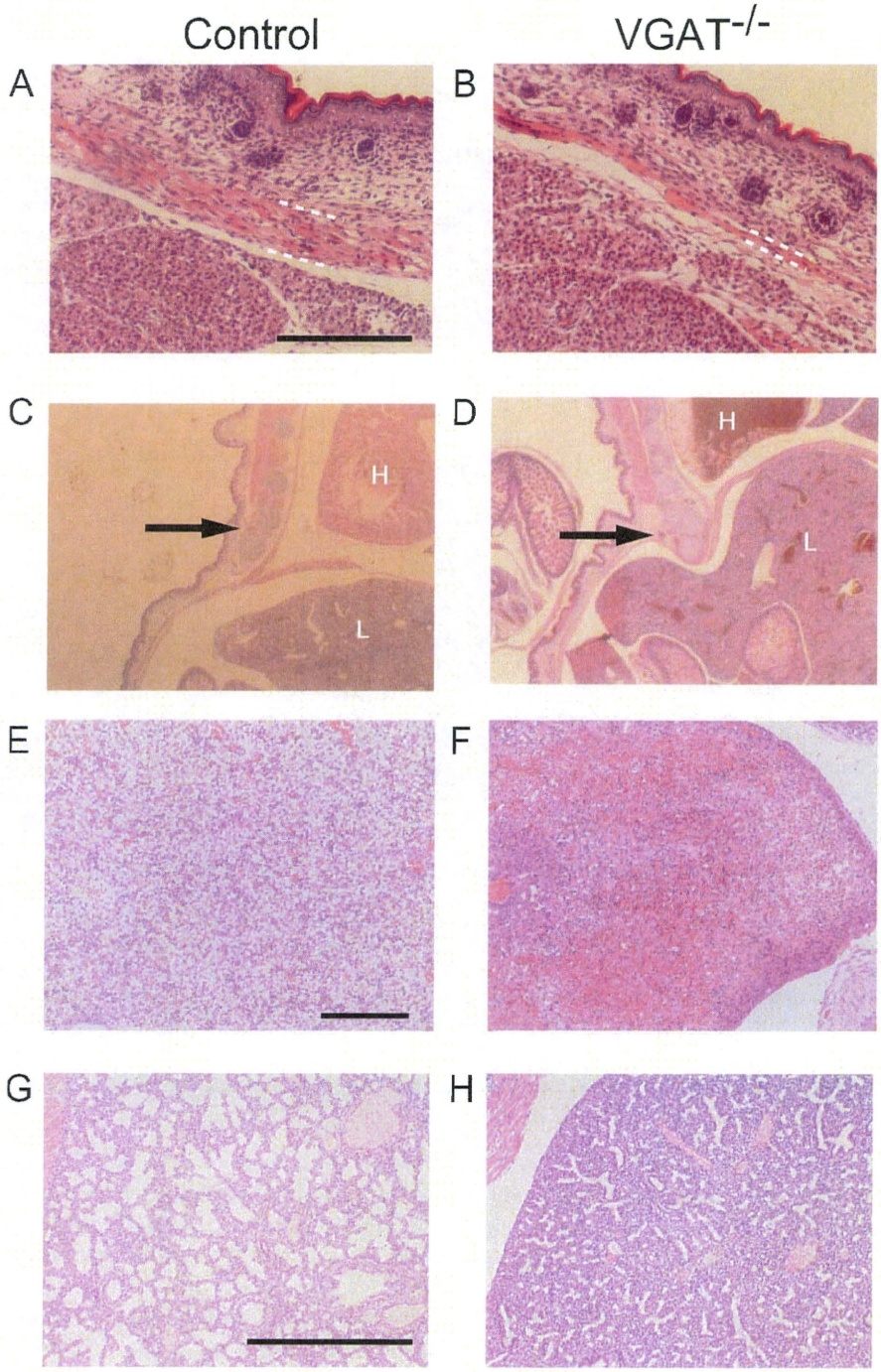

Figure 4 Histological analysis of VGAT$^{-/-}$ mice. Histological analysis (hematoxylin and eosin staining) of trapezius muscle (A, B), sagittal sections (C, D), liver (E, F) and lung (G, H) from E18.5 VGAT$^{-/-}$ mice (B, D, F, H) and control mice (A, C, E, G). (A, B) The trapezius muscle (bounded partly by white dashed lines) of VGAT$^{-/-}$ mouse (B) was thinner than the control mouse (A). Scale bar: 200 μm. (C, D) The VGAT$^{-/-}$ ribs (arrow in D) in the lower part were depressed, and their position was inside compared to the control ribs (arrow in C). H, heart; L, liver. (E, F) Red blood cell congestion was characteristic of VGAT$^{-/-}$ liver, but not control liver. Scale bar: 200 μm. (G, H) The VGAT$^{-/-}$ lung contained much less alveolar space than the control lung. Scale bar: 500 μm.

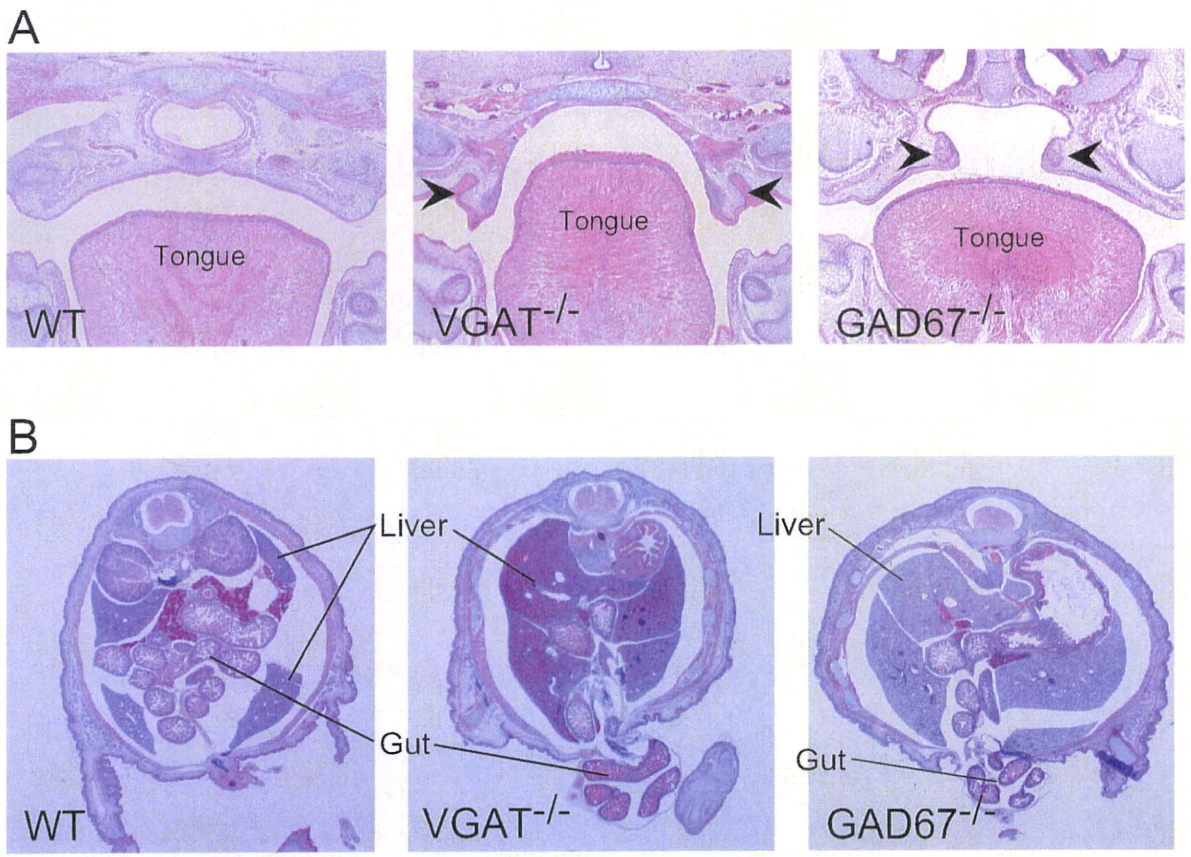

Figure 5 Histological comparison of omphalocele and cleft palate between VGAT$^{-/-}$ and GAD67$^{-/-}$ mice. (A) Hematoxylin-eosin stained coronal sections of the facial region of E18.5 wild-type (left panel), VGAT$^{-/-}$ (middle panel) and GAD67$^{-/-}$ (right panel) mice. Histological examination revealed that the secondary palatal shelves (arrowhead) of VGAT$^{-/-}$ mice were directed vertically down along the side of tongue in contrast to the fused palate of wild-type mice. In the cleft palate of GAD67$^{-/-}$ mice (right panel), palatal shelves failed to fuse but were elevated horizontally unlike those in VGAT$^{-/-}$ mice. (B) Hematoxylin-eosin stained coronal sections of the umbilical region of E18.5 wild-type (left panel), VGAT$^{-/-}$ (middle panel) and GAD67$^{-/-}$ (right panel) embryos. The ventral body wall of the VGAT$^{-/-}$ mice did not close and the gut protruded from the peritoneal cavity. In contrast to VGAT$^{-/-}$ mice, the ventral body wall of wild-type mice closed around the umbilicus, and the gut had already returned to the peritoneal cavity. The omphalocele in GAD67$^{-/-}$ mice was less severe than in VGAT$^{-/-}$ mice because a large amount of gut was observed at the umbilical level in VGAT$^{-/-}$ mice compared to GAD67$^{-/-}$ mice.

allele allowed for Cre-mediated conditional inactivation of the VGAT gene, and mice carrying these alleles will be useful in examining VGAT function at different developmental stages and in distinct cell types.

Increase of overall GABA and glycine contents in VGAT$^{-/-}$ forebrains

We showed that both GABA and glycine contents were increased in the VGAT$^{-/-}$ forebrain, but not an excitatory neurotransmitter glutamate, which is transported into synaptic vesicles by vesicular glutamate transporters. An increase in GABA content in VGAT$^{-/-}$ mice is similar to that in ACh contents in VAChT knockout mice [16], but it is opposite of the decrease in monoamine contents in vesicular monoamine transporter 2 knockout mice. In *C. elegans*, the mutational inactivation of VGAT also leads to an increase in GABA immunoreactivity in GABAergic neurons [24]. In VAChT knockout mice the amount of the ACh-synthesizing enzyme choline acetyltransferase (ChAT) is increased at the mRNA and protein levels compared to their wild-type littermates, suggesting that the change in ChAT expression may be related to a compensatory mechanism due to the lack of ACh release [16]. Conversely, the amounts of the GABA-synthesizing enzymes GAD65 and GAD67 were not different in between the brains of VGAT$^{-/-}$ mice and their control littermates (Figure 2B). Therefore, it is possible that the increased GABA in VGAT$^{-/-}$ brain was due to a reduction in their degradation. GABA and glycine are released from presynaptic

neurons into the synaptic cleft and are retrieved in neurons and glial cells by plasma membrane transporters [25,26]. GABA and glycine taken up in glial cells are further metabolized, but the GABA and glycine taken up in neurons are directly recycled into synaptic vesicles [27,28]. Because degradation systems for both GABA and glycine are mainly localized to glial cells [27,29], the transport into glial cells from the synaptic cleft is important for their degradation. Because the synaptic release of GABA and glycine was absent in VGAT$^{-/-}$ mice, the deletion of VGAT may result in little or no transport of GABA and glycine into glial cells. GABA and glycine then accumulate in the GABAergic and glycinergic neurons, respectively, but they are not degraded in the glial cells of VGAT$^{-/-}$ mice.

Contribution of VGAT to motor function

In the embryonic spinal cord of rodents, synaptic transmission to MNs mediated by GABA and glycine is prominent from the early fetal period [30,31]. Our results from the electrophysiological recordings of spinal cord MNs indicate that the inhibitory synaptic transmission was clearly absent in the VGAT knockout MNs, but that the excitatory synaptic transmission was present. Our results also suggest the absence of other functional mechanisms that transport GABA and/or glycine into synaptic vesicles in these synapses. VGAT$^{-/-}$ fetuses at E17.5-18.5 not only were completely immobile and stiff, but also none of them responded to mechanical stimuli by pinching the limb or the tail. Therefore, it is probable that the lack of inhibitory transmission onto MNs in VGAT$^{-/-}$ fetuses resulted in defects in the spontaneous and stimulus-induced movements *in vivo* despite the presence of excitatory synaptic transmission.

In addition to the defect in motor movement, trapezius muscle displayed atrophy in VGAT$^{-/-}$ mice. Embryonic myogenesis progresses by the proliferation of myoblasts and fusion of myotubes, but it requires substantial cell death [32]. Physical forces play a significant role in the development and maintenance of skeletal muscle [33]. In cultured myoblasts, chronic and cyclic stretch results in an increase in cell death, including apoptosis [34]. Therefore, a possible explanation for the atrophy in VGAT$^{-/-}$ trapezius muscle is that stretching of the trapezius muscle due to the hunched posture caused an increase in apoptosis during development.

Phenotypes of VGAT and GAD67 knockouts outside of the brain

Ventral body wall closure abnormalities, such as omphalocele, are common human birth defects, but their molecular and cellular bases are poorly understood [35]. The mouse provides a model system to study the genetic defects and environmental insults that can lead to ventral body wall closure abnormalities [20]. In this study, omphalocele was observed in VGAT$^{-/-}$ and GAD67$^{-/-}$ mice, indicating that the lack of GABA signaling was involved in its onset. Omphalocele has been observed in K^+-Cl^--cotransporter 2 (KCC2) knockout mice [36], and KCC2 is required for GABA- and/or glycine-induced hyperpolarizing responses [37]. In the KCC2 knockout mice, GABA and/or glycine signals continue to act in an excitatory, but not an inhibitory, manner. Therefore, the omphaloceles observed in both VGAT$^{-/-}$ mice and GAD67$^{-/-}$ mice resulted from defects in the inhibitory neurotransmission derived from the hyperpolarizing response. A lack of inhibitory transmission in VGAT$^{-/-}$ mice may lead to motor deficits, such as a hunched posture. It is likely that the hunched posture resulted in increases in both intrathoracic and intraabdominal pressures and this increased pressure caused omphalocele.

Concerning the mechanism of onset of cleft palate, studies using knockout mice have revealed associations between cleft palate and mutation of genes related to GABA signaling, such as *GAD67* and *GABRB3* [8,23,38]. Because the lack of the GAD67 gene leads to a reduction in tongue movement [39], the sluggish tongue may be an obstacle to development of the palatal shelves.

The cleft palate and omphalocele phenotypes were more severe in VGAT$^{-/-}$ mice than in GAD67$^{-/-}$ mice. Glycinergic transmission is present in embryonic spinal cord and brainstem [40]. Hyperekplexia is a neurogenetic disorder caused mostly by mutations in the gene encoding the α1 subunit of glycine receptor and is characterized by an exaggerated startle response and neonatal hypertonia. In patients with hyperekplexia, the recurrent abdominal muscle contraction from the exaggerated startle response can increase the abdominal pressure and lead to omphalocele and inguinal hernia [41,42]. These reports suggest that a defect in glycinergic transmission is involved in the onset of omphalocele. A small amount of GABA is synthesized by another GAD isoform, GAD65, at the embryonic stage [8,43]. The differences in the severity between VGAT$^{-/-}$ and GAD67$^{-/-}$ mice must be due to the presence of both glycinergic and GAD65-produced GABAergic transmission in GAD67$^{-/-}$ fetuses, but not in VGAT$^{-/-}$ fetuses.

Conclusion

In the present study, we established a VGAT knockout mouse, with which we demonstrated that VGAT is fundamental for GABAergic and/or glycinergic transmission. We also showed that VGAT is important for fetal growth and the development of muscle, liver and lung. The VGAT knockout mice described here may provide a useful tool for the study of specific functions of VGAT-dependent GABAergic and/or glycinergic

transmission in mice. GABAergic neurons are classified into several subtypes according to the expression of chemical markers, such as parvalbumin and somatostatin [44,45]. Therefore, our floxed VGAT mice will be useful for conditional knockout studies to further investigate the role of VGAT in GABAergic neuronal subtypes.

Methods

Animals

All animal procedures were conducted in accordance with the guiding principles of the NIH under the review and approval of the Animal Care and Experimentation Committee, Gunma University, Showa Campus (Maebashi, Japan). Every effort was made to minimize the number of animals used and their suffering.

Construction of the Targeting Vector

Genomic BAC clones containing the mouse VGAT (mVGAT) locus were purchased, and the regions covering the entire VGAT gene were subcloned [13]. A genomic fragment spanning exons 1-3 of the mVGAT gene was used for the targeting vector (Figure 1; targeting vector). The HindIII (in the 5'-flanking region) - KpnI (in the 3'-flanking region) fragment (7.5 kb) was subcloned into pBluescript II KS(-), and the 5'-loxP site was introduced into the XbaI site (in intron 1). The 5'-loxP site was flanked by a KpnI site artificially introduced for Southern blot analysis. The 7.5 kb fragment was used as the 5' homologous region containing the 5'-flanking region, exons 1-3 and the 3'-flanking region. The frt-flanked PGK-Neo cassette for positive selection of ES clones and the 3'-loxP site were inserted into the KpnI site (in the 3'-flanking region). The KpnI-BstEII fragment in the 3'-flanking region (3.5 kb) was added as the 3' homologous region. An MC1-DT-ApA cassette for negative selection [46] was ligated to the 3' end of the homologous region.

Creation of a VGAT knockout allele

The linearized targeting vector was introduced by electroporation into ES cells (CCE) of 129/SvEv mouse origin, and G418-resistant colonies were screened by Southern blot analysis using probes outside of the targeting vector. KpnI-digested genomic DNA prepared from ES cell colonies was hybridized with 5' probes. The correctly targeted ES clones were injected into C57BL/6 blastocysts to produce germline chimeras. The germline chimeras were mated with C57BL/6 mice to establish the $VGAT^{floxneo/+}$ mouse line. $VGAT^{floxneo/+}$ mice were crossed with CAG-Cre mice [14] to excise exons 2 and 3 (VGAT knockout allele), and $VGAT^{+/-}$ mice were obtained. We then intercrossed $VGAT^{+/-}$ mice to generate $VGAT^{-/-}$ mice. When we performed timed matings of the $VGAT^{+/-}$ mice, the morning on the day of vaginal plug detection was designated E0.5.

Genotypes of $VGAT^{+/+}$, $VGAT^{+/-}$ and $VGAT^{-/-}$ mice were determined by PCR using the following oligonucleotides: primer P1 (5'-AGTCTGATCCCGTGGCACTTCCAACTC-3') corresponding to intron 1 of the VGAT gene and primers P2 (5'-TCAGAGGCTTCT TCCTAGGGCTGCTG-3') and P3 (5'-GACCTCCCCCATTGCATAGAATGGCAC-3') corresponding to the 3'-flanking region of the VGAT gene. The primer set of P2 and P3 amplified a 183-bp fragment specific for the wild-type allele, and the primer set of P1 and P3 yielded a 430-bp fragment specific for the knockout allele.

GAD67 knockout mice

We used homozygous GAD67-GFP (□neo) ($GAD67^{GFP/GFP}$) mice as GAD67 knockout ($GAD67^{-/-}$) mice. The generation of the GAD67-GFP (□neo) mice and their genotyping by PCR were described previously [47,48]. In the GAD67-GFP (□neo) mice, a cDNA encoding enhanced green fluorescent protein (EGFP) followed by an SV40 polyadenylation signal was targeted to the locus encoding GAD67 by homologous recombination, and the GAD67 gene was disrupted.

Western blotting and measurement of neurotransmitter contents

For Western blotting, homogenates prepared from E18.5 mouse brain were separated by 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Whatman, Maidstone, UK), and probed with antibodies specific for VGAT (1:1000) [49], GAD65/67 (1:1000) [50], synaptophysin (1:1000) (Synaptic Systems, Goettingen, Germany), and β-actin (1:10000) (Abcam, Cambridge, UK). After the membranes were washed with Tris-HCl buffered saline containing 0.05% (w/v) Tween 20, the bound antibodies were visualized with horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) using the ECL Western blotting detection system (GE Healthcare, London, UK). Protein levels were quantified using Light Capture and its quantification software (ATTO, Tokyo, Japan). Expression levels were normalized to β-actin or synaptophysin levels, and the values are expressed as means ± SE. Statistical significance was assessed using Student's *t*-test.

GABA, glycine, and glutamate contents in the E18.5 mouse forebrain were measured according to previously described method [21,47].

Electrophysiological recording in spinal cord

Embryos (E17.5-18.5) of control ($VGAT^{+/+}$; n = 3, $VGAT^{+/-}$; n = 5) and $VGAT^{-/-}$ (n = 12) mice were

decapitated and eviscerated, and the spinal cord was removed by ventral laminectomy. The isolated spinal cord was placed in a recording chamber perfused with oxygenated Ringer's solution (118.4 mM NaCl, 3 mM KCl, 2.52 mM $CaCl_2$, 1.25 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.18 mM KH_2PO_4, and 11.1 mM D-glucose aerated with 5% CO_2 in O_2) at room temperature. Recordings from MNs in the isolated spinal cord were performed as described previously [17]. Briefly, visually guided whole-cell tight-seal recording of MNs was performed with patch electrodes pulled from thick walled borosilicate glass to a final resistance of 5-8 MΩ. The electrode tips were filled with (in mM) 138 K-gluconate, 10 HEPES, 1 $CaCl_2$, 5 ATP-Mg, and 0.3 GTP-Li. Intracellular signals were amplified with a Multiclamp 700B amplifier (Molecular Devices, Union City, CA), digitized at 5 kHz with the Digidata 1440A data acquisition system (Molecular Devices) and saved on a hard disk for off-line analysis. Electrical stimulations of lumbar ventral roots (VRs) were performed using glass suction electrodes. MNs were identified visually as cells with large soma in the ventral horn and by observing the antidromic firing activated by the electrical stimulation of the adjacent VR. All drugs (CNQX, AP5, strychnine and picrotoxin; Sigma-Aldrich, St. Louis, MO) were dissolved in Ringer's solution and bath-applied to the preparation. Analysis was performed using pClamp 10 software (Molecular Devices).

Histology

$VGAT^{-/-}$, $VGAT^{+/-}$ and $VGAT^{+/+}$ mice at E18.5 were investigated. Samples were fixed in 10% (vol/vol) formaldehyde, dehydrated with a graded series of ethanol solutions, and embedded in paraffin. Three-micrometer sections were prepared, subjected to paraffin removal by immersion in xylene, rehydrated, and stained with hematoxylin-eosin. $VGAT^{+/-}$ and $VGAT^{+/+}$ mice were mixed together and are referred to as control mice.

Additional material

Additional file 1: Supplementary Figure S1. VGAT expression levels in VGAT mutant mice. (A, B) VGAT expression level was normal in adult $VGAT^{floxneo/floxneo}$ mice. Western blot of whole brain homogenates from adult $VGAT^{+/+}$ (+/+) and $VGAT^{floxneo/floxneo}$ (floxneo/floxneo) mice is shown (A). VGAT expression level normalized to β-actin was not different between $VGAT^{+/+}$ (+/+) and $VGAT^{floxneo/floxneo}$ (floxneo/floxneo) mice (B). (C, D) VGAT expression level was reduced by about half in adult $VGAT^{+/-}$ mice. Western blot of whole brain homogenates of adult $VGAT^{+/+}$ (+/+) and $VGAT^{+/-}$ (+/-) mice is shown (C). VGAT expression level normalized to synaptophysin was significantly different between $VGAT^{+/+}$ (+/+) and $VGAT^{+/-}$ (+/-) mice (D). Significance was tested by Student's t-test (*P < 0.05).

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Authors' contributions
Conceived and designed the experiments: KS, TK, HN, YN, MY, SK, AF, MF, KN, KO, YY. Performed the experiments: KS, TK, HN, TF, RH, ST, SE, MU, KI, MF, KN, KO, YY. Analyzed the data: KS, TK, HN, TF, RH, KI, AF, MF, KN, KO, YY. Contributed new reagents/analytical tools: ST, MM, JM. Wrote the paper: KS, TK, HN, AF, MF, KN, KO, YY. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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