1521-0111/12/8104-610–619$25.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 81:610–619, 2012

Vol. 81, No. 4
76083/3760320

Impaired Glycinergic Synaptic Transmission and Enhanced Inflammatory Pain in Mice with Reduced Expression of Vesicular GABA Transporter (VGAT)

Makiko Hardy Yamada, Koichi Nishikawa, Kazuhiro Kubo, Yuchio Yanagawa, and Shigeru Saito

Departments of Anesthesiology (M.H.Y., K.K., S.S.) and Genetic and Behavioral Neuroscience (Y.Y.), Gunma University Graduate School of Medicine, Maebashi City, Japan; and Department of Anesthesiology, Wakayama Medical University, Wakayama City, Japan (K.N.)

Received September 26, 2011; accepted January 9, 2012

ABSTRACT

Loading of GABA and glycine into synaptic vesicles via the vesicular GABA transporter (VGAT) is an essential step in inhibitory neurotransmission. As a result of the evidence linking alterations in GABAergic and/or glycinergic neurotransmission to various pain disorders, we investigated the possible influence of down-regulation of VGAT on pain threshold and behavioral responses in mice. The phenotypes of heterozygous VGAT knockout [VGAT(+/−)] mice were compared with 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, high-performance liquid chromatography 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 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 superficial laminae I and II, the first sites 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 (Zeilhofer, 2005; Price et al., 2009). 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 (Ahmadi 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 neu-

This research was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan [Grant-in-Aid 22791416] (to M.H.Y.), [Grants-in-Aid 20390412, 23659736] (to K.N.), [Grant-in-Aid 21890032] (to K.K.), [Grants-in-Aid 22300105, #23115503] (to Y.Y.); the Japan Medical Association (to K.N.); Japan Science and Technology Agency Core Research for Evolutional Science and Technology; and Takeda Science Foundation (to Y.Y.)

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

http://dx.doi.org/10.1124/mol.111.076083.

ABBREVIATIONS: CNS, central nervous system; VGAT, vesicular GABA transporter; GAD, glutamate decarboxylase; WT, wild type; HPLC, high-performance liquid chromatography; LORR, loss of righting reflex; ACSF, artificial cerebrospinal fluid; IPSC, inhibitory postsynaptic currents; mIPSC, miniature IPSC; HPLC, high-performance liquid chromatography; GlyT, glycine transporter; TTX, tetrodotoxin; RRP, readily releasable pool; GlyR, glycine receptor; PGE2, prostaglandin E2.

rotransmission. 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 (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 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 down-regulation 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 (Nishikawa and MacIver 2000; Mihic et al., 1997; Nishikawa et al., 2002). In this context, we recently reported that *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,b; Nishikawa et al., 2011). We then investigated how reduced VGAT levels affected GABAergic and glycinergic inhibition in dorsal horn neurons of the spinal cord using patch-clamp methods.

Although 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 glycinergic inhibition may be advantageous in situations in which a more localized enhancement of inhibition is needed (i.e., some pain disorders that are caused by 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 according to National Institutes of Health 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 to 16 weeks old weighing 23 to 28 g were used for behavioral experiments and young male mice from 14 to 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-h 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, and 1 mM PMSF). Homogenates were centrifuged at 3000 rpm for 10 min at 4°C to obtain the supernatant. The protein concentrations were determined by BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA) with bovine serum albumin as a standard. Equal amounts of proteins were separated by 7% SDS-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 1 h with 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20, the trans-blots were reacted with rabbit anti-VGAT antibody (1:1000; 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, Chalfont St. Giles, Buckinghamshire, UK) and imaged by light capture (ATTO, Tokyo, Japan). For quantification of the protein levels, the images were scanned by Scion Image software (Scion Corporation, Frederick, MD).

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 to 5 ml of saline (saline volume was approximately 10 times tissue weight) and then homogenized in phosphate-buffered saline containing 0.2% protease inhibitor using a homogenizer (24,000 rpm, 15 s, 2–3 times; Polytron; Kinematica, Littau-Lucerne, Switzerland). After removal of cell debris by centrifugation at 3000 rpm (20 min, 4°C), the supernatant (500 μl), which was added to sulfosalicylic acid (750 μl), was centrifuged again at 3000 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 SRL (Tokyo, Japan). Neurotransmitter content (nanomoles per gram) was calculated as follows: measured neurotransmitter concentration (nanomoles per milliliter) × saline volume added (milliliters)/tissue weight (grams).

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 6:00 PM and 10:00 PM.

Tail-Immersion Test. The spinally mediated nociceptive thresholds were determined using a thermoregulated 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 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 microsyringe. Each mouse was returned immediately to the observation place after injection. Animal behaviors after formalin injection were continuously recorded by a video recorder for later analysis. The amount of time the injected hind-paw was lifted, 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 was used as a surrogate measure for general anesthetic hypnosis. Each animal received an intraperitoneal injection of propofol or ketamine and was then placed on its back in a chamber (20 × 28 × 15 cm), and the ability of the mouse to right itself was observed. Mice were judged to have lost this reflex when unable to right themselves within 10 s. The time from intraperitoneal injection of the drug to LORR was considered the latency, and the time between the LORR and the time at which mice regained the ability to right themselves within 2 s was considered the duration of LORR.

Rotarod. To test motor coordination and equilibrium, animals were placed on the rotating RotaRod apparatus (Panlab, S.L., Barcelona, Spain), where the rotational speed of the rod was kept constant at 10 rpm. Mice were trained to remain on the RotaRod until they could perform three consecutive 180-s 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 (LE 8811; Panlab, S.L.). This system allowed us to analyze animal trajectories with 16 × 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 × 5 cm, surrounded by a 0.25-cm high border) and two closed arms (25 × 5 cm, surrounded by 15-cm high walls), with the two pairs of identical platform, which emerged from a central platform (5 × 5 cm), positioned opposite each other (EP-3002; Ohara Medical Co., Tokyo, Japan). The apparatus was 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. A trained experimenter who was blind to genotype performed behavioral analysis. Several parameters were collected during the session: 1) open arm duration; 2) closed arm duration; 3) central platform duration; 4) open arm frequency; and 5) 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 diameter circular pool was filled with water kept at 26°C. A 10-cm diameter platform was hidden 1 cm beneath the surface of the water at a fixed position. Mice that failed to find the platform within 60 s 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 (14–21 days old) male mice were used in this study. In brief, mice were decapitated under deep isoflurane anesthesia, and thoracolumbar laminectomy was performed. The spinal cord at spinal level T12-L1 was removed and then placed in preoxygenated Krebs' solution at 1–3°C. A block of the spinal cord was quickly dissected out and glued to a microslicer tray (DTK-1000; Dosaka EM, Tokyo, Japan) using oxygenated ice-cold modified Ringer's solution. The spinal cord was mounted on a microslicer; a transverse slice (300 μm) was cut from the center of the spinal segment and then kept in the prechamber (Brain Slice Chamber System; Harvard Apparatus, Holliston, MA) filled with artificial cerebrospinal fluid (ACSF) consisting of 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 11 mM glucose, bubbled with 95% O_2 and 5% CO_2 at room temperature (22–24°C). Slices were allowed at least 1 h for recovery in the prechamber, which was designed to keep six to eight 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; solution heater SH-27A; Warner Instruments, Hamden, CT) at a rate of approximately 6 ml/min. Patch electrodes were made from borosilicate thin-walled capillaries (Harvard Apparatus, Holliston, MA). Recording electrodes (4–6 MΩ) were filled with $CsSO_4$-based solution (110 mM $CsSO_4$, 5 mM TEA,0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, and 5 mM MgATP, 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 microscope (Axioskop2 FS plus; Zeiss, Jena, Germany). The magnified image was collected by an intensified charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, 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. An Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) was used for whole-cell recordings. Whole-cell currents were filtered at 2 to 5 kHz and digitized at 10 kHz (Digidata 1322A; Molecular Devices) and stored on a Pentium-based personal computer. Addition of bicuculline (10 μM; Sigma-Aldrich, St. Louis, MO) or strychnine (300 nM; Sigma-Aldrich) 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 (Molecular Devices) and Mini-analysis 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 3 times 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

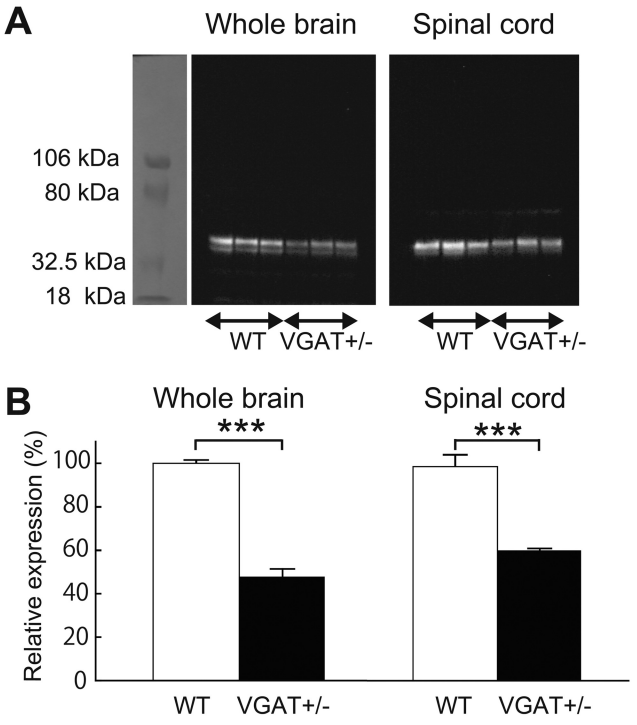

Fig. 1. Abundance of VGAT protein in the whole brain and spinal cord as measured by Western blotting. A, representative immunoblots of VGAT from WT mice and VGAT(+/−) mice. Relative signal intensities were normalized to β-actin. B, results are expressed as percentages of WT values. Data are shown as mean ± S.E.M. (n = 3 each) here and in subsequent figures. ***, $P < 0.001$; **, $P < 0.01$ versus corresponding age-matched WT mice (Student's t test).

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 injection of lipofundin MCT/LCT 10% (10 μl/g i.p.) 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 ± S.E.M. The results were analyzed by using Student's t test or one-way analysis of variance. 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. Despite reduced expression of VGAT protein levels in VGAT(+/−) mice, there were 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 the rotating bar for more than 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 (21.0 ± 0.9 s, n = 20) compared with WT mice (24.4 ± 1.1 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 sought to determine whether 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

TABLE 1
GABA, glycine, and glutamate levels in the whole brain and spinal cord from WT mice and VGAT(+/−) mice
Data represent means ± S.E.M. of five mice per group.

Region and Neurotransmitter	Neurotransmitter Levels	
	WT	VGAT(+/−)
	nmol/g	
Whole brain		
GABA	4052.1 ± 308.2	3899.3 ± 147.5
Glycine	1628.8 ± 139.8	1571.3 ± 65.3
Glutamate	7137.9 ± 202.9	7575.6 ± 156.1
Spinal cord		
GABA	1035.6 ± 57.0	1078.0 ± 91.7
Glycine	4294.5 ± 128.6	4176.3 ± 140.1
Glutamate	4214.0 ± 52.6	4056.4 ± 70.7

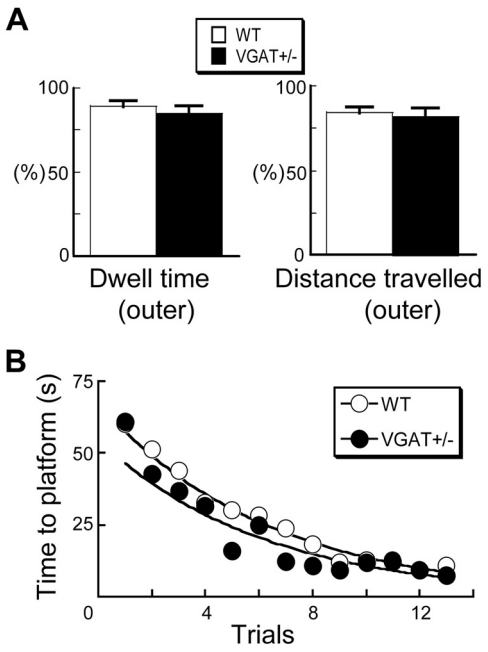

Fig. 2. A, VGAT(+/−) mice exhibit normal anxiety-like behaviors in the open field test. Regional dwell time in outer region (left) and regional distance traveled in outer region (right) was compared between genotypes (Student's *t* test, $n = 5$). B, performance of WT mice and VGAT(+/−) mice in the Morris water maze ($n = 10$ each). Learning curves for both genotypes show latencies to find the platform during trials.

that GlyT1 is involved in glycine supply for the activation of NMDA receptors. Intraperitoneal injection of *N*-[(3*R*)-3-([1,1′-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine hydrochloride (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 after 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 supraspinal 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 within 10 min of and 10 to 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 as a result of tissue damage, whereas the responses in phase 2 are a response to subsequent inflammation. A behaviorally quiescent interphase, in which 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 ($P < 0.05$ versus 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 $GABA_A$ receptors. The latency to LORR produced by propofol (100 mg/kg i.p.) was similar between both genotypes ($n = 20$ each; Fig. 5A, left). The latency to LORR produced by ketamine (75 mg/kg i.p.), 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 i.p.) and ketamine (75 mg/kg i.p.) between genotypes (Fig. 5B). These data suggest that VGAT(+/−) mice have normal sensitivity to anesthetic drugs that 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 $GABA_A$ receptor-mediated IPSCs [miniature IPSCs (mIPSCs)] and glycine receptor-mediated mIPSCs were recorded at 0 mV using Cs_2SO_4-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 were not detectable because the holding potential of 0 mV is near the reversal potential for excitatory postsynaptic currents.

Figure 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

TABLE 2
Elevated plus-maze performance of WT mice and VGAT(+/−)
Data represent mean ± S.E.M. of five mice. The mouse was placed in the center of the maze facing one of the enclosed arms and observed for 5 min.

Parameter	WT	VGAT(+/−)
Open arm duration, s	30.3 ± 3.2	35.8 ± 4.8
Closed arm duration, s	238.7 ± 13.8	230.9 ± 14.6
Central platform duration, s	30.6 ± 4.4	33.0 ± 3.4
Open arm frequency	6.3 ± 1.8	7.2 ± 1.4
Closed arm frequency	4.5 ± 1.2	6.5 ± 1.8

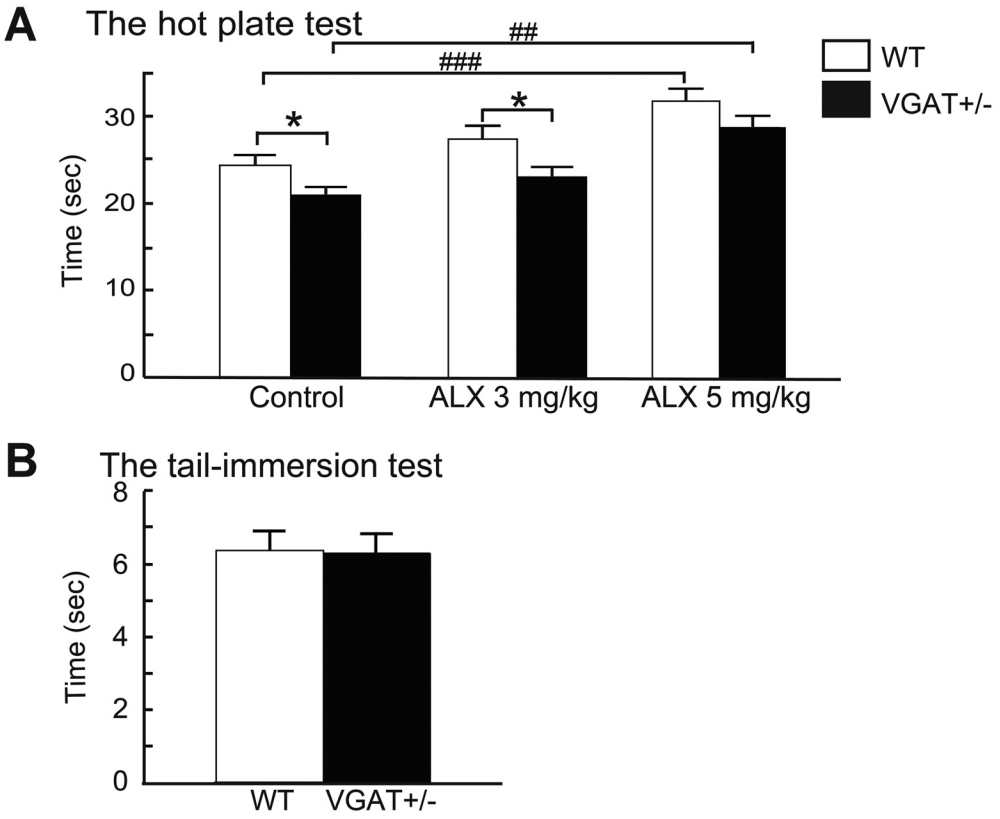

Fig. 3. Antinociceptive responses of WT mice and VGAT(+/−) mice in the hotplate test (53°C). A, the hotplate test revealed that the latency was significantly reduced in VGAT(+/−) mice ($n = 20$) compared with WT mice ($n = 20$; *, $P < 0.05$, Student's t test). ALX-5407, GlyT1 inhibitor, was injected intraperitoneally 24 h before the hotplate test. ALX-5407 dose dependently increased the latency in both genotypes. *, $P < 0.05$, comparison between genotypes (Student's t test); ##, $P < 0.01$ versus control in VGAT(+/−) mice; ###, $P < 0.001$ versus control in WT mice (one-way analysis of variance 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).

mIPSCs were observed in neurons of young mice (14–21 days old). These mixed GABAergic/glycinergic mIPSCs are the result from corelease of GABA and glycine at the same inhibitory synapse in spinal laminas I and 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 ($n = 7$, $P < 0.01$ versus WT mice, $n = 6$, Fig. 6C), indicating that reduction of VGAT proteins affected the probability of glycine release.

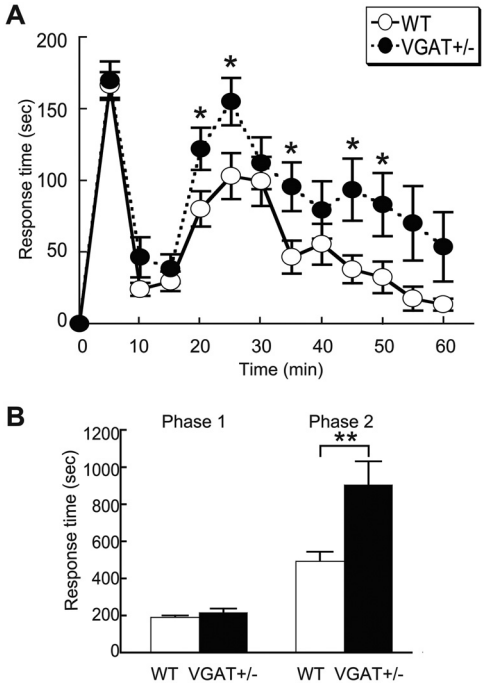

Fig. 4. The effects of partial reduction of VGAT on formalin-induced persistent pain. A, the time courses of the formalin-induced nociceptive responses in WT mice (○) and VGAT(+/−) mice (●) ($n = 12$, each). Total time of nociceptive response (seconds) per 5 min was measured. *, $P < 0.05$ between genotypes, Student's t test. B, the formalin-induced nociceptive responses of the initial 10-min period (phase 1) and the last 50-min period (phase 2) were compared between genotypes (**, $P < 0.01$).

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 Ca^{2+} in lamina II neurons using the whole-cell patch-clamp technique. Recordings were performed in the presence of bicuculline (10 μM), 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (20 μM), D,L-2-amino-5-phosphonovalerate (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, respectively (Mozhayeva et al., 2002; Moulder and Mennerick, 2005). 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

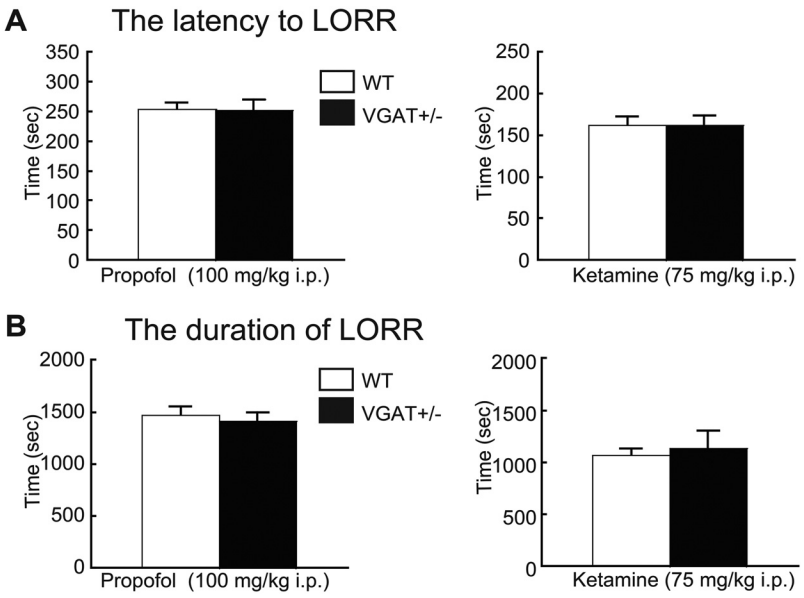

Fig. 5. Behavioral sensitivities to general anesthetics. A, the latency to LORR recorded from the time of propofol (100 mg/kg) or ketamine (75 mg/kg) injection. B, the duration of LORR produced by propofol (100 mg/kg) or ketamine (75 mg/kg).

($P < 0.05$, 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. On the basis of 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 kilobase pairs 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 predominantly 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, because 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 reported previously that *GAD65* gene knockout and the resulting reduction in GABAergic inhibition altered anesthetic sensitivity to propofol and short-term 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 glycinergic inhibition enhanced short-term 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, whereas 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, whereas bicuculline, a competitive antagonist of the $GABA_A$ 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 laminae II, III, and 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 glycinergic inputs, suggesting that lam-

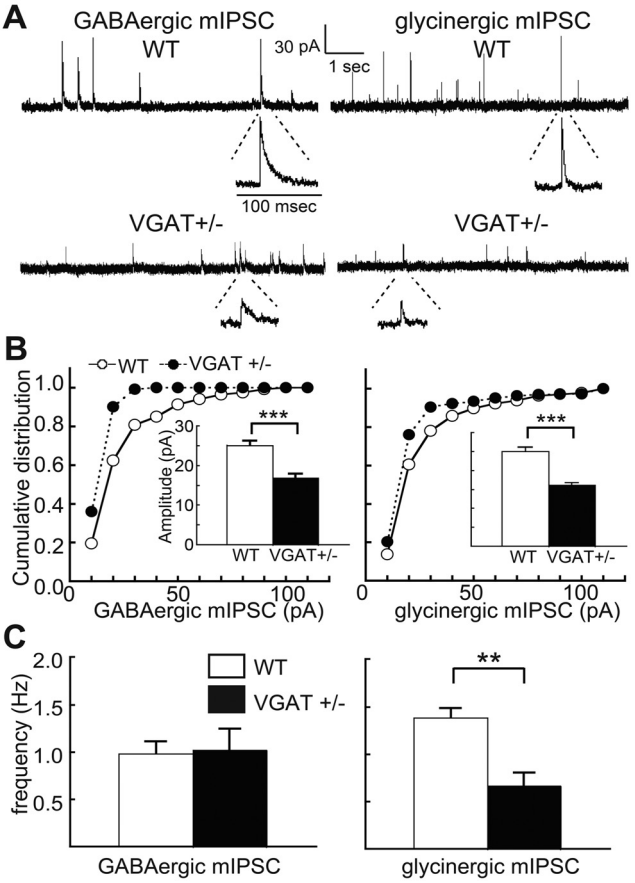

Fig. 6. Isolation of GABAergic and glycinergic mIPSCs in lamina II neurons of young WT mice and VGAT(+/−) mice. A, sample traces show GABAergic mIPSCs (left) and glycinergic mIPSCs (right) from WT mice (top) and VGAT(+/−) mice (bottom). Neurons were voltage-clamped at 0 mV using Cs_2SO_4-based internal solutions. Enlarged mIPSCs are also shown to compare the decay phase. B, cumulative distribution and mean amplitude of mIPSCs were compared between genotypes. ***, $P < 0.001$ versus corresponding WT mice (Student's t test). C, the frequency of mIPSCs was compared between genotypes. **, $P < 0.01$ versus corresponding WT mice (Student's t test).

ina II neurons, which play a crucial role in nociceptive processing, are regulated by glycinergic inhibition.

The Role of Glycine and Glycine Receptors in Inflammatory Pain. The amplitude of GABAergic and glycinergic mIPSCs was significantly reduced in lamina II neurons of VGAT(+/−) mice, suggesting that reduced VGAT protein levels led to a reduced content of GABA and glycine in the vesicles. The total GABA and glycine content per vesicle may therefore 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 because the tight seal was broken after sucrose application, presumably as a result of 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 that 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

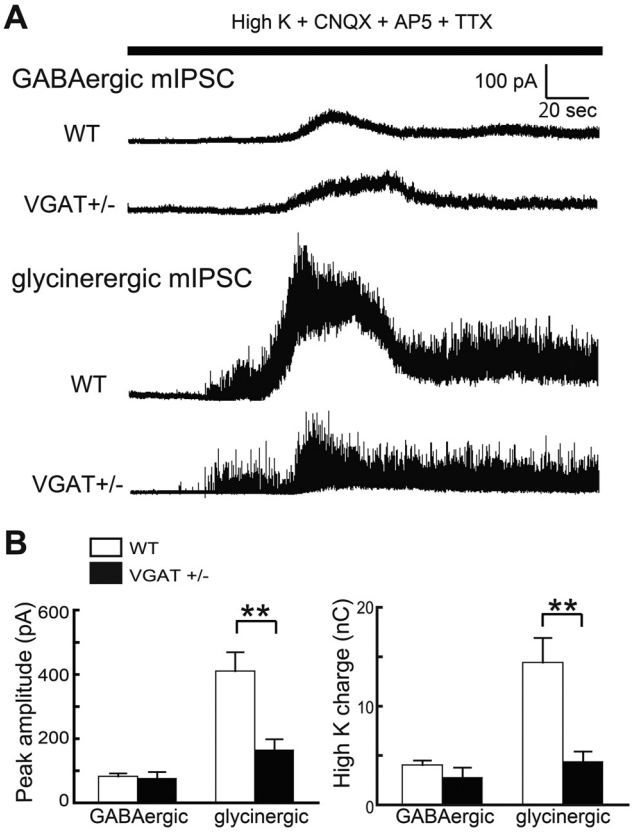

Fig. 7. The high potassium-induced potentiation of glycinergic, but not GABAergic, mIPSCs in lamina II neurons of young WT mice and VGAT(+/−) mice. A, representative traces of isolated GABAergic and glycinergic mIPSCs of WT mice and VGAT(+/−) mice. Currents were recorded in the presence of 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), D,L-2-amino-5-phosphonovalerate (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(+/−), $n = 9$]. **, $P < 0.01$ versus 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(+/−), $n = 9$]. **, $P < 0.01$ versus corresponding WT mice (Student's t test).

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 to 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 as a result of 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 seem 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, whereas 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

We 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, and M. Bruce MacIver and Yasuhiko Saito for critical comments on the manuscript. We also thank the staff at Institute of Experimental Animal Research, Gunma University Graduate School of Medicine, for assistance in animal care.

Authorship Contributions

Participated in research design: Yamada, Nishikawa, Kubo, Yanagawa, and Saito.

Conducted experiments: Yamada, Nishikawa, and Kubo.

Performed data analysis: Yamada and Nishikawa.

Contributed to the writing of the manuscript: Yamada and Nishikawa.

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Address correspondence to: Koichi Nishikawa, Department of Anesthesiology, Wakayama Medical University, 811-1 Kimiidera, Wakayama City 641-0012, Japan. E-mail: gaba-gly@wakayama-med.ac.jp