excitability of the brain area that is accessible to glycine. In hyperexcitable states, glycine is considered to elicit neuronal inhibition via strychnine-sensitive glycine receptors by overcoming any potential increase in glycine-mediated NMDA receptor–induced excitability (13, 18). In contrast, increased endogenous extracellular glycine in hypoglutamatergic states plausibly generates a net excitatory influence on neuronal excitability, as we demonstrated in this study using mice with a hypoglutamatergic cerebellum under conditions of spinocerebellar ataxia (6, 15).

Exogenously applied glycine did not markedly ameliorate motor dysfunction and had differential dose-dependent effects. Although glycine at 3 μg tended to reduce ataxic movement, a higher dose (10 μg) had no ameliorating effects. Stimulation of the glycine-recognition site of the NMDA receptor with higher concentrations of glycine facilitates NMDA-receptor internalization (19), possibly explaining the present lack of amelioration observed with 10 μg glycine. However, this is unlikely since D-serine, which has also been demonstrated to prime NMDA-receptor internalization (19), dose-dependently ameliorated ataxic movement. Rather, it is plausible that inhibition mediated by synaptic and/or extrasynaptic strychnine-sensitive glycine receptors exceeded or masked NMDA receptor–mediated excitation even in hypoglutamatergic states, when the glycine concentration was excessively increased via an exogenous route. It would be of interest to assess whether the endogenous increase of extracellular glycine by blockade of GlyT2 in the cerebellum deteriorates ataxic movement through preferential stimulation of co-localized strychnine-sensitive glycine receptors. This issue warrants investigation in the near future.

As our recent study (13) and a study by Morita et al. (20) have demonstrated, GlyT1 blockers have analgesic effects at the spinal level in various pain models in mice. The analgesic action of GlyT1 inhibitors seems to be beneficial for patients with spinocerebellar ataxia, as pain including generalized muscle and joint pain is reported to be a common feature of Machado-Joseph disease, the most common spinocerebellar ataxia that is inherited in an autosomal dominant manner (21, 22).

Since, in this study, drugs were administered intracerebroventricularly, it is possible that they indirectly restored excitatory transmission of the cerebellum by acting on other brain regions. Further studies to clarify their precise sites of action are necessary. Nevertheless, the present study has provided a novel therapeutic strategy for treating patients with spinocerebellar atrophy by recruiting endogenous glycine with GlyT1 inhibitors to restore glutamatergic excitatory transmission mediated by NMDA receptors.

References