Blockade of Glycine Transporter (GlyT) 2, but Not GlyT1, Ameliorates Dynamic and Static Mechanical Allodynia in Mice With Herpetic or Postherpetic Pain

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Abstract. Glycine is an inhibitory neurotransmitter in the spinal dorsal horn and its extracellular concentration is regulated by glial glycine transporter (GlyT) 1 and neuronal GlyT2. This study was conducted to elucidate the effects of intrathecal injections of GlyT1 and GlyT2 inhibitors on two distinct types of mechanical allodynia, dynamic and static allodynia, in mice with herpetic or postherpetic pain. The GlyT2 inhibitor ALX1393, but not the GlyT1 inhibitor sarcosine, suppressed dynamic and static allodynia at the herpetic and postherpetic stages. Intrathecal ALX1393 suppressed dynamic allodynia induced by intrathecal strychnine and N-methyl-D-aspartate (NMDA). Intrathecal sarcosine suppressed dynamic allodynia induced by intrathecal strychnine, but not NMDA. Expression level of GlyT1, but not GlyT2, mRNA in the lumbar dorsal horn was decreased at the herpetic and postherpetic stages. Glycine receptor α1-subunit mRNA was decreased in the lumbar dorsal horn at the herpetic, but not postherpetic stage, without alteration in α3-subunit mRNA. The results suggest that GlyT2 is a potential target for treatment of dynamic and static allodynia in patients with herpetic zoster and postherpetic neuralgia. The lack of efficacy of GlyT1 inhibitor may be explained by activation of NMDA receptors and the down-regulation of GlyT1 in the lumbar dorsal horn.

Keywords: dynamic allodynia, herpetic pain, postherpetic neuralgia, glycine transporter, glycine receptor

Introduction

Herpes zoster is caused by the reactivation of varicella-zoster virus in the sensory ganglion and is characterized by clustered vesicles and severe pain (1). In some herpes zoster patients, pain persists long after healing of the skin lesions, which is postherpetic neuralgia (1). Once established, postherpetic neuralgia is particularly difficult to treat and is often resistant to conventional analgesics (2). Patients with herpes zoster or postherpetic neuralgia complain of various types of pain, including a spontaneous pain and mechanical allodynia, pain elicited by normally innocuous mechanical stimulation. Two distinct types of mechanical allodynia, static and dynamic alloyd
findings suggest that these two kinds of mechanical allodynia are mediated by discrete pathophysiological mechanisms.

Several lines of evidence suggest that peripheral neuronal mechanisms, such as nociceptor sensitization, and changes in spinal neuronal function play significant roles in the development of mechanical allodynia (10). Our recent study has demonstrated that the excitatory response of spinal dorsal horn neurons to brush stimulation of the lesional skin was increased in herpetic mice, while the response of the tibial nerve, a branch of the sciatic nerve innervating the affected skin, to brush stimulation was decreased (11). These findings suggest that dynamic allodynia in the affected dermatome is due to the increased excitability of spinal dorsal horn neurons, but not primary afferents, to brush stimulation.

Dysfunction of inhibitory synaptic transmission in the spinal dorsal horn is considered to be involved in the development of neuropathic pain (12). Inhibitory glycine-ergic neurons and glycine receptors are abundant in the spinal dorsal horn (13–15). The glycine-receptor antagonist strychnine elicits mechanical allodynia that is caused by glycine-glycinergic disinhibition in the spinal dorsal horn (16, 17). Thus, an enhancement of glycinegic inhibition in the spinal dorsal horn is a potential strategy for relief of neuropathic pain. The extracellular concentration of glycine is regulated by its re-uptake into glycine-ergic presynaptic terminals and uptake into glial cells adjacent to inhibitory and excitatory synapses via Na+/Cl−-dependent glycine transporters (GlyTs) (18). Two GlyT subtypes encoded by distinct genes, GlyT1 and GlyT2, have been identified in the mammalian central nervous system (19, 20). Some recent studies have demonstrated that intrathecal injections of GlyT1 and GlyT2 inhibitors produce anti-allodynic and anti-hyperalgesic effects in animal models of neuropathic and inflammatory pains (21–23). The present study was conducted to elucidate the effects of the blockade of GlyT1 and GlyT2 in the spinal cord on mechanical, especially dynamic, allodynia in mice with herpetic or postherpetic pain.

**Materials and Methods**

**Animals**

Female C57BL/6j mice (6-week-old at the start of experiments; Japan SLC, Shizuoka) were used. They were housed 4–6 per cage under controlled temperature (22 ± 1°C) and humidity (55 ± 10%). The room was lighted from 07:00 to 19:00 h. Food and water were freely available. Experiments were conducted with the approval of the Animal Care Committee at University of Toyama and according to the guidelines for investigations of experimental pain in animals published by the International Association for the Study of Pain (24).

**Virus inoculation**

The mice were inoculated with HSV, as described previously (8). Briefly, 5 μl of a suspension of HSV (7401H strain, 1 × 10^6 plaque-forming units) was administered topically on the scarified skin of the femur of the right hind paw.

**Intrathecal injection**

The GlyT1 inhibitor sarcosine (18), the GlyT2 inhibitor ALX1393 (25), glycine hydrochloride, and N-methyl-D-aspartate (NMDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and strychnine nitrate was from Wako Pure Chemical Ind., Ltd. (Osaka). Sarcosine, strychnine, glycine, and NMDA were dissolved in 0.9% saline. ALX1393 was dissolved in 25% 2-hydroxypropyl-β-cyclodextrin (Nacalai Tesque, Kyoto) and pH was adjusted to about 6 with 1 N sodium hydroxide. A 30-gauge stainless steel needle attached to a microsyringe was inserted between the L5 and L6 vertebrae in animals under diethyl ether anesthesia, and the agents were administered slowly through a lumbar puncture in a volume of 5 μl (26). Weights of glycine and strychnine refer to the salts.

**Assessment of mechanical alldynia**

The mice were acclimated to an observation cage for at least 30 min immediately before the behavioral test. In mice with herpetic or postherpetic pain, two types of mechanical allodynia (static and dynamic allodynia) of the affected hindpaw were assessed as described previously (8). The mice were individually put in an observation cage (8 × 8.5 × 8 cm, width × depth × height) with a wire mesh bottom. Static allodynia was assessed by punctate stimulation of the plantar region of the hindpaw with von Frey filament of 1.6-mN strength (North Coast Medical, Inc., Morgan Hill, CA, USA). Dynamic alldynia was assessed by light stroking of the plantar surface of the hindpaw from the toe to the heel with an art paint-brush [Artjetje (round) Calmon-Pro 720TM #4/0; Chugoku Art Material, Inc., Okayama], the hairs of which were trimmed with 10 hairs left. Responses to the punctate or stroking stimulation of the hindpaw were ranked as follows: 0, no response or moving the stimulated paw aside; 1, lifting of the stimulated paw toward the abdomen; 2, flinching or licking of the stimulated hind paw. In mice given intrathecal injection of strychnine or NMDA, alldynia of the flank was assessed as described (27), with slight modifications. Immediately after intrathecal injection, the mice were individually put in an observation cage (17 × 25 × 12 cm, width × depth × height). The flank was stroked with an untrimmed
paintbrush and responses to the stroking stimulation were ranked as follows: 0, no response; 1, moving away from the stroking paintbrush; 2, biting at the probe, or strong efforts to escape from the paintbrush. The stimulation was applied six times at intervals of several seconds and the average served as pain-related score. Mice that showed 0.5 or higher pain-related score were considered to have allodynia (8).

**Determination of mRNA**

After decapitation under diethyl ether anesthesia, the ipsilateral spinal dorsal horn at the level of L4–L5 were rapidly removed on day 7 and day 35 after inoculation and were stored at −80°C until assay. After extraction of total RNA, quantitative reverse transcription-polymerase chain reaction was performed using the Mx3000P™ real-time PCR system (Stratagene Japan K.K., Tokyo), as described previously (28). cDNA was amplified with the following primers: 5′-AAC TGG GGC AAC CAG ATC GA-3′ (sense) and 5′-GTA CAT GAT ACC CGT GAA GGC-3′ (antisense) for GlyT1; 5′-TCT GCA GGG ATT GAA TAT CC-3′ (sense) and 5′-GTT TGT AGT GCT GTT TGG TGC-3′ (antisense) for GlyT2; 5′-AGG CCC AAC TTC AAA GGT CC -3′ (sense) and 5′-AGT GTT GCC CTC-3′ (antisense) for glycine receptor α1-subunit; 5′-AGT GAC ATT AAC ACT CTC TTG GCC CTC-3′ (sense) and 5′-CCA TCC AGA TGT CAA TTG CCT T-3′ (antisense) for glycine receptor α3-subunit; 5′-TGA CCA AGG TCA GCC ATG ACA AC-3′ (sense) and 5′-TTA CTC CTT GGA GCC CAT GT-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR was performed with 40 cycles (denaturation at 95°C for 30 s; primer annealing for 30 s at 45°C for glycine receptor α1, 50°C for glycine receptor α3, 55°C for GlyT1 and GlyT2, 60°C for GAPDH; and elongation at 72°C for 50 s). Target mRNA expression levels were normalized to GAPDH mRNA expression in each sample.

**Data analyses**

Data are expressed as the mean ± S.E.M. Data on the time course of anti-allodynic effects were analyzed with Friedman repeated measures analysis of variance on ranks followed by the post hoc Dunnett’s test. Data on the area under the curve of anti-allodynic effects and the mRNA expression were analyzed with one-way analysis of variance followed by the post hoc Dunnett’s test. A value of $P < 0.05$ was considered significant.

**Results**

**Effects of GlyT inhibitors on dynamic allodynia at the herpetic and postherpetic stages**

Since dynamic allodynia is marked at both herpetic and postherpetic stages (8), the effects of intrathecal injections of the GlyT1 inhibitor sarcosine and the GlyT2 inhibitor ALX1393 were examined on day 7 (herpetic stage) and day 35 – 40 (postherpetic stage) after herpes virus inoculation. Dynamic allodynia was tested by stroking the affected hindpaw with a trimmed paintbrush; pain-related score is described in Materials and Methods. The data presented are means ± S.E.M. (n = 6 – 8).

![Fig. 1. Effects of intrathecal injections of the GlyT1 inhibitor sarcosine on dynamic allodynia produced by herpes virus inoculation. Sarcosine (10 and 30 µg/site; gray circle and triangle, respectively) and vehicle (open circle) were injected intrathecally on day 7 (A, herpetic stage) and day 35 – 40 (B, postherpetic stage) after herpes virus inoculation. Dynamic allodynia was tested by stroking the affected hindpaw with a trimmed paintbrush; pain-related score is described in Materials and Methods. The data presented are means ± S.E.M. (n = 6 – 8).](image)
Effects of Glycine Transporter and Allodynia

**Effects of GlyT inhibitors on static allodynia at the postherpetic stage**

Static allodynia is observed in about half of mice inoculated at the postherpetic stage but not obvious at the herpetic stage (8). Therefore, the effects of intrathecal sarcosine and ALX1393 were examined at the postherpetic stage (day 35 – 40 after inoculation), and mice that showed 0.5 or higher pain-related score were used. Intrathecal sarcosine (10 and 30 μg/site) did not affect static allodynia (Fig. 3A). Intrathecal injections of ALX1393 (1, 3, and 5 μg/site) produced a dose-dependent inhibition of static allodynia; the effects peaked from 30 min to 1 h after injection (Fig. 3B).

**Effects of Glycine on mechanical allodynia at the herpetic and postherpetic stage**

Intrathecal injections of glycine produced only partial inhibition of mechanical allodynia at the herpetic and postherpetic stages (Fig. 4). There was a decreased tendency of static allodynia at the herpetic stage after intrathecal doses of 3 and 10 μg/site (Fig. 4A). An intrathecal dose of 10, but not 3, μg/site produced a partial but significant inhibition of dynamic allodynia at the postherpetic stage; the effect peaked at 15 min and subsided 45 min after injection (Fig. 4B). There was a decreased tendency of static allodynia at the postherpetic stage after intrathecal doses of 10 and 30 μg/site (Fig. 4C).

**Effects of GlyT inhibitors on dynamic allodynia induced by strychnine and NMDA**

Glycine is a major inhibitory transmitter acting on the strychnine-sensitive glycine receptors in the spinal cord and the brainstem (29). Glycine also acts as co-agonist for NMDA glutamate receptors to facilitate excitatory synaptic transmission (30, 31). Since intrathecal injection of either strychnine or NMDA induces dynamic allodynia (27, 32), manipulation of synaptic glycine concentrations with GlyT inhibitors may affect dynamic allodynia by influencing both glycine-dependent inhibitory and glutamatergic excitatory transmission. To address this question, we
examined the effects of sarcosine and ALX1393 on dynamic allodynia induced by intrathecal injections of strychnine and NMDA. Strychnine (1 μg/site) caused dynamic allodynia, which was inhibited by pre-treatment with either ALX1393 (5 μg/site) or sarcosine (30 μg/site) (Fig. 5: A and B). Intrathecal NMDA also caused dynamic allodynia, which was inhibited by pre-treatment with ALX1393 (5 μg/site), but not sarcosine (30 μg/site) (Fig. 5: C and D).

Expression of mRNAs encoding GlyTs and glycine receptor subunits in the lumbar dorsal horn

The validity of real-time PCR was confirmed by the melting temperature analysis and a standard curve. The melting temperature analysis showed single peaks for mRNAs encoding GlyT1, GlyT2, glycine receptor α1-and α3-subunits, and GAPDH (Fig. 6A). Standard curves for these mRNAs were linear over the range of 0.01 – 100 relative cDNA concentration (Fig. 6B).

The expression level of GlyT1 mRNA in the lumbar dorsal horn was significantly decreased at the herpetic and postherpetic stages (Fig. 6C). There was a decreased tendency of the GlyT2 mRNA expression at the herpetic stage, while there was no alteration at the postherpetic stage (Fig. 6D). With regard to glycine receptors in the lumbar dorsal horn, the expression level of mRNA encoding glycine receptor α1-subunit was decreased at the herpetic stage, without significant changes at the postherpetic stage (Fig. 6E). No significant alterations were observed in the expression of glycine receptor α3-subunit mRNA at the herpetic and postherpetic stages (Fig. 6F).

Discussion

In the present study, intrathecal injection of the GlyT2 inhibitor ALX1393 dose-dependently suppressed dynamic allodynia in mice at the herpetic and postherpetic stages. On the other hand, intrathecal injection of the GlyT1 inhibitor sarcosine was without effects on the dynamic allodynia. An important finding in the present study is that ALX1393 inhibited dynamic allodynia as well as static allodynia; it almost abolished both types of allodynia at an intrathecal dose of 5 μg/site. Our previous studies have shown that dynamic allodynia is more resistant to the analgesics and analgesic adjuvants than is static allodynia. Systemic administration of mexiletine and ketamine inhibit static, but not dynamic, allodynia (8). Morphine hydrochloride at a subcutaneous dose of 3 mg/kg partially inhibited static allodynia, but the same dose did not affect dynamic allodynia (Sasaki et al., unpublished observation). Gabapentin inhibits both types of allodynia, but the inhibition is more marked in static allodynia than dynamic allodynia (8). In many patients with herpes zoster and postherpetic neuralgia, dynamic allodynia is so afflictive that the quality of life is decreased and dynamic allodynia is a hallmark symptom. GlyT2, but not GlyT1, inhibitors may be effective against dynamic allodynia in patients with herpes zoster and postherpetic neuralgia.

GlyT2 is restricted to glycinergic synapse–rich regions in the central nervous system including the spinal cord (33, 34). Based on the localization, GlyT2 is thought to function as a glycine re-uptake transporter at inhibitory
Glycinergic synapses (33, 35). Glycinergic transmission is increased by the pharmacological blockade of GlyT2 in lamina X neurons of rat spinal cord slices (36). An increase in extracellular glycine was also demonstrated by microdialysis perfusion of the dorsal spinal cord of rats with the GlyT2 inhibitor ORG25543 (37). Taken together, these findings suggest that anti-allodynic effect of intrathecal ALX1393 observed in the present study results from the accumulation of glycine at the glycinergic synaptic cleft and subsequent suppression of excitatory neuronal activities in the spinal dorsal horn. Glycinergic inhibitory postsynaptic currents are markedly reduced in motoneurons from GlyT2-deficient mice (38). This may be due to reduced glycine content in the synaptic vesicles and hence insufficient glycine release, suggesting that GlyT2 has an essential role in replenishment of glycine into glycinergic nerve terminals. If this occurred in glycinergic neurons in the spinal dorsal horn, the long-term use of GlyT2 inhibitors may lead to the reduction of glycinergic inhibition and the anti-allodynic effect. However, this possibility seems unlikely. Repeated systemic administration of ORG25543 has been shown to inhibit allodynia without apparent tolerance in mice which underwent partial sciatic nerve ligation (22). In this case, the inhibition of GlyT1 function is expected to inhibit pathological pain due to the accumulation of glycine at the site of inhibitory glycine receptors. The present study demonstrated that intrathecal strychnine-induced dynamic allodynia was inhibited by intrathecal sarcosine (and also by ALX1393), supporting the ability of GlyT1 inhibitor to enhance glycinergic inhibition. However, the expression of GlyT1 is very high in the laminae I and II of the dorsal horn (33), an area where the expression of NMDA receptors is rich (41, 42). Glycine acts as an essential co-agonist of glutamate at NMDA receptors (30), and its binding to the modulatory site of NMDA receptor is necessary for ion-channel opening (31). Based on these localizations, GlyT1 is thought to play a role in controlling glycine levels around glutamatergic synapses and thereby the regulation of NMDA receptor activity. The inhibition of this GlyT1 role would counteract the ability of the GlyT1 inhibitor to suppress dynamic allodynia. Intrathecal NMDA–induced dynamic

Fig. 5. Effects of intrathecal injections of GlyT inhibitors on dynamic allodynia induced by intrathecal injection of strychnine (STR) or NMDA. STR (A and B; 1 μg/site, open circle and column) and NMDA (C and D; 100 ng/site, open circle and column) were injected intrathecally and dynamic allodynia was tested for 2 h; (A and C) Time course and (B and D) area under the curve (AUC) of pain-related score during the 2-h period are shown. Sarcosine (30 μg/site, gray circle and column) and ALX1393 (5 μg/site, closed circle and column) were injected intrathecally 15 min before STR or NMDA. Dynamic allodynia was tested as described in the Fig. 1 legend. The data presented are means ± S.E.M. (n = 6). *P < 0.05, as compared with STR or NMDA alone (Dunnett’s test).
signaling cascade (37). The GlyT2 inhibitor ORG25543 induces a similar increase in glycine levels without affecting citrulline release (37). With these findings taken into account, the present results suggest that the inhibitory effects of GlyT1 inhibitor are masked by excitatory effects via glycine-mediated activation of NMDA receptors in mice with herpetic or postherpetic allodynia.

In the present study, the level of GlyT2 mRNA was not significantly altered in the lumbar dorsal horn at the herpetic and postherpetic stages, whereas the level of GlyT1 mRNA was significantly decreased at the herpetic and postherpetic stages. The results raise the possibility of the reduced action of GlyT1 inhibitor and this may be another explanation for the absence of the action of intrathecal sarcosine. Intrathecal injection of the GlyT1 inhibitor ALX5407 has been reported to suppress static allodynia in rats which underwent chronic constriction injury of the sciatic nerve (21), in which the expression level of GlyT1 is decreased in the lumbar spinal cord (43). There are at least six GlyT1 splice variants (18, 44).

Since the functional roles of these splice variants and the splice variant selectivity of the GlyT1 inhibitors including sarcosine are unknown, the present results do not deny the possibility that GlyT1 inhibitors other than sarcosine affect allodynia induced by herpes virus inoculation. Further experiments are needed to address this issue.

Glycine receptor α1-subunit is a component of the major glycine receptor isoform (α1β) in adult spinal cord (14). Glycine receptor α3-subunit is distinctly expressed in the superficial laminae of the mouse dorsal horn (15), suggesting that it is involved in the regulation of spinal nociceptive processing. The present study demonstrated that the expression level of mRNA encoding glycine receptor α1-subunit was decreased in the lumbar dorsal horn at the herpetic stage, but not postherpetic stage, and α3-subunit mRNA was not significantly altered at the herpetic and postherpetic stages. Therefore, global (not specific for nociceptive transmission) glycineric function may be reduced in the dorsal horn at the herpetic stage.

In the present study, intrathecal glycine (3 – 30 μg/site) produced weak inhibition of dynamic and static allodynia in mice at the herpetic and postherpetic stages. Considering that exogenous glycine does not produce the same effects as endogenous glycine, the results are consistent with the effects of the GlyT2 inhibitor ALX1393. Intrathecal glycine (1 and 3 μg/site) has been reported to produce marked inhibition of static allodynia in mice that underwent partial sciatic nerve ligation (23). The difference in anti-allodynic efficacy of intrathecal glycine between pain models produced by herpes infection and surgical injury of the sciatic nerve may be partly due to

![Graphs and Tables]

**Fig. 6.** Expression levels of mRNAs encoding glycine transporters and glycine receptor subunits in the lumbar dorsal horn. The dorsal horn of L4–L5 spinal cord was isolated from naive mice inoculated with herpes virus at the herpetic (HS, 7 days after inoculation) and postherpetic stages (PHS, 35 days after inoculation). Expression levels of mRNAs encoding glycine transporters GlyT1 and GlyT2 and glycine receptor α1- and α3-subunits (GlyRα1 and GlyRα3, respectively) were determined by real-time PCR and normalized to that of GAPDH mRNA in each sample. A) Melting temperature analysis, B) standard curves, C) GlyT1, D) GlyT2, E) GlyRα1, and F) GlyRα3. Results are shown as the ratio of expression level in herpes-inoculated mice to that of naive mice. The data presented are means ± S.E.M. (n = 6). *P < 0.05 as compared with the control (Dunnett’s test).

Allodynia was inhibited by intrathecal ALX1393, but not sarcosine, supporting the idea that blockade of GlyT1, but not GlyT2, can enhance excitatory effects via glycine-mediated activation of NMDA receptors. In fact, a study using microdialysis perfusion of the lumbar spinal cord of rats has demonstrated that the GlyT1 inhibitor ORG24598 increases extracellular glycine accompanied by a progressive increase in citrulline release, an index for the activation of the NMDA/nitric oxide synthase...
differences in expresionnal and functional changes of GlyT1, GlyT2, glycine receptors, and/or glycine binding site of the NMDA receptor.

Changes in the balance between local excitatory and inhibitory synaptic inputs in the spinal dorsal horn, and therefore a net increase in spinal excitation, is considered to be a crucial mechanism involved in developing pathological pain (12). The decrease of glycine receptor α1-subunit mRNA suggests that dysfunction of inhibitory glycinergic transmission is a factor in the pathogenesis of herpetic allodynia. The expression level of GlyT1 mRNA was significantly decreased in the lumbar dorsal horn at the herpetic stage and postherpetic stage. It is possible that a decrease in the expression level of GlyT1 in the spinal dorsal horn is involved in the pathogenesis of herpetic and postherpetic pain via the activation of NMDA receptors.

In summary, intrathecal injection of the GlyT2 inhibitor ALX1393 produced the potent inhibition of dynamic and static allodynia in mice with herpetic or postherpetic pain, suggesting that GlyT2 is a potential target for treatment of intractable dynamic allodynia as well as static allodynia in patients with herpetic zoster or postherpetic neuralgia. The GlyT1 inhibitor sarcoine did not suppress dynamic and static allodynia. This ineffectiveness can be in part explained by the masking of the inhibitory effect by NMDA-receptor activation and the down-regulation of GlyT1 and glycine receptor.

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