Impaired glycinergic synaptic transmission and enhanced inflammatory pain in mice with reduced expression of vesicular GABA transporter (VGAT)

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Running title: VGAT and inflammatory pain

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Document statistics:

The number of text pages 33
The number of tables 2
The number of figures 7
The number of references 43
The number of words in Abstract 250
The number of words in Introduction 604
The number of words in Discussion 1,484
Abstract

Loading of GABA and glycine into synaptic vesicles via the vesicular GABA transporter (VGAT) is an essential step in inhibitory neurotransmission. Due to the evidence linking alterations in GABAergic and/or glycinergic neurotransmission to various pain disorders, we investigated the possible influence of downregulation of VGAT on pain threshold and behavioral responses in mice. The phenotypes of heterozygous VGAT knockout (VGAT+/-) mice were compared to wild-type (WT) mice using behavioral assays. In addition, GABAergic and glycinergic miniature inhibitory postsynaptic currents (mIPSCs) were recorded in dorsal horn neurons. Western blot analysis confirmed significant reduction of VGAT protein levels in VGAT+/- mice. However, HPLC revealed that glutamate, GABA, and glycine contents in the whole brain and spinal cord were normal in VGAT+/- mice. Behavioral analysis of VGAT+/- mice showed unchanged motor coordination, anxiety, memory performance and anesthetic sensitivity to propofol and ketamine, although thermal nociception and inflammatory pain were enhanced. Patch-clamp recordings revealed that the frequency and amplitude of glycinergic mIPSCs in lamina II neurons were reduced in VGAT+/- mice. Genotype differences in glycinergic mIPSCs were more evident during sustained stimulation by solutions with high potassium levels, suggesting that the estimated size of the readily releasable pool (RRP) of glycine-containing vesicles was reduced in VGAT+/- mice. These results provide genetic, behavioral and electrophysiological evidence that VGAT-mediated inhibitory drive alters very specific forms of sensory processing: those related to pain processing. More close examination will be needed to verify the possibility of VGAT as a new therapeutic target for the treatment of inflammatory pain.
Introduction

GABA and glycine are the primary inhibitory neurotransmitters in the central nervous system (CNS). Glycine mediates synaptic inhibition in the spinal cord, brain stem and other regions (Lynch, 2004; Betz and Laube, 2006). In the dorsal horn of the spinal cord, nociceptive afferents coming from the periphery make synaptic connections with neurons located in the superficial laminae I and II, the first site of synaptic integration in the pain pathway. Glycinergic and GABAergic inhibition in the spinal cord are known to regulate propagation of nociceptive signals to higher brain regions (Price et al., 2009; Zeilhofer, 2005). In fact, changes in spinal inhibitory neurotransmission have been associated with various pain disorders (Furue et al., 2004). However, investigation of glycinergic inhibitory mechanisms in pain processing has largely focused on postsynaptic glycine receptors (Ahamadi et al., 2002; Harvey et al., 2004); the physiological roles of glycine release machinery from presynaptic terminals have received less attention.

Loading of GABA and glycine into synaptic vesicles via the vesicular transporter is an essential step in inhibitory neurotransmission. Vesicular transporters regulate the uptake and type of neurotransmitter sequestered in synaptic vesicles and, therefore, the amount and type of neurotransmitter released (Masson et al. 1999). Only one vesicular transporter for GABA and glycine has been identified; this having been separately identified by two researchers as a vesicular GABA/glycine transporter (VGAT) (McIntire et al., 1997) and as a vesicular inhibitory amino acid transporter (VIAAT) (Sagné et al., 1997). VGAT is expressed mainly in the CNS (McIntire et al., 1997), where it localizes to the synaptic vesicles of GABAergic and glycinergic neurons (Chaudhry et al., 1998; Dumoulin et al. 1999; Takamori et al. 2000). VGAT belongs to a eukaryotic-specific superfamily of H+-coupled amino acid transporters, and exchanges GABA or glycine for protons. VGAT defines the GABAergic and glycinergic phenotypes of neurons, in addition to the biosynthetic enzymes (glutamate decarboxylase: GAD) and the plasma membrane transporters (SLC6) of these transmitters (Gasnier, 2004).

This study was performed to elucidate whether genetic manipulation of VGAT would affect
behavior, anesthetic sensitivity and pain thresholds in mice. Unfortunately, VGAT knockout leads to embryonic lethality between embryonic day 18.5 (E18.5) and birth (Wojcik et al., 2006), which prevents us from being able to analyze the influence of complete inactivation of VGAT in the mature CNS. We therefore investigated how downregulation of VGAT might influence behavioral performance and pain thresholds. Anesthetic sensitivity to intravenous general anesthetics, propofol and ketamine, were also investigated, because GABA and glycine receptors are potential targets of anesthetic drugs (Mihic et al. 1997, Nishikawa and MacIver 2000; Nishikawa et al., 2002). In this context, we recently reported that glutamate decarboxylase 65 (GAD65) gene knockout and the resulting changes in GABAergic inhibition altered anesthetic sensitivity and acute thermal nociception without affecting inflammatory pain (Kubo et al., 2009a; Kubo et al., 2009b; Nishikawa et al., 2011). We then investigated how reduced VGAT levels affected GABAergic and glycinergetic inhibition in dorsal horn neurons of the spinal cord using patch-clamp methods.

While GABA mediates fast inhibitory neurotransmission throughout the CNS, glycine acts as an inhibitory neurotransmitter in spatially restricted areas such as the spinal cord, brain stem, and the cerebellum (Lynch, 2004; Betz and Laube, 2006). This spatially more restricted distribution of glycinergetic inhibition may be advantageous in situations when a more localized enhancement of inhibition is needed (i.e. some pain disorders due to diminished inhibition). This is the first study to use genetic, biochemical, behavioral and electrophysiological approaches to investigate the role of VGAT in behavioral performance including sensory processing. The present study provides evidence that VGAT would be a new potential target for future analgesic drugs, acting via a novel and potentially more selective pathway.
Materials and Methods

Mice

All animal procedures and protocols used in this study were approved by the Animal Care Committee of Gunma University Graduate School of Medicine (protocol # 06-47) and performed through NIH guidelines. We generated heterozygous mice lacking exons 2 and 3 on one VGAT allele (VGAT+/- mice) (Saito et al., 2010). Adult male wild-type (WT) mice and VGAT+/- mice from 12-16 weeks old weighing 23-28 g were used for behavioral experiments and young male mice from 14-21 days old for electrophysiological recordings. Genotypes were determined by polymerase chain reaction, shortly after weaning. VGAT+/- mice exhibited no apparent phenotypic abnormalities during development and adulthood. Mice were group-housed (four to six per cage) in a pathogen-free transgenic facility (12-hr light/dark cycle, room temperature 27 ± 2 °C), and water and food were available ad libitum. The behavioral studies were performed by a single experimenter who was blinded to the genotypes of the mice. Mice were kept warm by a heat lamp before and throughout the behavioral experiments to avoid hypothermia. None of the animals were used for more than two experiments and at least 1 week was allowed between the two treatments for the mice to recover.

Western blot analysis

VGAT+/- mice and WT mice at 12 weeks old were decapitated under anesthesia, and the whole brain or spinal cord was rapidly dissected and homogenized in ice-cold homogenization buffer (320 mM sucrose, 50 mM Tris–HCl pH 7.2, 5 mM EDTA, 1 mM PMSF). Homogenates were centrifuged at 3,000 rpm for 10 min at 4 °C to obtain the supernatant. The protein concentrations were determined by BCA protein assay reagent (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Equal amounts of proteins were separated by 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis for detection of VGAT and β-actin. The proteins were transferred onto nitrocellulose membranes using a semidry transfer method. After blocking for 1h with 5% non-fat milk in Tris-buffered saline plus 0.1% Tween 20, the tans-blots were reacted with rabbit anti-VGAT antibody
(1:1,000) (Takamori et al. 2000), or mouse anti-β-actin antibody (1:10,000; Abcam, Cambridge, UK), followed by reaction with secondary anti-rabbit horseradish peroxidase or anti-mouse horseradish peroxidase. Peroxidase activity was detected visually by chemiluminescence using Western Blotting Analysis System (GE Healthcare Life Sciences, Buckinghamshire, UK) and imaged by light-capture (ATTO, Tokyo, Japan). For quantification of the protein levels, the images were scanned by NIH IMAGE software.

**Measurement of neurotransmitter content**

For analysis of neurotransmitter tissue content, WT mice and VGAT+/− mice at 12 weeks of age were sacrificed by decapitation under deep isoflurane anesthesia. Tissue samples of the whole brain and the whole spinal cord were removed quickly and tissue weight was measured. The tissue was added to 3-5 ml of saline (saline volume was approximately ten times tissue weight), and then homogenized in phosphate-buffered saline (PBS) containing 0.2% protease inhibitor using a polytron homogenizer (24,000 rpm, 15 sec, 2-3 times). Following removal of cell debris by centrifugation at 3,000 rpm (20 min, 4°C), the supernatant (500 μl), which was added to sulfosalicylic acid (750 μl), was centrifuged again at 3,000 rpm (20 min, 4°C). The supernatant after pH adjustment was analyzed using high-performance liquid chromatography (HPLC) and fluorescence detection. HPLC was performed by the company (SRL, Tokyo, Japan). Neurotransmitter content (nmol/g) was calculated as follows: measured neurotransmitter concentration (nmol/ml) × saline volume added (ml) / tissue weight (g).

**Behavioral assays**

**Hotplate test:** An animal was placed on an aluminum plate (25 × 20 cm) maintained at 53 ± 0.5 °C and a Plexiglas cage (24 cm in height) was used to restrict the movements of the animal (Hot plate analgesia meter MK-350C, Muromachi Kikai Co., Ltd, Tokyo, Japan). Mice remained on the plate until they performed a behavior indicative of nociception: hind-paw licking and/or jumping. The latency to responses was measured. Only hind-paw responses were used, because forepaw responses are
components of normal grooming behavior. To prevent tissue damage, the cut-off latency was set at 60 s in control experiments or 80 s in drug treated experiments. Animals not responding within the cut-off time were removed and assigned a score of the cut-off time. In these experiments, mice did not show any behavior to conclude that they had been injured in any way. All measurements were carried out between 18:00 and 22:00 hours.

**Tail-immersion test:** The spinally-mediated nociceptive thresholds were determined using a thermo regulated water circulating pump (NTT-20S, Tokyo Rikakikai Co., Ltd, Tokyo, Japan). The mouse was maintained in a mouse holder, and the distal tail was then immersed in the water bath, which was thermostatically controlled at 48 ± 0.5 °C. The tail was rapidly immersed in the bath and the latency to respond to the heat stimulus with vigorous flexion of the tail was measured to the nearest 0.1 s using a manual stopwatch. Animals were removed immediately after responding, and the tail was wiped off with a cloth. The maximum latency allowed was 20 s in order to prevent tissue damage.

**Formalin test:** The test was carried out in individual transparent containers. The mice were placed in the test chambers for 30 min. After this adaptation period, 20 µl of 5 % formalin (dissolved in distilled water) was injected into the dorsal surface of the right hind-paw of the mouse, using a 30-gauge needle connected to a micro syringe. Each mouse was returned immediately to the observation place after injection. Animal behaviors after formalin injection were continuously recorded by the video recorder for later analysis. The amount of time the injected hind-paw was lifted, or licked or flinched by the animal was measured for 60 min starting immediately after formalin injection (Dubuisson and Dennis, 1977).

**Loss of righting reflex (LORR):** LORR was used as a surrogate measure for general anesthetic hypnosis. Each animal received an intraperitoneal (ip) injection of propofol or ketamine, and then placed on their backs in a chamber (20 × 28 × 15 cm), and the ability to right themselves was observed. Mice were judged to have lost this reflex when unable to right themselves within 10 sec. The time from ip injection of the drug to LORR was considered as the latency, and the time between the LORR and the time mice regained the ability to right themselves within 2 sec was considered the duration of LORR.
Rotarod: To test motor coordination and equilibrium, animals were placed on the rotating rotarod apparatus (Letica, Barcelona, Spain), where the rotational speed of the rod was kept constant at 10 rpm. Mice were trained to remain on a rotarod until they could perform three consecutive 180 sec trials. The latency to fall from the rod was recorded and compared between genotypes.

Open field analysis: Spontaneous activity of mice was measured in the Plexiglas transparent open field. Their movements were tracked for 20 min by using an infrared photo-beam detection system for locomotor activity (LSI LETICA, LE 8811, Panlab, S.L., Barcelona, Spain). This system allowed us to analyze animal trajectories with $16 \times 16$ infrared beams for optimal subject detection. Behavioral data were stored on a computer for later analysis.

Elevated plus maze: Plus-maze test consisted of two open arms ($25 \times 5$ cm, surrounded by a 0.25-cm-high border) and two closed arms ($25 \times 5$ cm, surrounded by 15-cm-high walls), with the two pairs of identical platform, which emerged from a central platform ($5 \times 5$ cm), positioned opposite each other (EP-3002, Ohara Medical Co., Japan). The apparatus was elevated 50 cm above the floor. Mice were tested on the maze in randomized order. The test was initiated by placing the mouse on the central platform of the maze, facing one of the open arms, and letting it move freely. Each session lasted 5 min and was recorded by a video-camera. All tests were carried out under dim red lighting between the second and seventh hour of the dark phase. Behavioral analysis was performed by a trained experimenter who was blind to genotype. Several parameters were collected during the session: (a) Open arm duration; (b) Closed arm duration; (c) Central platform duration; (d) Open arm frequency; and (e) Closed arm frequency.

Morris water maze: Spatial memory abilities were examined in the standard hidden-platform acquisition learning versions of the Morris water maze (Schenk and Morris, 1985). A 125 cm circular pool was filled with water, and kept at 26°C. A 10 cm round platform was hidden 1 cm beneath the surface of the water at a fixed position. Mice that failed to find the platform within 60 sec were guided to the platform, where they remained for 30 s before being returned to their cages. The mean latency to the platform was recorded.
Patch-clamp recordings from spinal cord slice preparations

The methods of electrophysiology were described previously (Yoshimura and Nishi, 1993; Nishikawa et al., 2011). Slices from young (P14-P21) male mice were used in this study. Briefly, mice were decapitated under deep isoflurane anesthesia, and thoracolumbar laminectomy was performed. The spinal cord at spinal level Th12-L1 was removed, and then placed in pre-oxygenated Krebs solution at 1-3 °C. A block of the spinal cord was quickly dissected out and glued to a DTK-1000 vibratome tray (Dosaka EM, Tokyo, Japan) using oxygenated cold modified Ringer solution. The spinal cord was mounted on a microslicer and then a transverse slice (300 μm thick) was cut from the center of the spinal segment, and then kept in the pre-chamber (Brain Slice Chamber System; Harvard Apparatus, Holliston, MA) filled with artificial cerebrospinal fluid (ACSF) consisting of (in mM), 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 11 glucose, bubbled with 95% O₂ and 5% CO₂ at room temperature (22-24 °C). Slices were allowed at least one hour for recovery in the pre-chamber, which was designed to keep 6-8 slices viable for several hours.

Slices were transferred to a recording chamber (2 ml in volume) perfused with an oxygenated ACSF (maintained at 30 ± 1 °C, SH-27A, Warner Instruments, Hamden, CT) at a rate of approximately 6 ml/min. Patch electrodes were made from borosilicate thin-walled capillaries (GC150F, Harvard Apparatus, Holliston, MA). Recording electrodes (4-6 MΩ) were filled with Cs₂SO₄-based solution (Cs₂SO₄ 110 mM, TEA 5 mM, CaCl₂ 0.5 mM, MgCl₂ 2 mM, EGTA 5 mM, HEPES 5 mM and MgATP 5 mM; pH 7.2) to investigate spontaneous IPSCs at a holding potential of 0 mV. Whole cell patch clamp recordings were made from lamina II neurons using an upright Axioskop2 FS plus microscope (Zeiss, Jena, Germany). The magnified image was collected by an intensified CCD camera (Hamamatsu Photonics, Japan) with contrast enhancement. The image of neurons was displayed on a video monitor, and glass patch pipettes were visually advanced using a micromanipulator (MWO3, Narishige Co., Ltd., Tokyo, Japan) through the slice to the surface of the neuron. Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA) was used for whole cell recordings. Whole cell currents were filtered.
at 2-5 kHz and digitized at 10 kHz (Digidata 1322A, Axon Instruments Inc., Union City, CA) and stored on a Pentium-based PC. Addition of bicuculline (BIC) (10 μM; Sigma, St. Louis, MO, USA) or strychnine (300 nM; Sigma) was used to isolate GABAergic and glycinergic IPSCs, respectively. Series resistances were generally between 10 and 25 MΩ, and were then compensated approximately 80%.

Data analysis: Data acquisition and analysis were performed with pCLAMP software version 8.1 (Axon instruments Inc., Union City, CA) and Minianalysis software (Synaptosoft Inc. Fort Lee, NJ). Synaptic currents were defined as current deflections with a fast rising phase and a relatively slower decay phase. The rise time was defined as time interval between 10% and 90% of the peak amplitude and synaptic currents having the rise time < 2 ms were included for analysis. The amplitude of synaptic current was measured from the initial inflection point (not from the baseline) to the peak, to avoid the effects of summation on amplitude distribution. Threshold-level crossing were set at approximately three times of baseline noise, which was measured during the period of no detectable events. As a result, synaptic currents larger than 6 pA in the amplitude were counted for analysis. This definition eliminated the infrequently observed single channel events or synaptic currents with slow rise time, but successfully detected most IPSCs. The decay phase was fitted with a single exponential curve and a time from peak to 36.8% of peak was defined as the decay time constant.

Chemicals: For behavioral studies, mice were treated with propofol (Maruishi Pharmaceuticals Co, Ltd., Osaka, Japan) or ketamine (Sankyo Co, Ltd., Tokyo, Japan) administered intraperitoneally with a volume of 10 μl/g of body weight. Vehicle solutions for behavioral studies were as follows: propofol, lipofundin MCT/LCT 10% (B. Braun Melsungen AG, Mulsungen, Germany); ketamine, 0.9 % saline. An intraperitoneal injection of lipofundin MCT/LCT 10% (10 μl/g) alone had no hypnotic/analgesic effect on mice behavior (n = 5, data not shown). Other drugs and reagent grade chemicals were purchased from Sigma-Aldrich Chemicals (Tokyo, Japan).

Statistical analysis: Results are expressed as mean ± SEM. The results were analyzed by using Student’s t-test or one-way analysis of variance (ANOVA). Post hoc comparisons between the individual groups were performed by means of the Tukey test. The level of statistical significance was
set at \( P < 0.05 \) in all tests.
Results

Western blot analysis: We first examined the expression of the VGAT protein in WT mice and VGAT+/- mice (fig.1). The VGAT protein level in the brain from VGAT+/- animals was significantly reduced to 47.8 ± 2.1 % of WT mice (n = 3, P < 0.001). The expression of VGAT in the VGAT+/- spinal cord was also reduced to 61.0 ± 0.5 % of WT mice (n = 3, P < 0.01). These data show that VGAT protein levels are considerably reduced in VGAT+/- mice.

GABA and glycine levels in the brain and spinal cord are normal in VGAT+/- mice: We measured neurotransmitter content in the whole brain and spinal cord in VGAT+/- mice at 12 weeks old. In spite of reduced expression of VGAT protein levels in VGAT+/- mice, there was no significant genotype differences in glutamate, GABA, and glycine levels (Table 1). Glycine levels in the spinal cord were approximately four times higher than GABA in both genotypes, whereas GABA levels were dominant in the brain. HPLC data include synaptic vesicular, intracellular, and extracellular (ambient) contents, indicating that the total amount of GABA and glycine are unchanged in the CNS.

Behavioral analysis of VGAT+/- mice: WT mice and VGAT+/- mice were tested in measurements of spontaneous activity in the home cage, rotarod, elevated plus maze, and Morris water maze. None of these assays showed significant difference between genotypes (fig 2, Table 2), indicating that VGAT+/- mice have normal motor coordination, anxiety level and memory performance. In the rotarod assay, both WT mice (n = 5) and VGAT+/- mice (n = 5) could stay on rotating bar over 180 s in three consecutive trials. In the elevated plus-maze test, no significant differences were found between WT mice and VGAT+/- mice in any parameters recorded (Table 2). In the Morris water maze test, genotype had no effect on ability to improve performance as result of training, although VGAT+/- mice reached the platform even faster than WT mice during early trials (fig 2B).

Thermal hyperalgesia in VGAT+/- mice: In the hot-plate test, licking or jumping responses were considered to be the result of supraspinal sensory integration (Caggiula et al., 1995; Rubinstein et al., 1996). To test the sensory performance at the supraspinal level, we first measured the latency to responses from the hot-plate set at 53 °C (fig. 3A). The cut-off latency was set at 60 s. A significant
reduction in the latency was observed in VGAT+/- mice (24.4 ± 1.1 s, n = 20) compared with WT mice (21.0 ± 0.9 s, n = 20, P < 0.05), suggesting that nociceptive perception via supraspinal sites is increased in VGAT+/- mice.

Transmitter spillover between synapses is often enhanced after blockade of transmitter transporters. Given the dominant role of glycine in the spinal cord, we asked if glycine uptake systems would play any role in acute thermal nociception. Glycine transporters have been cloned and are classified into two distinct gene families, glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2). Cellular expression studies have revealed that GlyT1 is distributed widely throughout the CNS, and that distribution correlates better with the localization of NMDA receptors than with the strychnine-sensitive glycine receptor (Borowsky et al., 1993), suggesting that GlyT1 is involved in glycine supply for the activation of NMDA receptors. Intraperitoneal injection of ALX-5407, a selective inhibitor of the GlyT1, dose-dependently prolonged the latency in both WT mice (n = 16) and VGAT+/- mice (n = 11, fig 3A). Thus, ALX-5407 had an analgesic effect, perhaps by increasing tonic inhibition mediated by glycine receptors.

To test the sensory performance at the spinal cord level, responses to thermal nociception were examined using the tail-immersion test which reflects spinally-mediated reactions (Caggiula et al., 1995). We measured tail withdrawal latencies following tail immersion at 48 ± 0.5 °C and results are shown in fig 3B. There was no significant difference in the latency of the tail-immersion test (n = 14 each).

*Enhanced inflammatory pain in VGAT+/- mice:* We examined whether the reduction of VGAT protein affected the behavioral responses of mice in an inflammatory pain model. Inflammatory pain involves an activity-dependent facilitation in excitability of both peripheral neurons (peripheral sensitization), and spinal and supra-spinal neurons (central sensitization), including thalamus and cortex. To examine the contribution of VGAT to inflammatory pain signaling, we used the formalin test. The subcutaneous injection of formalin (5% formalin, 20 µl) produced a well-known biphasic pattern of nociceptive behaviors in both genotypes (fig 4A). The nociceptive responses were mainly observed
during 0-10 min and 10-60 min after injection, which correspond to the early (phase 1) and late (phase 2) phases. Total time of lifting, licking or flinching in phase 1 represents a response to chemical nociception due to tissue damage, while these responses in phase 2 is a response to subsequent inflammation. A behaviorally quiescent inter-phase, where the animals showed very little nociceptive responses, was observed between two phases. Although no genotype difference was observed in total time of phase 1 responses (n = 12 each), phase 2 responses were significantly enhanced in VGAT+/− mice (P < 0.05 vs. WT mice). We conclude that partial reduction of VGAT protein levels are involved in formalin-induced inflammatory pain signaling.

Behavioral responses to intravenous general anesthetics, propofol and ketamine, are unchanged in VGAT+/− mice: We then compared anesthetic sensitivity to an intravenous anesthetic propofol, a positive allosteric modulator of GABA<sub>A</sub> receptors. The latency to LORR produced by propofol (100 mg/kg, ip) was similar between both genotypes (n = 20 each, fig 5A left). The latency to LORR produced by ketamine (75 mg/kg, ip), a NMDA receptor open channel blocker, was also similar between both genotypes (n = 12 each, fig 5A right). In addition, there was no significant difference in the duration of LORR produced by propofol (100 mg/kg, ip) and ketamine (75 mg/kg, ip) between genotypes (fig 5B). These data suggest that VGAT+/− mouse have normal sensitivity to anesthetic drugs which produce hypnosis by enhancing GABAergic inhibition or by inhibiting NMDA receptors.

Glycinergic, but not GABAergic, mIPSCs in the spinal cord is diminished in VGAT+/− mice: Given behavioral effects of reduction of VGAT protein on acute nociception and inflammatory pain in VGAT+/− mice, we next tried to provide physiological evidence for the role of VGAT in inhibitory synaptic transmission. We tried to identify a functional correlation of reduction of VGAT protein for the phenotype observed in VGAT+/− mice. To study inhibitory synaptic transmission, we then looked at the influence of reduction of VGAT protein on spontaneous GABAergic and glycinergic synaptic currents in lamina II in the spinal cord. Action potential-independent components of GABA<sub>A</sub> receptor-mediated IPSCs (miniature IPSCs: mIPSCs) and glycine receptor-mediated mIPSCs were recorded at 0 mV using Cs2SO4-based internal solutions in the presence of the sodium channel blocker
tetrodotoxin (TTX, 1 μM). Under these conditions, mIPSCs were recorded as outward currents and excitatory postsynaptic currents (EPSCs) were not detectable because the holding potential of 0 mV is near the reversal potential for EPSCs.

Fig 6A shows representative traces of GABAergic (left) and glycinergic (right) mIPSCs from lamina II neurons in both genotypes. Bicuculline (10 μM) or strychnine (300 nM) was used to isolate glycinergic and GABAergic mIPSCs, respectively. Kinetic analysis revealed that the decay phase of GABAergic mIPSCs (19.9 ± 2.9 ms, n = 6) was significantly slower than that of strychnine-sensitive glycinergic mIPSCs (7.7 ± 1.1 ms, n = 6, P < 0.01). As reported earlier (Jonas et al., 1998; Keller et al., 2001), both GABAergic and glycinergic mIPSCs were observed in neurons of young mice (P14-P21). These mixed GABAergic/glycinergic mIPSCs are the result from co-release of GABA and glycine at the same inhibitory synapse in spinal laminas I-II. The amplitude of GABAergic and glycinergic mIPSCs either displayed as the mean amplitude or as a cumulative probability distribution was significantly reduced in VGAT+/- mice (P < 0.001, fig 6B). Although the frequency of GABAergic mIPSCs was similar between genotypes, the frequency of glycinergic mIPSCs was significantly reduced in VGAT+/- mice (n = 7, P < 0.01 vs. WT mice, n = 6, fig 6C), indicating that reduction of VGAT proteins affected the probability of glycine release.

Glycinergic, but not GABAergic, synaptic transmission stimulated by high potassium solutions is also impaired in VGAT+/- mice: We next compared the high potassium-induced potentiation of spontaneous glycine release in ACSF solutions including 2 mM Ca²⁺ in lamina II neurons using the whole-cell patch-clamp technique. Recordings were performed in the presence of bicuculline (10 μM), CNQX (20 μM), AP5 (100 μM), and TTX (1 μM), so that spontaneous responses observed in this condition were glycinergic mIPSCs. Bath application of high potassium (50 mM) evoked transient outward currents by direct depolarization of terminals and a secondary steady-state component in WT mice (fig 7A). These are thought to represent the massive release of glycine from the readily releasable pool (RRP) and the reserve pool (RP), respectively (Moulder and Mennerick, 2005; Mozhayeva et al. 2002). On the other hand, hyperkalemic solution (50 mM) application failed to evoke an initial rapid
peak response in VGAT+/- mice, although a secondary steady-state response was observed (fig 7A). As a result, the peak amplitude of evoked responses was significantly reduced in VGAT+/- mice compared with WT mice ($P < 0.05$, fig 7B), and the charge was also significantly reduced in VGAT+/- mice (fig 7C).
Discussion

Using mice with reduced expression of VGAT, we found that VGAT+/- mice showed enhanced sensitivity to thermal stimulation and chemical inflammation in an animal model. However, motor coordination, anxiety, memory performance and behavioral responses to the anesthetics of VGAT+/- mice were unchanged. Glycinergic mIPSCs in the lamina II neurons during sustained stimulation by high potassium levels were diminished in VGAT+/- mice. From this evidence, we conclude that partial reduction of VGAT-mediated inhibitory drive alters very specific forms of sensory processing.

VGAT protein levels, neurotransmitter content and behavior: The VGAT gene is remarkably compact, spanning ~5 kb in mice (Ebihara et al., 2003), and is expressed mainly in the CNS, where it localizes to the synaptic vesicles of GABAergic and glycinergic neurons (McIntire et al, 1997). It is also expressed in some peripheral tissues, including the pituitary and pineal glands and the pancreas (Gasnier, 2004). Thus, reduction of VGAT protein may affect not only behavioral performance but also higher brain function. In our tests, however, motor performance and spatial memory function were normal in VGAT+/- mice. In concurrence with previous observations (Saito et al., 2010), VGAT protein levels were reduced in VGAT+/- mice. HPLC assays revealed that, in both genotypes, glycine levels were dominant in the spinal cord, whereas GABA levels were dominant in the brain. These data are consistent with previous findings that glycine levels are highest in the spinal cord, pons and medulla oblongata, regions in which glycine receptors (GlyRs) are predominately expressed (Legendre, 2001).

The pain threshold resulting from glycinergic neuromechanisms is not always constant, because glycine content can be drastically up-regulated or down-regulated by various factors. For example, presynaptic membrane glycine transporters also play an important role in the control of neuronal excitability by modulating tonic glycinergic inhibition. We found that ALX-5407, a selective inhibitor of the glycine transporter GlyT1, prolonged the hotplate latency in both genotypes, suggesting that the sensitivity of mice to acute thermal pain is influenced by variation in GlyT1 function. However, since HPLC analysis cannot distinguish between synaptic vesicular, intracellular and extracellular (ambient) content of glycine, the exact role of tonic glycinergic inhibition on the pain threshold should be further
studied using a microdialysis assay.

**The distinct role of GABA and glycine in sensory processing:** We previously reported that GAD65 gene knockout and the resulting reduction in GABAergic inhibition altered anesthetic sensitivity to propofol and acute thermal nociception without affecting the inflammatory pain threshold (Kubo et al., 2009a; Kubo et al., 2009b). In contrast, in the present study, we found that VGAT-mediated reduction of glycinerergic inhibition enhanced acute thermal nociception and inflammatory pain without affecting behavioral sensitivity to propofol and ketamine. In the formalin test, genotype differences were evident only in phase II, which was enhanced in VGAT+/- mice, although reduction of VGAT protein did not affect responses in phase I. Phase I responses are evoked by direct chemical activation of peripheral C-fibers, while phase II responses depend on local inflammation and/or subsequent sensitization of nociceptive neurons (Rosland et al., 1990). These data support the hypothesis that GABA and glycine may have distinct roles in pain signal transduction. As an example to support this notion, strychnine, a selective competitive antagonist of glycine at the postsynaptic membrane, induces morphine-resistant dynamic, but not static, mechanical allodynia in rats, while bicuculline, a competitive antagonist of GABA_A receptor, induces static, but not dynamic, allodynia (Miraucourt et al. 2009). In addition, Inquimbert et al. (2007) compared the properties of inhibitory synaptic transmission in lamina II and laminae III-IV of the dorsal horn, which are involved in the processing of nociceptive and non-nociceptive sensory information. Fifty-five percent of lamina II neurons received both GABAergic and glycinerergic inputs, suggesting that lamina II neurons, which play a crucial role in nociceptive processing, are regulated by glycinerergic inhibition.

**The role of glycine and glycine receptors in inflammatory pain:** The amplitude of GABAergic and glycinerergic mIPSCs was significantly reduced in lamina II neurons of VGAT+/- mice, suggesting that reduced VGAT protein levels lead to a reduced content of GABA and glycine in the vesicles. The total GABA and glycine content per vesicle may, thus, be sensitive to VGAT protein levels. In addition, significant genotype differences in glycine release were observed in the presence of solutions containing high levels of potassium. These results suggest that, during sustained stimulation, the
increase in glycine release is probably not maintained in VGAT⁺⁻ mice. The loading of transmitters, mobilization of vesicles, and/or replenishment of vesicles at release sites that normally occur in WT mice during sustained stimulation, such as with inflammatory pain, may be impaired in VGAT⁺⁻ mice. A frequently used technique to evaluate the size of the RRP is hypertonic sucrose application (0.5 mM) in autaptic synapses in cell culture. We also tried to apply hypertonic sucrose solution (0.5 mM, n = 3) instead of high potassium solutions to deplete the RRP. Unfortunately, we could not complete these experiments in slice preparations, since the tight seal was broken after sucrose application, presumably due to changes in the osmotic pressure of neurons.

Tonic inhibition is a key regulator of inhibitory tone in several brain regions (Farrant and Nusser, 2005) and in the dorsal horn (Mitchell et al., 2007). GlyRs are pentameric ion channels composed of α1-α4 and β subunits (Legendre, 2001; Lynch, 2004). RNA edited α2- and α3-GlyRs may serve a particular function as extrasynaptic high affinity GlyRs in the hippocampus (Meier et al., 2005), little is known about the role of tonic inhibition mediated by GlyRs in the dorsal horn. GlyRα3 is an adult glycine receptor subunit which is much less abundant than GlyRα1, but plays an important role in inflammatory pain sensation, because GlyRα3 is predominantly expressed in superficial layers of the spinal cord dorsal horn, where nociceptive afferents terminate. The central component of sensitization to inflammatory pain is disinhibition of dorsal horn neurons, which are relieved from glycinergic inhibition by the inflammatory mediator prostaglandin E2 (PGE2) (Ahmadi et al., 2002). PGE2 activates prostaglandin E receptors of the EP2 subtype and leads to protein kinase A-dependent phosphorylation and inhibition of synaptic GlyRs containing the α3 subunit (Harvey et al., 2004). Mice deficient in GlyR α3 not only lack the inhibition of glycinergic neurotransmission by PGE2 seen in WT mice, but also show a reduction in pain sensitization induced by spinal PGE2 injection or peripheral inflammation. These results suggest that therapies aimed at glycinergic inhibition mediated by α3 subunits of GlyR may prove effective in the management of chronic inflammatory pain.

Study limitations: Although we tried to elucidate the relative contributions of VGAT on in vivo pain and anesthetic sensitivity, this study has some limitations. First, mice in different developmental stages
were used in this study. Although adult mice 12-16 weeks of age were used for behavioral assays, young mice (postnatal days 14-21) were used for electrophysiological analysis, because neurons in slice preparations could be visually identified by their relative translucency, due to the lack of myelination in young mice (Yoshimura and Nishi, 1993). However, chloride homeostasis and the relative expression of GABA and glycine receptors in lamina II neurons may be changing during this stage (Jonas et al., 1998; Keller et al., 2001). These two studies used rats, not mice; this prevents direct comparison in terms of developmental stage of GABAergic and glycinergic neurotransmission. Therefore, our data lack convincing evidence that altered glycinergic transmission in young mice is directly linked with the behavioral observations in adult mice. Second, western blot analysis showed that VGAT protein of VGAT+/− mice was reduced compared with WT mice, but there is no evidence for a change in GABA or glycine levels. In this context, it seems critical to establish that GABA/glycine uptake into synaptic vesicles is actually affected by reduced VGAT expression. However, it is technically difficult to quantify GABA/glycine uptake in a synaptosome-enriched fraction of the spinal cord (Hell and Jahn 1998). Finally, recent evidence shows that neurons in the CNS appear to contain an as yet unknown vesicular transport system for GABA and glycine with VGAT-like substrate specificity, because some VGAT-deficient neurons still show measurable vesicular release of the two transmitters (Wojcik et al. 2006). Further examination will be needed to confirm the role of VGAT to behavioral responses in vivo.

Possible indications of VGAT as a therapeutic target: GABA receptors contribute successful drug targets in the treatment of anxiety, sleep disorders, epilepsy, and general anesthesia, while drugs specifically targeting glycine are currently lacking. Previous studies have revealed that an increase in the extracellular concentrations of glycine in the spinal cord by blocking its uptake via membrane glycine transporters generates analgesic effects on neuropathic pain (Morita et al., 2008; Tanabe et al., 2008). The present study provides new evidence that VGAT, which belongs to a eukaryotic-specific superfamily of H⁺-coupled amino acid transporters and regulates the uptake of neurotransmitter sequestered in synaptic vesicles (McIntire et al., 1997; Sagné et al., 1997), may be a therapeutic drug target for the treatment of inflammatory pain.
Acknowledgments

The authors thank Masae Iino, Toshikazu Kakizaki, and Ying Wang for technical assistance in patch-clamp recordings or Western blot analysis, Shigeo Takamori for providing antibodies against VGAT, M. Bruce MacIver and Yasuhiko Saito for critical comments on the manuscript. The authors also thank the staff at Institute of Experimental Animal Research, Gunma University Graduate School of Medicine, for assistance in animal care.

Author contributions

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Conducted experiments: Makiko Hardy Yamada, Koichi Nishikawa, Kazuhiro Kubo

Performed data analysis: Makiko Hardy Yamada, Koichi Nishikawa

Contributed to the writing of the manuscript: Koichi Nishikawa, Makiko Hardy Yamada
References


during embryonic development: a study using knockout mice. Mol Brain 3:40


Footnotes

This research was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M.H.Y. [#22791416], K.N. [#20390412, #23659736], K.K. [#21890032], and Y.Y. [#22300105, #23115503]. This work was also supported by a Grant-in-Aid from the Japan Medical Association, Tokyo, Japan to K.N., JST, CREST and Takeda Science Foundation to Y.Y.
Legends for figures

**Figure 1** Abundance of VGAT protein in the whole brain and spinal cord as measured by Western blotting. (A) Representative immunoblots of VGAT from WT mice and VGAT+- mice. Relative signal intensities were normalized to β-actin. (B) Results are expressed as percentages of WT values. Data are shown as mean ± SEM (n = 3 each) here and in subsequent figures. *** P < 0.001 and ** P < 0.01 vs. corresponding age-matched WT mice (Student’s t-test).

**Figure 2** (A) VGAT+- mice exhibit normal anxiety-like behaviors in the open field test. Regional dwell time in outer region (left) and regional distance traveled in outer region (right) was compared between genotypes (Student’s t-test, n = 5). (B) Performance of WT mice and VGAT+- mice in the Morris water maze. Learning curves for both genotypes showing latencies to find the platform during trials.

**Figure 3** Antinociceptive responses of WT mice and VGAT+- mice in the hotplate test (53 °C). (A) The hotplate test revealed that the latency was significantly reduced in VGAT+- mice (n = 20) compared with WT mice (n = 20, * P < 0.05, Student’s t-test). ALX-5407, GlyT1 inhibitor, was injected intraperitoneally 24 hours prior to the hotplate test. ALX-5407 dose-dependently increased the latency in both genotypes. * P < 0.05, comparison between genotypes (Student’s t-test); ## P < 0.01 vs. control in VGAT+- mice, ### P < 0.001 vs. control in WT mice (one-way ANOVA followed by the post hoc Tukey method). (B) Antinociceptive responses of WT mice and VGAT+- mice in the tail-immersion test (48 °C). The tail-immersion test, in which the response is considered to be a spinal reflex, is normal in VGAT+- mice (Student’s t-test).

**Figure 4** The effects of partial reduction of VGAT on the formalin-induced persistent pain. (A) The time courses of the formalin-induced nociceptive responses in WT mice (open circle) and VGAT+- mice (closed circle) (n = 12, each). Total time of nociceptive response (s) per 5 min was measured. * P
Figure 5 Behavioral sensitivities to general anesthetics. (A) The latency to LORR recorded from the time of propofol (100 mg/kg) or ketamine (75 mg/kg) injection. (B) The duration of LORR produced by propofol (100 mg/kg) or ketamine (75 mg/kg).

Figure 6 Isolation of GABAergic and glycinergic mIPSCs in lamina II neurons of young WT mice and VGAT+/- mice. (A) Sample traces show GABAergic mIPSCs (left) and glycinergic mIPSCs (right) from WT mice (top) and VGAT+/- mice (bottom). Neurons were voltage-clamped at 0 mV using Cs2SO4-based internal solutions. Enlarged mIPSCs are also shown to compare the decay phase. (B) Cumulative distribution and mean amplitude of mIPSCs were compared between genotypes. *** P < 0.001 vs. corresponding WT mice (Student’s t-test) (C) The frequency of mIPSCs was compared between genotypes. ** P < 0.01 vs. corresponding WT mice (Student’s t-test).

Figure 7 The high potassium-induced potentiation of glycinergic, but not GABAergic, mIPSCs in lamina II neurons of young WT mice and VGAT+/- mice. (A) Representative traces of isolated GABAergic and glycinergic mIPSCs of WT mice and VGAT+/- mice. Currents were recorded in the presence of CNQX, AP5, TTX, and strychnine (for GABAergic mIPSC) or bicuculline (for glycinergic mIPSC). (B) Peak amplitudes of potassium-evoked currents were compared between genotypes (GABAergic: WT, n = 8, VGAT+/-, n = 6; glycinergic: WT, n = 6, VGAT+/-, n = 9). ** P < 0.01 vs. corresponding WT mice (Student’s t-test). (C) Charges of potassium-evoked currents were compared between genotypes (GABAergic: WT, n = 8, VGAT+/-, n = 6; glycinergic: WT, n = 6, VGAT+/-, n = 9). ** P < 0.01 vs. corresponding WT mice (Student’s t-test).
Table 1 GABA, glycine, and glutamate levels in the whole brain and spinal cord from WT mice and VGAT+/- mice

<table>
<thead>
<tr>
<th>Region</th>
<th>Neurotransmitter levels (nmol/g)</th>
<th>Neurotransmitter</th>
<th>WT</th>
<th>VGAT +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GABA</td>
<td>4052.1 ± 689.2</td>
<td>3899.3 ± 329.8</td>
</tr>
<tr>
<td>Whole brain</td>
<td></td>
<td>Glycine</td>
<td>1628.8 ± 312.7</td>
<td>1571.3 ± 146.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutamate</td>
<td>7137.9 ± 453.8</td>
<td>7575.6 ± 349.0</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>GABA</td>
<td>1035.6 ± 127.4</td>
<td>1078.0 ± 205.0</td>
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</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>4294.5 ± 287.5</td>
<td>4176.3 ± 313.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>4214.0 ± 117.7</td>
<td>4056.4 ± 158.1</td>
<td></td>
</tr>
</tbody>
</table>

Data represents means ± SEM (nmol/g) of five mice per group.
Table 2 Elevated plus-maze performance of WT mice and VGAT+/- mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>VGAT+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open arm duration (s)</td>
<td>30.3 ± 3.2</td>
<td>35.8 ± 4.8</td>
</tr>
<tr>
<td>Closed arm duration (s)</td>
<td>238.7 ± 13.8</td>
<td>230.9 ± 14.6</td>
</tr>
<tr>
<td>Central platform duration (s)</td>
<td>30.6 ± 4.4</td>
<td>33.0 ± 3.4</td>
</tr>
<tr>
<td>Open arm frequency</td>
<td>6.3 ± 1.8</td>
<td>7.2 ± 1.4</td>
</tr>
<tr>
<td>Closed arm frequency</td>
<td>4.5 ± 1.2</td>
<td>6.5 ± 1.8</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM of five mice. The mouse was placed in the center of the maze facing one of the enclosed arms and observed for five min.
Figure 2

**A**

- **Dwell time (outer)**
  - WT: Open square
  - VGAT+/-: Filled square

- **Distance travelled (outer)**
  - WT: Open square
  - VGAT+/-: Filled square

**B**

- **Time to platform (s)**
  - WT: Open circle
  - VGAT+/-: Filled circle

Graphs showing the comparison of dwell time and distance travelled between WT and VGAT+/- genotypes in outer regions, and the time to platform across trials.
Figure 3

A  The hot plate test

![Bar chart showing the hot plate test results.](chart)

B  The tail-immersion test

![Bar chart showing the tail-immersion test results.](chart)
Figure 4

A

Response time (sec)

Time (min)

WT
VGAT+/-

B

Response time (sec)

Phase 1
Phase 2

WT VGAT+/- WT VGAT+/-

**
Figure 5

A  The latency to LORR

B  The duration of LORR
Figure 6

A  GABAergic mIPSC

WT  30 pA  1 sec

VGAT+/-

B  Cumulative distribution

WT  VGAT+/-

GABAergic mIPSC (pA)

B  Amplitude (pA)

WT  VGAT+/-

C  frequency (Hz)

WT  VGAT+/-

GABAergic mIPSC

glycinergic mIPSC
Figure 7

A

High K + CNQX + AP5 + TTX

GABAergic mIPSC

WT

VGAT+/-

glycinergic mIPSC

WT

VGAT+/-

B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>VGAT +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak amplitude (pA)</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>High K charge (nC)</td>
<td></td>
<td>**</td>
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