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Full paper

Electrophysiological evidence of increased glycine receptor-mediated phasic and tonic inhibition by blockade of glycine transporters in spinal superficial dorsal horn neurons of adult mice

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ABSTRACT

To understand the synaptic and/or extrasynaptic mechanisms underlying pain relief by blockade of glycine transporter subtypes GlyT1 and GlyT2, whole-cell recordings were made from dorsal horn neurons in spinal slices from adult mice, and the effects of NFPS and ALX-1393, selective GlyT1 and GlyT2 inhibitors, respectively, on phasic evoked or miniature glycinergic inhibitory postsynaptic currents (eIPSCs or mIPSCs) were examined. NFPS and ALX-1393 prolonged the decay phase of eIPSCs without affecting their amplitude. In the presence of tetrodotoxin to record mIPSCs, NFPS and ALX-1393 induced a tonic inward current that was reversed by strychnine. Although NFPS had no statistically significant influences on mIPSCs, ALX-1393 significantly increased their frequency. We then further explored the role of GlyTs in the maintenance of glycinergic IPSCs. To facilitate vesicular release of glycine, repetitive high-frequency stimulation (HFS) was applied at 10 Hz for 3 min during continuous recordings of eIPSCs at 0.1 Hz. Prominent suppression of eIPSCs was evident after HFS in the presence of ALX-1393, but not NFPS. Thus, it appears that phasic and tonic inhibition may contribute to the analgesic effects of GlyT inhibitors. However, reduced glycinergic inhibition due to impaired vesicular refilling could hamper the analgesic efficacy of GlyT2 inhibitors.

1. Introduction

Pain signals are conducted along primary afferent fibers and enter the spinal dorsal horn, where the signals are transmitted to secondary neurons that ascend to the upper brain. The spinal dorsal horn receives descending projections and also excitatory and inhibitory modulations from spinal intrinsic neurons. Thus, the spinal dorsal horn plays a crucial role in the transmission and modulation of pain signals. In chronic pain conditions, excitatory and inhibitory balance in the spinal dorsal horn is thought to be impaired (1–3), and therefore, one potential therapeutic approach could be to normalize this balance by increasing inhibitory influences such as GABA and glycine, the two major inhibitory neurotransmitters in the central nervous system (CNS).

The extracellular concentration of glycine is regulated by the sodium/chloride-dependent glycine transporters GlyT1 and GlyT2 (4,5). GlyT1 is expressed widely in the CNS and localized mostly in glial cells (6,7) and also in a subpopulation of glutamatergic terminals (8). GlyT2 is localized in axons and presynaptic terminals of inhibitory glycinergic neurons in the lower brain regions, including the spinal cord, brainstem, and cerebellum (7). Both GlyT subtypes eliminate glycine near strychnine-sensitive glycine receptors. In addition, GlyT1 removes glycine near N-methyl-D-aspartate (NMDA) receptors where glycine acts as a coagonist of glutamate to facilitate excitatory synaptic transmission (9–11). GlyT2 is essential for providing glycine at presynaptic terminals of inhibitory glycinergic neurons to refill the synaptic vesicles and maintain inhibitory transmission (12,13). So far, the therapeutic potential of GlyT1 inhibitors in the treatment of schizophrenia, due to their role in facilitating excitatory neurotransmission, has been tested in clinical

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trials (14). However, there are yet no GlyT1 inhibitors available for clinical use.

Our previous studies and others have demonstrated that intrathecal application of glycine or an increase in its endogenous concentration by blocking the reuptake of glycine via GlyTs results in relief of acute pain in naive animals and also chronic pain in animal models of neuropathy and inflammation (15–19). This strategy for increasing the endogenous concentration of glycine appears to have therapeutic promise for relief of pain. The temporally increased glycine concentration at the release event and the low ambient glycine concentration participate in phasic and tonic inhibition of neurons via strychnine-sensitive glycine receptors, respectively. However, little is known about what is taking place at phasic glycinergic synaptic transmission mediated via synaptic glycine receptors when reuptake of glycine via GlyTs is hampered. Moreover, no previous study has addressed whether the extrasynaptic glycine receptors participating in the tonic inhibition of neurons (20–23) are activated by GlyT inhibitors in the spinal dorsal horn. In the present study, we investigated how blockade of glycine reuptake affects phasic and tonic inhibition mediated via glycine receptors in the spinal dorsal horn. Moreover, with regard to the essential role of GlyT2 in the refilling of vesicles with glycine, we tried to clarify how glycinergic transmission could be affected after frequent use of glycinergic synapses under GlyTs blockade.

2. Materials and methods

All of the experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of Kitasato University, and carried out in accordance with the guidelines of the National Institutes of Health and the Japanese Pharmacological Society.

2.1. Slice preparation

Transverse slices of the spinal cord were prepared as reported previously (24). Briefly, 5–7-week-old male ddY-strain mice (SLC, Shizuoka, Japan) were anesthetized with urethane (1.6 g/kg, i.p.) and α-chloralose (15 mg/kg, i.p.), and a dorsal laminectomy was performed in the lumbosacral region. After the spinal cord had been isolated, spinal cord slices (300 μm) were prepared using a linear slicer (PRO7, Dosaka, Kyoto, Japan) in ice-cold low-sodium artificial cerebrospinal fluid (low-sodium ACSF, pH 7.4, after bubbling with 95% O_2 and 5% CO_2) containing (in mM): sucrose 212.5, KCl 3, NaH_2PO_4 1, $NaHCO_3$ 25, D-glucose 11, $CaCl_2$ 1, and $MgCl_2$ 5, and then maintained for at least 60 min in standard ACSF (at 30–32 °C, pH 7.4 after bubbling with 95% O_2 and 5% CO_2) containing (in mM): NaCl 113, KCl 3, NaH_2PO_4 1, $NaHCO_3$ 25, D-glucose 11, $CaCl_2$ 2, and $MgCl_2$ 1. The slices were then transferred to a recording chamber on the stage of a microscope (BX51-WI; Olympus, Tokyo, Japan), and superfused with standard ACSF kept at 30–32 °C at a rate of 2.0–2.5 ml/min.

2.2. Patch-clamp electrophysiology

Whole-cell voltage-clamp recordings were made from substantia gelatinosa (SG) neurons visually identified using an upright microscope (BX51WI, Olympus) with infrared differential interference contrast (IR-DIC) optics. The patch electrodes (2.5–3 μm tip diameter) were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) and had a resistance of 2.5–4 MΩ when filled with the internal solution containing (in mM): CsCl 140, HEPES 10, EGTA 1.1, $MgCl_2$ 2, MgATP 3, TrisGTP 0.3, pH 7.2, adjusted with CsOH.

Glycinergic inhibitory post-synaptic currents (IPSCs) were evoked by stimulating the neighboring area (within a 40–100 μm radius of the recorded neuron) via a glass pipette filled with 1 M NaCl. A voltage pulse 0.2 ms in duration at 0.1 Hz was applied at suprathreshold intensity via the stimulating electrode. In some experiments, repetitive high-frequency stimulation at 10 Hz for 3 min was applied to facilitate exocytosis (13). These evoked IPSCs (eIPSCs) were recorded in the presence of (-)-bicuculline methobromide [10 μM (referred to simply as bicuculline)] and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) (10 μM), respectively, at a holding potential of −70 mV. When spontaneous miniature IPSCs (mIPSCs) were recorded, tetrodotoxin (TTX; 0.5 μM) was further added to the extracellular solution. Under these experimental conditions, eIPSCs and mIPSCs were detected as inward deflections (EPC800; HEKA, Darmstadt, Germany), which were low-pass filtered at 3 kHz, and digitized at 10 kHz for computer analysis with pClamp10 software (Molecular Devices, Union City, CA, USA). The access resistance was monitored by measuring capacitative transients obtained in response to a hyperpolarizing voltage step (10 mV, 10 ms) from a holding potential of −70 mV. All experiments were performed at 30–32 °C.

The effects of the GlyT1 inhibitor *N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS) or the GlyT2 inhibitor ALX-1393 on eIPSCs were evaluated by comparing averaged eIPSCs taken during the responses to NFPS or ALX-1393 (18 traces for 3 min before starting washout) with those before drug application (18 traces for 3 min). mIPSCs were analyzed offline using a peak detection program (MiniAnalysis; Synaptosoft, Fort Lee, NJ, USA), and the frequency and amplitude distribution of the events (ranging from 200 to 238 events) before the end of NFPS or ALX-1393 application were compared with those (ranging from 130 to 300 events) obtained before application of the drug. GlyT inhibitors were applied only once for each slice in this study. The current decays of eIPSCs and mIPSCs were fitted by exponential functions embedded in Origin (Microcal Software, Northampton, MA, USA) to obtain the time constant.

2.3. Drugs

NFPS was purchased from Tocris Cookson (Bristol, UK). ALX-1393, CNQX and strychnine hydrochloride (referred to simply as strychnine) were obtained from Sigma-Aldrich (St Louis, MO, USA), and bicuculline and TTX were from WAKO (Osaka, Japan). NFPS, ALX-1393 and CNQX were dissolved in dimethyl sulfoxide, and bicuculline, strychnine and TTX were dissolved in distilled water, then administered by bath application at 1000-fold dilution with standard ACSF.

2.4. Statistical analysis

All data are expressed as the mean ± SEM. Statistical significance was evaluated by a two-tailed *t* test with Bonferroni correction after one-way analysis of variance (ANOVA) for multiple comparisons, two-tailed Student's *t* test (25) or two-tailed paired *t* test. Differences at $p < 0.05$ were considered significant.

3. Results

3.1. Effects of GlyT1 and GlyT2 blockers on evoked glycinergic IPSCs

We obtained glycinergic eIPSCs from spinal inner SG neurons by electrically stimulating the neighboring deeper dorsal area via a glass pipette, because glycinergic neurons are known to be abundant in the deep dorsal horn and intermediate area (26). Indeed, the recorded eIPSCs were mediated by strychnine-sensitive glycine

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