

## Glycine Transporter Inhibitors as a Potential Therapeutic Strategy for Chronic Pain with Memory Impairment

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**Background:** Impaired excitatory and inhibitory balance in the spinal dorsal horn has a crucial role in the pathophysiology of chronic pain. The authors addressed the therapeutic impact of increasing spinal glycine applied exogenously or *via* blockade of glycine transporter 1 using its selective inhibitors sarcosine and *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine on neuropathic and inflammatory pain in mice.

**Methods:** Mice with thermal and mechanical hypersensitivity after partial ligation of the sciatic nerve (Seltzer model) or mice with mechanical hypersensitivity after streptozotocin injection received intrathecal injection of glycine, sarcosine, and *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine. These drugs were also intrathecally injected in mice to assess their effects on formalin-evoked nociceptive behaviors. The supraspinal effect of blockade of glycine transporter 1 was studied on tetanus-induced long-term potentiation of the Schaffer-collateral synapses in hippocampal slices prepared from Seltzer model mice.

**Results:** Glycine, sarcosine, and *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine ameliorated thermal and mechanical hypersensitivity in Seltzer model mice, and reduced mechanical hypersensitivity in streptozotocin-injected diabetic mice. Moreover, they selectively inhibited the second phase of formalin-evoked licking/biting behavior. In hippocampal slices prepared from Seltzer model mice, long-term potentiation was maintained at a significantly lower level than that in sham-treated mice. Such impairment of long-term potentiation was never observed when it was induced in the presence of *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine.

**Conclusions:** An increase in endogenous glycine *via* glycine transporter 1 blockade not only results in a net inhibitory influence on pain transmission at the spinal level but also supraspinally relieves decreased synaptic efficacy presumably related to cognitive disturbance often described in patients with chronic pain.

IN neuropathic pain and inflammatory pain, enhanced efficacy of synaptic transmission in the dorsal horn of the spinal cord underlies central major aspects of hyperalgesia and tactile allodynia.<sup>1,2</sup> Such increases in synaptic efficacy are ascribed to short- and long-term changes in the excitability of primary sensory afferent terminals and spinal dorsal horn neurons,<sup>3,4</sup> including alterations in the function and/or expression of various receptors and ion

channels that play a crucial role in the determination of membrane potential.

Moreover, changes in balance between local excitatory and inhibitory synaptic inputs in the spinal dorsal horn may profoundly influence pain signal transmission. For example, removal of spinal glycinergic or  $\gamma$ -aminobutyric acid-mediated (GABAergic) inhibitory synaptic transmission by the selective antagonist strychnine (for strychnine-sensitive glycine receptors) or bicuculline (for GABA type A receptors) elicits pain hypersensitivity states.<sup>5,6</sup> Peripheral nerve injury and long-lasting peripheral inflammation lead to reduced glycinergic and GABAergic inhibitory control in the superficial dorsal horn of the spinal cord, respectively.<sup>7,8</sup> The fact that intrathecal application of glycine prevents mechanical hyperalgesia in a rat model of neuropathic pain<sup>9</sup> suggests the therapeutic importance of establishing excitatory and inhibitory balance in the spinal dorsal horn.

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.<sup>10,11</sup> Two GlyT subtypes, GlyT1 and GlyT2, have been identified. GlyT1 is expressed widely in the central nervous system and localized mostly in glial cells. GlyT2 is present in axons and presynaptic terminals of inhibitory glycinergic neurons in the central nervous system, including the spinal cord, brainstem, and cerebellum. Recent accumulating evidence indicates that GlyT2 has an essential role in the refilling of synaptic vesicles with glycine and therefore in the maintenance of glycinergic inhibitory synaptic transmission.<sup>12</sup> By contrast, GlyT1 reduces glycine concentration near *N*-methyl-D-aspartate (NMDA) receptors, where glycine acts as a coagonist of glutamate to facilitate excitatory synaptic transmission mediated by NMDA receptors.<sup>13-15</sup> Moreover, GlyT1 eliminates glycine from the synaptic cleft to terminate glycinergic neurotransmission.<sup>11</sup> Hence, blockade of glycine uptake mediated *via* GlyT1 potentially facilitates both glutamatergic excitation and glycinergic inhibition. In the study presented here, using the competitive GlyT1 inhibitor sarcosine, which acts as a substrate,<sup>11</sup> and/or the noncompetitive GlyT1 inhibitor *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS),<sup>16</sup> we demonstrate that an increase in glycine due to blockade of GlyT1 produces a net inhibitory effect on spinal pain processing in chronic pain states and also supraspinally restores impaired hippocampal synaptic plasticity of excitatory transmission after peripheral nerve injury<sup>17</sup> presumably related to

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cognitive disturbance, which is often described in patients with chronic pain.<sup>18,19</sup> Some preliminary data have been published elsewhere in abstract format.<sup>20</sup>

## Materials and Methods

All of the experimental protocols used here were approved by the Animal Care and Use Committee of Nagoya City University (Nagoya, Japan) and were conducted in accordance with the guidelines of the National Institutes of Health (Tokyo, Japan) and the Japanese Pharmacological Society (Tokyo, Japan).

### *Preparation of the Neuropathic Animal Models*

**Partial Nerve Ligation Model.** The surgical procedure was based on that described by Seltzer *et al.*<sup>21</sup> In brief, 4-week-old, male ddY-strain mice were anesthetized by intraperitoneal administration of pentobarbital sodium (60 mg/kg). One third to one half of the dorsal aspect of the right sciatic nerve was ligated just distal to its branch to the posterior biceps and semitendinosus muscles. Thermal and mechanical hypersensitivity was assessed 7 days after ligation.

**Diabetic Neuropathy.** Three- to 4-week-old male ddY mice were injected with streptozotocin (200 mg/kg, intraperitoneal) dissolved in saline after a 14- to 17-h fast. All animals were housed in solid floored cages with a deep layer of paper chips that was changed daily. A 12:12 h light-dark cycle was used, and the animals were allowed free access to sufficient food and water. The blood glucose level was measured 1 day before and 2 weeks after streptozotocin injection by measuring the glucose concentration in a blood sample obtained by tail prick using an Accu-Chek blood glucose monitoring system (Roche Diagnostics, Indianapolis, IN). The mice were defined as diabetic when their blood glucose concentration exceeded 350 mg/dl. Mechanical hypersensitivity was assessed 2 weeks after streptozotocin administration.

### *Assessment of Thermal Hypersensitivity*

Thermal hypersensitivity was assessed by the plantar test (Ugo Basile, Comerio, Italy) following a modification of the method of Hargreaves *et al.*<sup>22</sup> Mice were placed in a clear plastic chamber with a glass floor and were allowed to acclimate to their environment before testing. During this time, the mice initially demonstrated exploratory behavior, but subsequently stopped exploring and stood quietly with occasional bouts of grooming. A mobile radiant heat source, which was located under the glass floor, was focused onto the plantar surface of the right hind paw, and paw withdrawal latencies (PWLs) were recorded. The intensity of radiant heat was adjusted to give a 7- to 8-s withdrawal latency in naive mice. A cutoff latency of 15 s was imposed to avoid

tissue damage. PWLs were measured in duplicate for the right hind paw of each animal, and the mean of the two values was used for analysis.

### *Assessment of Mechanical Hypersensitivity*

Mice were placed in individual transparent Perspex cubicles with a wire mesh bottom, and a series of calibrated von Frey filaments (Semmes-Weinstein monofilaments; Stoelting, Wood Dale, IL) was used to determine the 50% likelihood of a paw withdrawal response (50% threshold) by the up-down method of Dixon.<sup>23</sup> Eight von Frey filaments, with approximately equal logarithmic incremental bending forces, were chosen (von Frey numbers 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, and 4.17; equivalent to 0.02, 0.03, 0.07, 0.17, 0.41, 0.69, 1.20, and 1.48 g force, respectively). Testing was initiated with the 0.17-g hair, and each hair was applied perpendicularly to the plantar surface of the right hind paw (partial nerve ligation model), with sufficient force to bend the filament, for 3-4 s. Lifting of the paw indicated a positive response and prompted the use of the next weaker (*i.e.*, lighter) filament. Absence of a paw withdrawal response prompted the use of the next stronger (*i.e.*, heavier) filament. This paradigm was continued until four measurements had been obtained after an initial change in behavior, or until four consecutive positive scores (score of 0.01 g) or five negative scores (score of 1.5 g) had been obtained. The resulting scores were used to calculate the 50% threshold.<sup>24</sup> In diabetic mice, testing was made on both the right and left hind paws of each animal, and the mean of the two 50% threshold values was used for analysis.

In the study presented here, mice that exhibited a PWL of less than 5 s in the plantar test and a 50% threshold of 0.1 g in the von Frey test 7 days after partial ligation of the sciatic nerve were considered to be developing thermal and mechanical hypersensitivity. In diabetic mice, a 50% threshold of 0.2 g in the von Frey test 14 days after streptozotocin administration was considered to indicate development of mechanical hypersensitivity.

### *Behavioral Testing after Formalin Injection*

In the formalin test, 3- to 4-week-old male ddY mice were used. Mice were placed individually in a clear plastic chamber and were allowed to acclimate to their environment before testing. A mirror was situated behind the chamber to allow an unobstructed view of the animals' paws. Ten minutes after administration of either drugs or vehicle, mice were gently restrained, and 20  $\mu$ l formaldehyde, 1% (2.7% formalin in distilled water), was subcutaneously injected into the plantar surface of the left hind paw using a 27-gauge needle. After formalin injection, each mouse was placed back in the testing chamber, and the incidence of formalin-induced noci-

ceptive behavior characterized by licking/biting of the affected paw was measured for 50 min. Formalin-induced nociceptive behavior is biphasic, with the first and second phases recorded during 0–10 min and 10–40 min after injection of formalin, respectively. Time spent performing the licking/biting behavior in each 5-min block was recorded continuously during the first and second phases.

#### *Effects on Acute Nociception*

The degree of antinociception was determined by the plantar test (see Assessment of Thermal Hypersensitivity) and the paw pressure test in normal (nonligated) mice.

In the plantar test, a higher intensity of radiant heat than that used in ligated animals was applied. Otherwise, at the weaker intensity used in ligated animals (including before ligation), mice occasionally exhibited PWL values above 10 s, which is close to the cutoff latency of 15 s and prevented us from making a proper evaluation of analgesic effects on acute nociception.

After the plantar test, mice were subjected to the paw pressure test (Pressure Analgesy-Meter; Muromachi Kikai, Tokyo, Japan) to assess their threshold for acute mechanical nociception. In brief, while the experimenter gently held the body of each mouse, the right hind paw was exposed to increasing mechanical pressure. The pressure level was increased at a rate of 10 mmHg/s, and the pressure (mmHg) required to elicit a response was determined for each mouse, that pressure being defined as the nociceptive threshold. Paw pressure measurements were made in duplicate, and the mean of the two values was used for calculations. The cutoff pressure was 200 mmHg.

#### *Electrophysiology in Hippocampal Slices*

Mice either developing mechanical hypersensitivity after partial nerve ligation or subjected to a sham operation were killed by cervical dislocation during ether anesthesia. The brain was then removed and both hippocampi were quickly dissected out in ice-cold low-sodium artificial cerebrospinal fluid (pH 7.4 after bubbling with 95% O<sub>2</sub> and 5% CO<sub>2</sub>) containing 215.5 mM sucrose, 3.5 mM KCl, 1.24 mM KH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 2.4 mM CaCl<sub>2</sub>, and 1.3 mM MgSO<sub>4</sub>. Transverse hippocampal slices 400 μm thick were prepared using a vibratome (DSK-2000; Dosaka, Kyoto, Japan) and then maintained for at least 60 min at 32°C. The slices were then transferred to a recording chamber and superfused with standard artificial cerebrospinal fluid (at 28°–29°C, pH 7.4 after bubbling with 95% O<sub>2</sub> and 5% CO<sub>2</sub>) containing 124 mM NaCl, 2 mM KCl, 1.24 mM KH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 2.4 mM CaCl<sub>2</sub>, and 1.3 mM MgSO<sub>4</sub>. Each slice was completely submerged in the medium and superfused continuously at a rate of 2.0–2.5 ml/min.

The field excitatory postsynaptic potentials (fEPSPs) were recorded from the striatum radiatum of the CA1 area with glass electrodes (1–5 MΩ) filled with 2 M NaCl after electrical stimulation of the Schaffer-collateral/commissural fibers with square wave pulses (0.1-ms duration at a baseline frequency of 0.033 Hz) *via* a bipolar enamel-coated stainless steel electrode. Test stimuli were applied to produce 30% of the maximum fEPSPs. After stable baseline fEPSPs had been recorded for approximately 60 min, long-term potentiation (LTP) was induced by high-frequency stimulation with a train of 100 shocks delivered at 100 Hz. The fEPSPs were acquired and their slopes were measured using the Measurement and Analysis System for Extracellular Potentials (Furusawa Lab Appliance, Kawagoe, Japan). The values of the measured slopes were normalized to the average of baseline responses obtained during 60 min before induction of LTP.

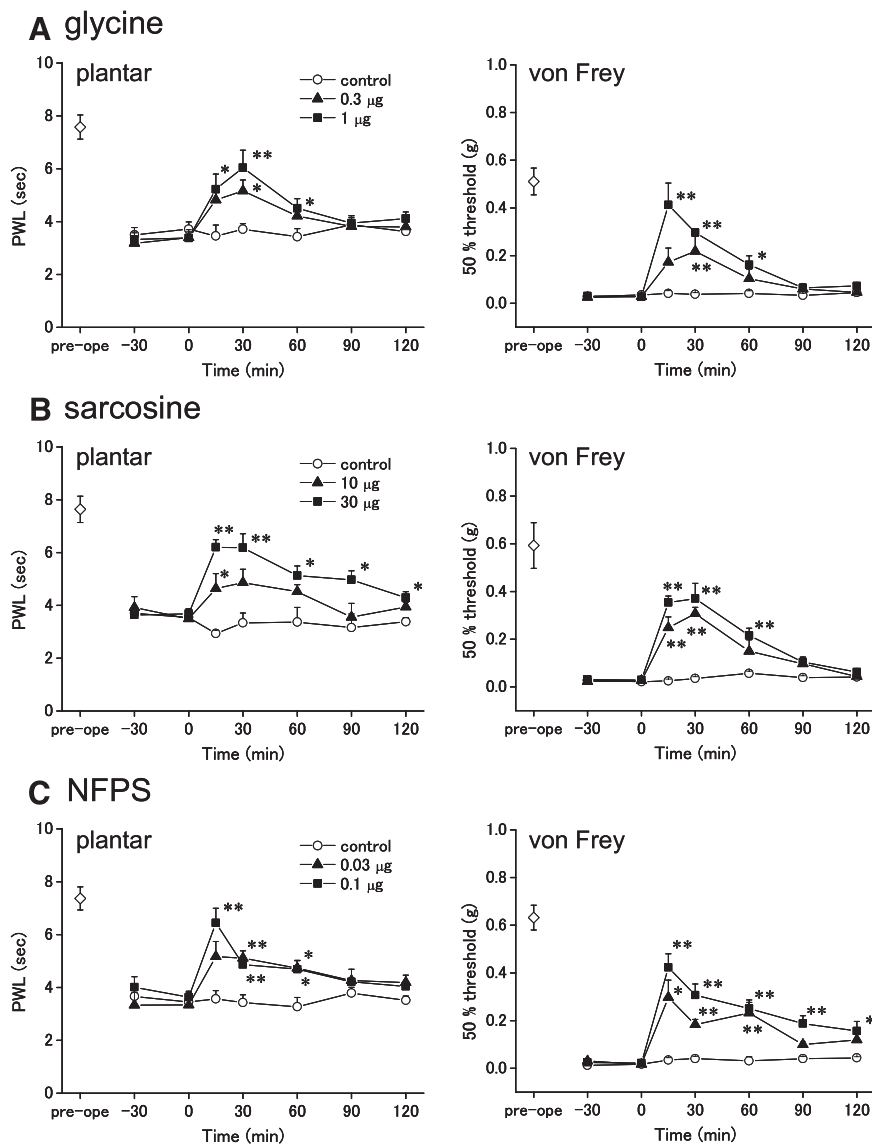
#### *Drugs*

Glycine and sarcosine were purchased from Nacalai (Kyoto, Japan) and Sigma Chemical (St. Louis, MO), respectively, and dissolved in 0.9% physiologic saline. NFPS was obtained from Tocris Cookson (Bristol, United Kingdom; *in vivo* experiments with the permission of NPS Pharmaceuticals Inc., Parsippany, NJ) and dissolved in 20% 2-hydroxypropyl-β-cyclodextrine. In the behavioral studies, glycine, sarcosine, and NFPS were injected intrathecally. These drugs or their vehicle were administered in a volume of 5 μl *via* a disposable 27-gauge needle, which was inserted into the subarachnoid space through the intervertebral foramen between L5 and L6 in accordance with the method described by Hylden and Wilcox.<sup>25</sup> In the electrophysiologic study, NFPS was dissolved in 20% 2-hydroxypropyl-β-cyclodextrine and then administered by bath application at 1,000-fold dilution with standard artificial cerebrospinal fluid.

#### *Statistical Analysis*

All data are expressed as mean ± SEM. The effects of drugs (glycine, sarcosine, and NFPS) on the nociceptive threshold in both the plantar and von Frey tests were evaluated with respect to time; the time of administration of drugs was defined as time 0. In the formalin test, glycine, sarcosine, or NFPS was administered 10 min before formalin injection, and the time of formalin injection was defined as time 0. The statistical analysis was made using Java Applets & Servlets for Biostatistics software (programmed by Hideki Ono, Ph.D., Professor, Nagoya City University, Nagoya, Japan). Two-tailed multiple *t* test with Bonferroni correction after one-way analysis of variance was used.<sup>26</sup> Differences with *P* < 0.05 were considered significant.





**Fig. 1.** Glycine, sarcosine, and *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS) exhibit analgesic effects on thermal and mechanical hypersensitivity in mice after partial ligation of the sciatic nerve. Thermal and mechanical hypersensitivity was assessed by the plantar and von Frey tests, respectively. Glycine (A; 0.3 and 1  $\mu\text{g}$ ), sarcosine (B; 10 and 30  $\mu\text{g}$ ), or NFPS (C; 0.03 and 0.1  $\mu\text{g}$ ) was administered intrathecally at time 0. Each point represents the mean  $\pm$  SEM of five or six separate mice. *Ordinates:* mean paw withdrawal latencies (PWLs; plantar test) and 50% thresholds (von Frey test) in the three groups of mice. The *clear diamond* in each graph shows the mean of pooled PWLs (plantar test in A, B, and C) or 50% thresholds (von Frey test in A, B, and C) obtained before ligation in the three groups of mice. The *asterisks* indicate data points for which a significant difference between the vehicle control (*clear circles*) and drug-treated groups (*solid triangles and squares*) was observed, as determined by two-tailed multiple *t* test with Bonferroni correction after one-way analysis of variance (two comparisons in three groups, \*  $P < 0.05$  and \*\*  $P < 0.01$ ).

## Results

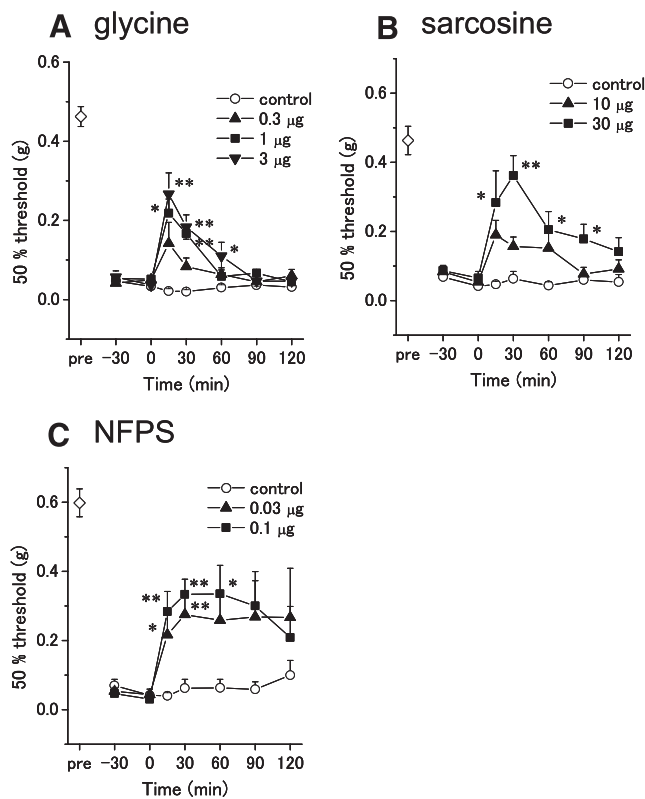
### *Analgesic Effects in Mice after Peripheral Nerve Injury*

We first injected glycine intrathecally to examine whether the increase in glycine actually reduces thermal and/or mechanical hypersensitivity developing after partial ligation of the sciatic nerve. As figure 1A shows, intrathecal glycine (0.3 and 1  $\mu\text{g}$ ) significantly increased the PWL in the plantar test and the 50% threshold in the von Frey test in a dose-dependent manner. Similar significant reduction of thermal and mechanical hypersensitivity was obtained after intrathecal injection of the GlyT1 inhibitors sarcosine (10 and 30  $\mu\text{g}$ ; fig. 1B) and NFPS (0.03 and 0.1  $\mu\text{g}$ ; fig. 1C). Maximal analgesic effects, which at the doses used were not increased beyond the averaged PWL or the 50% threshold values measured before ligation, were generally observed within 30 min after injection of

these drugs, and the effects mostly returned to the predrug level within 120 min.

### *Analgesic Effects in Mice Developing Diabetic Neuropathy*

Sixty mice developing diabetic mechanical hypersensitivity 2 weeks after streptozotocin administration were used. Their mean blood glucose concentrations and 50% thresholds before and 2 weeks after streptozotocin administration were  $138.4 \pm 3.4$  mg/dl and  $0.503 \pm 0.021$  g, and  $552.9 \pm 8.2$  mg/dl and  $0.060 \pm 0.004$  g, respectively. Intrathecal injection of glycine (0.3, 1, and 3  $\mu\text{g}$ ), sarcosine (10 and 30  $\mu\text{g}$ ), and NFPS (0.03 and 0.1  $\mu\text{g}$ ) reduced mechanical hypersensitivity dose dependently and significantly, and the effect of NFPS was relatively long-lasting (fig. 2). Again, these drugs at the doses used did not generally produce analgesic effects beyond the nociceptive withdrawal levels obtained before ligation.



**Fig. 2.** Glycine, sarcosine, and *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS) reverse the reduced withdrawal threshold to mechanical stimuli developing in streptozotocin-injected diabetic mice. Streptozotocin was injected into 3- to 4-week-old mice, and mechanical hypersensitivity was assessed 2 weeks later by the von Frey test. Glycine (A; 0.3, 1, and 3  $\mu\text{g}$ ), sarcosine (B; 10 and 30  $\mu\text{g}$ ), or NFPS (C; 0.03 and 0.1  $\mu\text{g}$ ) was administered intrathecally at time 0. Each point represents the mean  $\pm$  SEM for six separate mice. *Ordinates*: mean 50% thresholds (von Frey test). *Abscissae*: 2 weeks before (pre = before streptozotocin administration) and time in minutes after drug administration. The clear diamond in each graph indicates the mean of pooled 50% thresholds obtained before streptozotocin administration in the four (A) or three (B and C) groups of mice. The asterisks indicate data points for which a significant difference between the vehicle control (clear circles) and drug-treated groups (solid triangles and squares) was observed, as determined by two-tailed multiple *t* test with Bonferroni correction after one-way analysis of variance (three comparisons in four groups [A] and two comparisons in three groups [B and C], \*  $P < 0.05$  and \*\*  $P < 0.01$ ).

#### Analgesic Effects in the Formalin Test

Glycine (0.3 and 1  $\mu\text{g}$ ), sarcosine (10 and 30  $\mu\text{g}$ ), and NFPS (0.03 and 0.1  $\mu\text{g}$ ), which were respectively intrathecally administered 10 min before formalin injection, reduced the second phase of formalin-induced nociceptive behavior. By contrast, none of these drugs had any significant influence on the first phase (fig. 3). Generally, mice pretreated with 20% 2-hydroxypropyl- $\beta$ -cyclodextrin (vehicle for NFPS) exhibited less nociceptive behavior after formalin injection compared with those pretreated with saline (vehicle for glycine and sarcosine). Nonetheless, NFPS revealed prominent inhibition of the second phase of formalin-induced nociceptive behavior (fig. 3C). Although the first phase of formalin-induced

nociceptive behavior (10–20 min after drug administration) was not influenced, these drugs generated obvious analgesic effects (both in the Seltzer and streptozotocin-induced diabetic neuropathy models) 15 min after drug administration, suggesting that the analgesic effects were not elicited secondarily *via* impairment of motor activity.

#### Antinociceptive Effects of NFPS

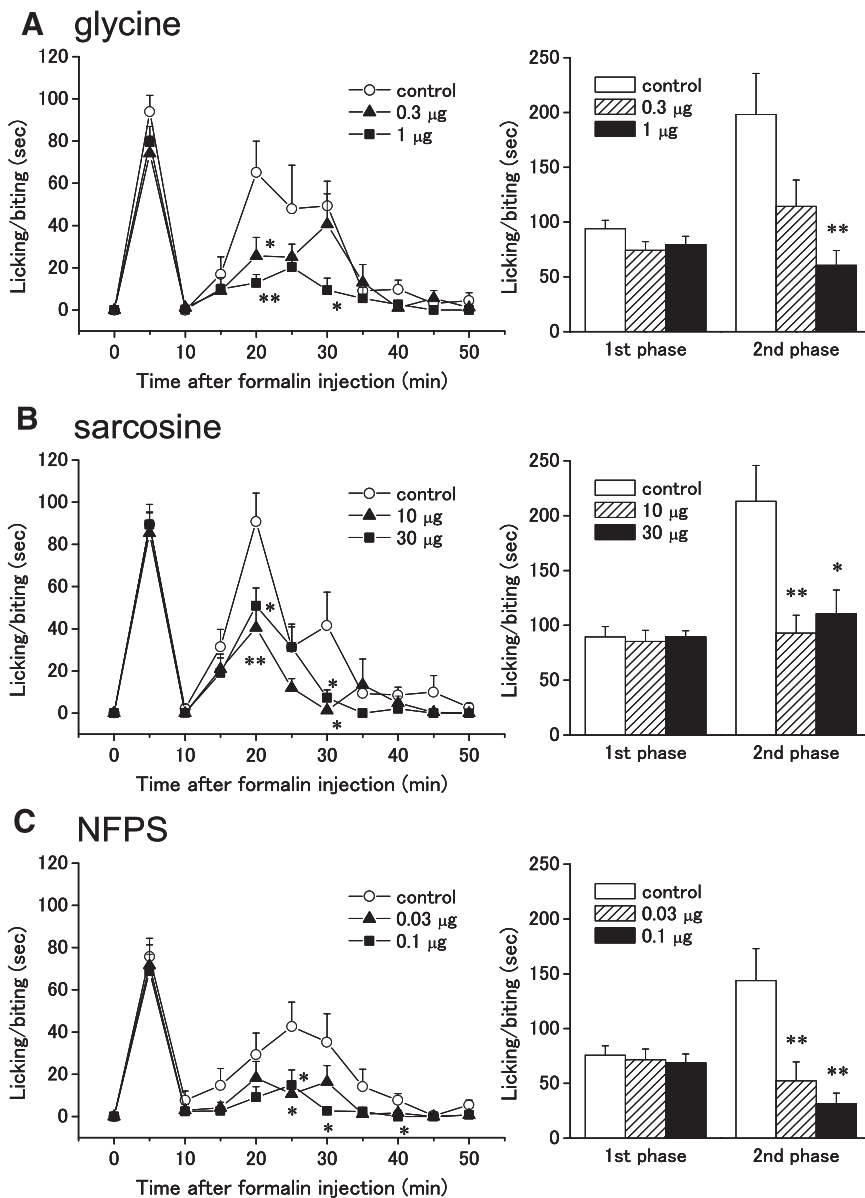
The experiment on acute nociception was in addition made to know whether blockage of GlyT1 by NFPS at the effective doses in chronic pain states generates analgesic effects on acute nociception. When assessing the effects on acute nociception in the plantar test, a higher intensity of radiant heat than that used in ligated animals was applied. Otherwise, at the weaker intensity used in ligated animals (including before ligation), mice occasionally exhibited PWL values above 10 s, which is close to the cutoff latency of 15 s and prevented us from making a proper evaluation of analgesic effects on acute nociception. In nonligated naive mice, intrathecally injected NFPS (0.03 and 0.1  $\mu\text{g}$ ) significantly increased the PWL in the plantar test and the nociceptive threshold in the paw pressure test (fig. 4). The antinociceptive effects elicited at 0.1  $\mu\text{g}$  seemed to be equivalent to those of morphine hydrochloride at 10  $\mu\text{mol/kg}$ , subcutaneous (approximately 3 mg/kg) assessed in the plantar test (see our previous data, fig. 1 of Sakaue *et al.*<sup>27</sup>).

#### Normalization of LTP in the Hippocampal CA1 Region by NFPS

We have recently demonstrated that LTP in the hippocampal CA1 region is impaired after peripheral nerve injury.<sup>17</sup> In agreement with this, the fEPSP slope for hippocampal slices prepared from mice after peripheral nerve injury was significantly less potentiated compared with that in mice given a sham operation (fig. 5;  $165.0 \pm 5.6\%$  in Seltzer *vs.*  $207.1 \pm 11.5\%$  in sham control [ $n = 8$  each,  $P < 0.05$ ], 60 min after high-frequency stimulation in the presence of vehicle for NFPS). By contrast, in the presence of NFPS (25 nM), which at this concentration alone did not significantly influence LTP in slices prepared from sham-treated mice, the fEPSP slope was equally potentiated in slices after nerve injury and a sham operation (fig. 5;  $211.3 \pm 10.0\%$  in Seltzer *vs.*  $223.2 \pm 9.8\%$  in sham control [ $n = 8$  each], 60 min after high-frequency stimulation). Therefore, NFPS improved the maintenance of LTP, which was impaired after peripheral nerve injury.

#### Discussion

A net predominance of excitatory over inhibitory influences on spinal neuronal networks related to pain

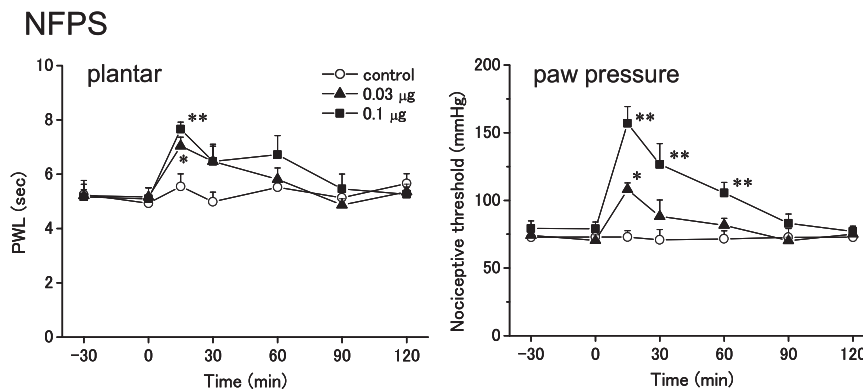


**Fig. 3.** Glycine, sarcosine, and *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]-sarcosine (NFPS) reduce formalin-induced nociceptive behaviors. Glycine (A; 0.3 and 1  $\mu$ g), sarcosine (B; 10 and 30  $\mu$ g), or NFPS (C; 0.03 and 0.1  $\mu$ g) was administered intrathecally 10 min before formalin injection (formalin administered at time 0), and the formalin-induced nociceptive behavior characterized by licking/biting of the affected paw was measured for 50 min. Time-course graphs showing licking/biting behavior (in seconds) in consecutive 5-min blocks and columns showing total licking/biting behavior (in seconds) during the first (0–10 min) and second (10–40 min) phase. Each point or column represents the mean  $\pm$  SEM for 10 separate mice. The asterisks indicate data points for which a significant difference between the vehicle control (clear circles or columns) and drug-treated groups (solid triangles and squares, or hatched and solid columns) was observed, as determined by two-tailed multiple *t* test with Bonferroni correction after one-way analysis of variance (two comparisons in three groups, \*  $P < 0.05$  and \*\*  $P < 0.01$ ).

signaling is likely to be one crucial factor that underlies chronic pain conditions. Hence, normalization of excitatory and inhibitory balance in the spinal dorsal horn by strengthening inhibition may have therapeutic consequences for neuropathic and/or inflammatory pain. Accordingly, the current study clearly demonstrated that administration of glycine or blockade of endogenous glycine reuptake by GlyT1, and therefore an increase of extracellular glycine in the spinal cord, reduced thermal and mechanical hypersensitivity developing after partial ligation of the sciatic nerve, mechanical hypersensitivity in diabetic neuropathy, and formalin-induced persistent nociceptive behavior. More importantly, blockade of glycine uptake *via* GlyT1 also normalized the weakened LTP in hippocampal CA1 hippocampal synaptic transmission, suggesting that an increase of endogenous glycine not only removes chronic pain at the spinal level

but also supraspinally ameliorates the decrease in synaptic efficacy presumably related to cognitive disturbance which is often described in patients with chronic pain.<sup>18,19</sup>

Dysfunction of inhibitory synaptic transmission in the spinal dorsal horn, and therefore a net increase in spinal excitation, is considered to be a crucial mechanism involved in the pathophysiology of chronic pain states.<sup>3</sup> However, it is currently unclear whether impairment of GABAergic and/or glycinergic inhibition is of presynaptic or postsynaptic origin. Peripheral nerve injury reduces presynaptic releasable GABA pool and induces functional loss of GABAergic inhibitory synaptic transmission in the superficial dorsal horn.<sup>7</sup> Moreover, peripheral inflammation also leads to a reduction of glycine release from glycinergic nerve terminals in the spinal superficial layer.<sup>8</sup> By contrast, Polgár *et al.*<sup>28,29</sup> revealed



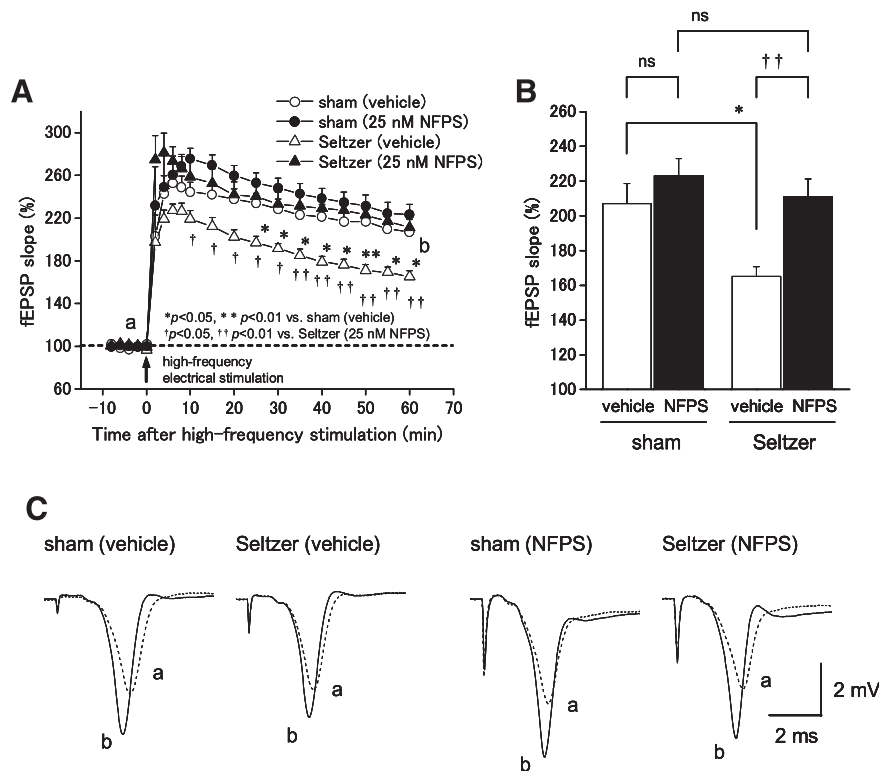
**Fig. 4.** *N*-[3-(4'-Fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS) produces antinociceptive effects against acute thermal and mechanical nociception in nonligated mice. Acute thermal and mechanical nociception was assessed by the plantar and paw pressure tests, respectively. Note that a higher intensity of radiant heat than that used in ligated animals was applied in the study of acute thermal nociception. NFPS (0.03 and 0.1  $\mu\text{g}$ ) was administered intrathecally at time 0. Each *point* represents the mean  $\pm$  SEM for five or six separate mice. *Ordinates*: mean paw withdrawal latencies (PWLs; plantar test) and nociceptive threshold (paw pressure test). *Abscissae*: time in minutes after NFPS injection. The *asterisks* indicate data points for which a significant difference between the vehicle control (*clear circles*) and NFPS-treated groups (*solid triangles* and *squares*) was observed, as determined by two-tailed multiple *t* test with Bonferroni correction after one-way analysis of variance (two comparisons in three groups, \*  $P < 0.05$  and \*\*  $P < 0.01$ ).

no evidence for a reduction of GABAergic or glycinergic neurons after peripheral nerve injury. While prostaglandin  $E_2$ , one of the important mediators of pain and inflammation, reduces glycinergic transmission by a postsynaptic mechanism involving the glycine receptor  $\alpha_3$  subunit in the spinal superficial dorsal horn,<sup>30,31</sup> this mechanism does not seem to contribute to pain after peripheral nerve injury or the formalin test.<sup>32</sup> Therefore, various presynaptic and/or postsynaptic mechanisms may contribute to spinal disinhibition in neuropathic and inflammatory pain conditions. Our study focusing on glycinergic inhibition has demonstrated that it is possible to normalize excitatory and inhibitory balance by using existing functional inhibitory mechanisms including presynaptic and postsynaptic components. As presented here, successful pain relief by blockade of glycine uptake both in neuropathic pain models including peripheral nerve injury and diabetic neuropathy and in the formalin test implies that GlyT1 blockers have potential for the treatment of chronic pain. In addition, pathophysiologically, it seems that glycine is functionally released presumably from spinal glycinergic interneurons even in these chronic pain conditions. Hence, the use of available extracellular glycine may provide analgesic effects. It was noteworthy that NFPS generated only analgesic effects below the basal nociceptive withdrawal levels obtained before ligation of the sciatic nerve or streptozotocin administration at doses that produced antinociceptive effects in naive animals. This may reflect reduced functional glycinergic inhibition after peripheral nerve injury or in diabetic neuropathy.

It has been generally accepted that there are many similarities in the synaptic plasticity underlying memory and central sensitization.<sup>1,2</sup> Hence, it should be borne in mind that drugs influencing central sensitization poten-

tially impair memory function. In fact, the benzodiazepine diazepam impairs memory function in human and animals<sup>33,34</sup> and blocks LTP in the rat hippocampus<sup>35</sup> and LTP of C fiber-mediated field potentials in the rat spinal dorsal horn,<sup>36</sup> a synaptic model that has been proposed to describe the hypersensitivity underlying increased efficacy of nociceptive transmission.<sup>37</sup> However, as we demonstrated here, GlyT1 blockade had different influences on synaptic plasticity in the fore-brain and spinal cord. In addition to the blockade of glycine reuptake near inhibitory glycinergic terminals in the spinal cord, glial GlyT1 regulates glycine levels close to NMDA receptors, where glycine acts as a coagonist of glutamate.<sup>11</sup> Hence, the analgesic effect in chronic pain models and the antinociception in naive animals may reflect a net neuronal inhibition at the spinal dorsal horn as a result of overcoming any excitatory effects *via* glycine-mediated activation of NMDA receptors. After blockade of GlyT1, glycine may reach strychnine-sensitive glycine receptors that are also expressed functionally in the hippocampus.<sup>38-40</sup> Nevertheless, excitatory effects of glycine on synaptic plasticity were observed at the hippocampus after peripheral nerve injury. We have recently demonstrated that the LTP of the Schaffer-collateral synapses in the hippocampal CA1 region elicited by high-frequency electrical stimulation in slices prepared from neuropathic mice was maintained at a significantly lower level than that in sham-treated mice.<sup>17</sup> Such impairment of LTP after peripheral nerve injury was never observed when the LTP was induced in the presence of the GlyT1 blocker NFPS. Although not significant, the LTP in the sham-treated groups was also enhanced by NFPS. These results are consistent with studies demonstrating facilitatory effects of GlyT1 blockers on LTP in the CA1 region<sup>41</sup> and dentate gyrus<sup>42</sup> of the hippocampus. Chattipakorn and McMahon *et al.*<sup>43</sup> have





**Fig. 5.** N-[3-(4'-Fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS) facilitates long-term potentiation in CA1 hippocampal synaptic transmission in slices prepared from Seltzer model mice. (A) Time course of long-term potentiation in slices prepared from sham-treated (circles) and Seltzer model (triangles) mice in the absence (clear circles and triangles; vehicle) or presence (solid circles and triangles) of NFPS (25 nM). High-frequency electrical stimulation was delivered at time 0. Each point represents the mean  $\pm$  SEM of eight separate experiments. (B) Long-term potentiation at 60 min after high-frequency electrical stimulation in slices from sham-treated and Seltzer model mice in the absence (clear columns; vehicle) or presence (solid columns) of NFPS (25 nM). (C) Representative averaged traces of two consecutive field excitatory postsynaptic potentials (fEPSPs) before (broken line) and 60 min after high-frequency electrical stimulation (solid line) during times on the time course graph were shown. The asterisks and daggers indicate data points for which a significant difference between the vehicle-treated Seltzer (clear triangles in A) and vehicle-treated sham (clear circles in A) groups and between the vehicle-treated Seltzer (clear triangles A) and NFPS-treated Seltzer (solid triangles in A) groups, respectively, was observed, as determined by two-tailed multiple *t* test with Bonferroni correction after one-way analysis of variance (four comparisons in four groups, \**P* < 0.05 and \*\**P* < 0.01, †*P* < 0.05 and ††*P* < 0.01). ns = not significant.

suggested that increases in glycine concentration can elicit neuronal inhibition particularly when excitability is high. This not only supports the analgesic effects produced by increasing the spinal glycine level but also implies decreased neuronal activities of the hippocampus after peripheral nerve injury. The latter may be plausibly ascribed to reduced activities of NMDA receptors that can be restored by blockade of glycine uptake. Further studies are ongoing in our laboratory to clarify whether peripheral nerve injury alters the intrinsic activities of hippocampal NMDA receptors or glycine uptake *via* GlyT1.

Because GlyT2 inhibitors are not commercially available, we were unable to examine their effects on chronic pain. Because GlyT2 exhibits exclusively neuronal expression and is located in inhibitory glycinergic nerve terminals,<sup>44</sup> its blockade may increase glycinergic inhibition and therefore potentially lead to pain relief. However, a recent study using GlyT2-deficient mice has revealed that GlyT2 has an essential role in the recycling of glycine at glycinergic nerve terminals for vesicular release.<sup>12</sup> Hence, long-term use of GlyT2 blockers may

possibly lead to diminished glycinergic inhibition with a consequent deterioration of chronic pain conditions, in contrast to the case of GlyT1-deficient mice that develop hyperglycinergic symptoms.<sup>45</sup>

In conclusion, the current study has demonstrated that either exogenously applied glycine or endogenously increased extracellular glycine *via* blockade of GlyT1 can lead to sufficient pain relief in neuropathic and inflammatory pain. In particular, GlyT1 blockers seem to elicit both supraspinal excitatory and spinal inhibitory actions of glycine in chronic pain conditions, suggesting that this could be a novel therapeutic approach for patients with chronic pain.

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