FLUORESCENT LABELING OF BOTH GABAERGIC AND GLYCINERGIC NEURONS IN VESICULAR GABA TRANSPORTER (VGAT)–VENUS TRANSGENIC MOUSE

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Abstract—Inhibitory neurons play important roles in a number of brain functions. They are composed of GABAergic neurons and glycinergic neurons, and vesicular GABA transporter (VGAT) is specifically expressed in these neurons. Since the inhibitory neurons are scattered around in the CNS, it is difficult to identify these cells in living brain preparations. The glutamate decarboxylase (GAD) 67–GFP knock-in mouse has been widely used for the identification of GABAergic neurons, but their GAD67 expression was decreased compared to the wild-type mice. To overcome such a problem and to highlight the function and morphology of inhibitory neurons, we generated four lines of VGAT–Venus transgenic mice (lines #40, #29, #39 and #49) expressing Venus fluorescent protein under the control of mouse VGAT promoter. We found higher expression level of Venus transcripts and proteins as well as brighter fluorescent signal in line #39 mouse brains, compared to brains of other lines examined. By Western blots and spectrofluorometric measurements of forebrain, the line #39 mouse showed stronger GFP immunoreactivity and brighter fluorescent intensity than the GAD67–GFP knock-in mouse. In addition, Venus was present not only in somata, but also in neurites in the line #39 mouse by histological studies. In situ hybridization analysis showed that the expression pattern of Venus in the line #39 mouse was similar to that of endogenous VGAT. Double immunostaining analysis in line #39 mouse showed that Venus-expressing cells are primarily immunoreactive for GABA in cerebral cortex, hippocampus and cerebellar cortex and for GABA or glycine in dorsal cochlear nucleus. These results demonstrate that the VGAT–Venus line #39 mouse should be useful for studies on function and morphology of inhibitory neurons in the CNS. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: vesicular GABA transporter, inhibitory neurons, label, fluorescent protein, promoter, transgenic mouse.

Inhibitory neurons play an important role in the regulation and stabilization of network activities and are essential for a number of brain functions such as cognition, perception, movement, and emotion. Inhibitory neurons are composed of GABAergic and glycinergic neurons, and their distributions are very different in the mammalian CNS. GABAergic neurons widely spread in the CNS, whereas glycinergic neurons are largely restricted to the brainstem and spinal cord (Esclapez et al., 1994; Legendre, 2001). Therefore, forebrain inhibitory neurons are GABAergic but not glycinergic. Inhibitory neurons are primarily scattered around throughout the CNS, and thus can be hardly identified in living brain preparations.

GABA is synthesized from glutamic acid by glutamate decarboxylase (GAD) (Roberts and Kuriyama, 1968) and is accumulated into synaptic vesicles by vesicular GABA transporter (VGAT) (McIntire et al., 1997; Sagné et al., 1997). Two isozymes of GAD, GAD65 and GAD67 are primarily expressed in GABAergic neurons with some exceptions (Bo et al., 1992; Esclapez et al., 1994; Parrish-Aungst et al., 2007). On the other hand, VGAT is present in both GABAergic and glycinergic neurons, and is also called vesicular inhibitory amino acid transporter (VIAAT) (Dumoulin et al., 1999; Sagné et al., 1997). In addition to its presence at GABAergic and glycinergic synapses, the role of VGAT/VIAAT in both GABA and glycine release is supported by electrophysiological evidence from VGAT-deficient mice (Wojcik et al., 2006) and from VGAT-transfected secretory cells (Aubrey et al., 2007).

To date, transgenic labelings of GABAergic cells in mice are produced using one of the two GAD promoters and pronuclear injection. The resultant GAD67–GFP transgenic mice expressed GFP in only subpopulations of GABAergic cells (Oliva et al., 2000; Chattopadhyaya et al., 2004; Ma et al., 2006). On the other hand, in GAD65–GFP transgenic mice, abundant GABAergic cells are fluorescently labeled, but the GAD65–GFP cells are a specific subgroup within the larger population of GAD67-expressing cells and the cells expressing only GAD67 are not labeled (López-Bendito et al., 2004; Parrish-Aungst et al., 2007; Young and Sun, in press).

We previously generated the GAD67–GFP knock-in mouse using a gene targeting method via homologous...
recombination in ES cells (Tamamaki et al., 2003). GFP fluorescence was specifically observed in most of GABAergic neurons in the GAD67–GFP knock-in mouse (Acuna-Goycolea et al., 2005; Brown et al., 2008; Ono et al., 2005). The GAD67–GFP knock-in mouse has helped to elucidate the anatomical profile of GABAergic neuronal network and its electrophysiological activity as well as the development of GABAergic neurons (Berghuis et al., 2007; Marowsky et al., 2005; Yamanaka et al., 2004). However, the overall GABA content was shown to be reduced because of destruction of the endogenous GAD67 gene. Furthermore, it was reported that heterozygous GAD67–GFP knock-in mice caused the impairment of synaptic innervations during development (Nakayama et al., 2007).

In order to generate a transgenic mouse, which has both a fluorescent labeling of inhibitory neurons and a normal level of GAD67, we used a bacterial artificial chromosome (BAC) construct that allows the VGAT gene promoter-driven expression of Venus, a fluorescent protein brighter than enhanced GFP (Nagai et al., 2002). We generated four lines of VGAT–Venus transgenic mice using the BAC construct. In this study, we examined the

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**Fig. 1.** Spatial expression of Venus and VGAT mRNAs among various lines of VGAT–Venus transgenic mice. Sagittal sections of brains from the wild-type mouse (A, B) and transgenic mouse line #04 (C, D), line #29 (E, F), line #39 (G, H) and line #49 (I, J) were hybridized with radioactive probes for either Venus (A, C, E, G, I) and VGAT (B, D, F, H, J), and were emulsion-autoradiographed. Dark-field photographs are shown.
expression of Venus transgene and the protein levels of GAD65, GAD67 and VGAT in VGAT–Venus mouse lines, and confirmed that there were virtually no changes in such protein levels compared to the wild-type controls, which is a clear contrast to the GAD67–GFP knock-in mouse. Moreover, we found that the fluorescence intensity was much brighter and the protein level of Venus was much higher in one mouse line #39 than that of GFP in the GAD67–GFP knock-in mouse. In the line #39 mouse, Venus expression was largely consistent with VGAT expression and Venus fluorescence was sufficiently bright to visualize inhibitory neurons. We show here that the VGAT–Venus mouse line #39 is a powerful tool for the research on both GABAergic and glycineergic inhibitory neurons in the CNS.

**EXPERIMENTAL PROCEDURES**

**Animal**

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.

**Generation of VGAT–Venus transgenic mice**

The DNA construct used for pronuclear injection was the same VGAT–Venus BAC vector as in Uematsu et al. (2008). The vector contained 5 kb of the mouse VGAT gene and 102 kb upstream and 25 kb downstream. In addition, the vector included Venus cDNA followed by SV40 polyadenylation signals placed in frame into the ATG translation initiation codon of exon one of the VGAT gene (Ebihara et al., 2003). To isolate the fragment for injection, we digested VGAT–Venus BAC vector with NruI to release the VGAT–Venus DNA from the BAC backbone. The linearized fragment was injected into fertilized eggs of C57BL/6 according to the procedure described by Brinster et al. (1985).

Among 445 pronuclear-stage zygotes injected, 365 could be transferred into pseudopregnant ICR mice. From them, 56 mice were born. Five transgenic founders were identified from the pups by PCR for the presence of Venus. Primers used for the PCR were Venus-F: 5′-ATGGTGAGCAAGGGCGAGGAGCTGT-3′ and Venus-R: 5′-TTACTTTGATACGCTGTCATGCGGA-3′. Four founders transmitted the transgene to their progeny. The lines (lines #04, #29, #39, and #49) derived from these founders were maintained on the C57BL/6 background by crossing transgenic mice with wild-type to generate heterozygous offspring, and heterozygous transgenic mice were characterized. Venus expression in line #39 of newborn mouse brains was detected by MOD.GFsP-5, a GFP visualization tool for macroscopy (Biological Laboratory Equipments, Maintenance and Services Ltd., Budapest, Hungary). Heterozygous GAD67–GFP (Δneo) mice expressing GFP under the control of the endogenous GAD67 promoter were used for comparison (Tamamaki et al., 2003). In this study these transgenic mice are referred to as GAD67–GFP knock-in mice.

**Genotyping by PCR**

To detect BAC transgene integration, PCR analysis is performed. The BAC transgene has small portions of BAC vector at both ends of the insert, and they were termed as 5′- and 3′-arms (Fig. 2A).

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**Fig. 2.** Characterization of transgene integration in VGAT–Venus mouse lines. (A) Schematic representation of a BAC construct for the generation of the VGAT–Venus transgenic mice. Venus cDNA followed by SV40 poly-A signal was inserted into exon 1 of the VGAT gene in frame with translation initiation codon. The modified BAC construct was digested with NruI. The resultant linearized BAC DNA for injection into mouse eggs contained VGAT gene, Venus gene, and 102 kb upstream and 25 kb downstream of the VGAT gene, in addition to 5′- and 3′-arms derived from pBeloBAC vector. The positions of PCR corresponding to the 5′-arm, VGAT–Venus and 3′-arm of the transgene are indicated by arrows. (B) PCR typing corresponding to the 5′-arm, VGAT–Venus and 3′-arm of the 133 kb transgene was used to analyze the presence of each sequence in each transgenic line. The DNA sequences corresponding to the GAD67 gene were amplified as an internal control. Genomic DNAs from three animals from each transgenic mouse line were used for PCR typing.
quence side) of the pBeloBAC11 vector were CAT-4: 5′-ATGACGCTGATCCTGATCGGCGCGATC-3′ and CAT-3b: 5′-CGGCGCGACGCTGATCGGCGCGATC-3′ and primers for the 3′-arm (T7 promoter sequence) side detection were Belo-1: 5′-ACGTGGTTGATTGCGCTCACGACG-3′ and Belo-2: 5′-TCGTATCTTGCTGATCGGCGCATG-3′ (PCR product size, 431 bp). Primers for detection of VGAT–Venus DNA were Venus–3tg: 5′-TGAGCTACCAGTCCGCCCTGAGCAA-3′ and VGAT–1tg: 5′-GGCTCACTTGGCCTGGACCTTTG-3′ corresponding to the Venus gene and VGAT gene exon one, respectively (PCR product size, 452 bp). Primers for detection of GAD67 as a control were primer #3: 5′-GGCAACAGTCCTCCCTTCTGGTCG-3′ and primer #4: 5′-GCTTCCTCTTGGCCCTGCACG-3′ corresponding to intron 0b and intron one of the GAD67 gene, respectively (PCR product size, 265 bp) (Kaneko et al., 2008; Yanagawa et al., 1997).

Western blots and spectrofluorometric analysis

Whole brain or forebrain was taken from heterozygous GAD67–GFP knock-in mice, three lines of VGAT–Venus mice (#04, #39 and #49) and wild-type mice at postnatal day (P) 21–35, and was 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 as S1 fraction (for Western blots). The S1 fraction was centrifuged at 12,000 rpm for 10 min at 4 °C to obtain the supernatant as S2 fraction (for spectrofluorometric analysis). The protein concentrations of S1 and S2 fractions were determined by BCA protein assay reagent (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard. Equal amounts of protein from the S1 fractions of whole brain or forebrain were separated by 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (for detection of GAD65, GAD67, VGAT and β-actin) and 15% SDS–PAGE (for detection of GFP, Venus and β-actin). Then, the proteins were transferred to nitrocellulose membrane using a semidry transfer method. The membrane was reacted with rabbit anti-GAD65/67 antibody (1:1,000; Asada et al., 1996), anti-VGAT antibody (1:1,000; Takamori et al., 2000), anti–GFP (1:1,000; Novus Biologicals, Littleton, CO, USA) antibody or mouse anti–β-actin antibody (1:10,000; Abcam, Cambridge, UK). Signals were visualized by enhanced chemiluminescence (ECL) Western Blotting Analysis System (GE Healthcare Life Sciences, Buckinghamshire, UK) and followed by imaging with light-capture (ATTO, Tokyo, Japan). For quantification of the protein levels, the images were scanned by NIH IMAGE software.

For spectrofluorometric analysis, forebrain S2 fractions of GAD67–GFP knock-in mice, three lines of VGAT–Venus mice and their wild-type littermates were diluted by phosphate-buffered saline (PBS, pH 7.4) to 5 μg/μl of protein concentration. The diluted S2 fractions were excited at 490 or 500 nm (VGAT–Venus mice and their wild-type littermates) or at 473 nm (GAD67–GFP knock-in mice and their wild-type littermates), and the fluorescence emission intensities of Venus and GFP were measured at 528 and 507 nm, respectively on a Hitachi F-4500 Fluorescence Spectrophotometer. Fluorescence intensities of GFP and Venus were determined by subtracting the arbitrary units (AU) of wild-type littermates from those of the transgenic mice.

In situ hybridization analysis

The antisense oligonucleotide probes for VGAT and Venus were 5′-GCTGAAATGCTGGAGACTGATATTCAAGAACGTGAATGTGCTGA-3′ and 5′-CCTCGGGACTGCCTGGGACATGAGATGCCTGGTCTCATG-3′, respectively (Nagai et al., 2002; Sagné et al., 1997). In situ hybridization was performed as described previously (Sakagami et al., 2004). Briefly, consecutive sagittal and coronal sections of brain at a thickness of 25 μm were hybridized with 35S-labeled oligonucleotide probes overnight at 42 °C. After hybridization, they were washed at a high stringent condition at 50 °C for 2 h. For autoradiographic detection, the sections were subsequently exposed to NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA) for 1 month at 4 °C and developed using Kodak D19 (Eastman Kodak).

Immunohistochemistry

Three GAD67–GFP knock-in and three VGAT–Venus line #39 mice at P21–35 were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) under deep anesthesia with Nembutal (50 mg/kg i.p.). After the brain was removed, it was post-fixed in the same fixative for 4 h, immersed in 15% and 30% sucrose in 0.1 M PB overnight for cryoprotection. The brain block including coecleia nucleus (CN) was trimmed and coronal sections were cut at 20–25 μm on a cryostat (CM 1510 S, Leica, Nussloch, Germany). The slices were rinsed in PBS containing 1% BSA for 30 min and incubated in PBS solution containing 5% normal goat serum (NGS), 1% BSA and 0.3% Triton X-100, and rabbit anti–GFP antibody (1:1,000; Novus Biologicals, Littleton, CO, USA) overnight at 4 °C. After rinsing with PBS, the slices were incubated in PBS solution containing 5% NGS, 1% BSA and 0.3% Triton X-100, and Alexa 594-conjugated goat anti-rabbit IgG (1:200; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After rinsing with PBS, they were mounted on saline-coated slides and coversliped with Prolong Gold antifade reagent (Molecular Probes). After observations with fluorescent microscope (BX-8000, Keyence, Osaka, Japan), the slices containing labeled CN cells were photographed.

For double immunostaining, coronal sections of line #39 mouse brains were made on a cryostat at a thickness of 25 μm. The sections were permeabilized in 0.3% Triton X-100 and incubated in 3% H2O2 in PBS for 30 min to inhibit endogenous peroxidase activity. The sections were incubated overnight with polyclonal guinea-pig anti–GFP (1:200, Tamamaki et al., 2000) and either rabbit anti–GABA (1:200,000, Sigma, Saint Louis, MO, USA) or anti–glycine (1:200,000, Sigma) antibodies. After extensive wash with PBS, they were incubated with Alexa Fluor 488-conjugated anti–guinea pig IgG (1:1,000, Molecular Probes) and biotinylated anti–rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) in PBS for 2 h, followed by the incubation with horseradish peroxidase-conjugated streptavidin (1:200, Molecular Probes) for 1 h. The immunofluorescent reaction for GABA and glycine was developed by the 10-min incubation with Alexa Fluor 568-labeled tyramide (1:200) in the amplification buffer containing 0.0015% H2O2 (Tyramide signal amplification kit, Molecular Probes). Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI). Immunoreaction was examined with a confocal laser scanning microscope (LSM710, Zeiss, Germany). Brightness and contrast of the final images were adjusted using the Photoshop CS4 software (Adobe Systems). To quantify the colocalization of the immunoreactive cells for GABA and GABA or glycine, immunofluorescent images taken with a confocal microscope using a ×20 plan-apochromat objective were transferred onto transparent over-head projector sheets and the number of immunolabeled neurons was counted.

RESULTS AND DISCUSSION

Generation of VGAT–Venus transgenic mice

We generated transgenic mice with Venus expression under the control of the mouse VGAT promoter. Venus is a yellow shifted variant of the GFP and brighter than enhanced GFP (Nagai et al., 2002). From 56 offspring, we identified five founder animals by PCR using their tail genomic DNA. Four lines (lines #04, #29, #39, and #49) transmitted the transgene to their progeny and were char-
characterized for transgene expression. To determine the expression pattern of Venus transgene in four lines of VGAT–Venus mice, sagittal section slices of whole brain were examined using in situ hybridization histochemistry (Fig. 1). Both regional expression and expression level of Venus mRNA were distinct among various lines of transgenic mice (Fig. 1C, E, G, I), while no significant hybridization signals were detected in brain of the wild-type, C57BL/6 (Fig. 1A). These results confirmed specificity of the probe. The expression pattern of VGAT transcripts in lines #04, #29, #39 and #49 mouse brains was very similar to that previously described (Fig. 1B, D, F, H, J) (Mcintire et al., 1997). It is notable that in line #39, the expression pattern of Venus is very similar to that of VGAT (Fig. 1G, H).

Although all four lines were generated by injection of the same VGAT–Venus DNA into the fertilized eggs, the spatial distribution of Venus transcripts in the brain was distinct in each line. A possible explanation of the different expression of Venus among the lines may be the integrity of the BAC transgene. The loss of sequence from the 5′-and/or 3′-ends of large transgenes is a common phenomenon (Raguz et al., 1998). To assess the integrity of the transgene into the mouse genome, we measured the presence of the ends of BAC (5′- and 3′-arms) and the VGAT–Venus gene by PCR analysis (Fig. 2A). The 5′- and 3′-arms of BAC DNA can be detected separately by PCR typing. If both arms are positive by PCR typing, it is likely that the entire BAC insert is integrated into the mouse genome without fragmentation (Takahashi et al., 2000). As shown in Fig. 2B, the line #39 mouse contained both 5′- and 3′-arms, while loss of 5′-arm was observed for the other transgenic lines (#04, #29, and #49). These results indicate that the BAC transgene was intact only in line #39, but was eroded at the 5′-end in lines #04, #29, and #49. As shown in Fig. 1, the expression pattern of Venus transcripts in the line #39 mouse was almost the same as that of VGAT transcripts among the four lines of transgenic mice. Taken together, the regulatory DNA elements in the 5′-flanking region of VGAT gene seems essential for the spatial expression of VGAT in the brain. Another possible explanation may be a positional effect (Bessis et al., 1995).

In the case of transgenic mice, a transgene is randomly inserted at non-specific positions of the chromosome. Sometimes, the expression of the transgene is not completely consistent with that of the endogenous gene due to the positional effects. Therefore, different expression patterns of Venus transgene among the four lines of transgenic mice would be due to the positional effects in addition to the degree of transgene integration.

Studies using GAD67 short promoter driving the expression of GFP resulted in the labeling of somatostatin-positive subtype of GABAergic neurons (Oliva et al., 2000). Chattopadhyaya et al. (2004) generated BAC transgenic mice expressing GFP under the control of GAD67 gene promoter, and in a line of the transgenic mice, GFP was selectively expressed in the parvalbumin-positive subtype of GABAergic neurons. Considering these reports, it is possible that VGAT–Venus lines #04, #29 or #49 express Venus in a subpopulation of GABAergic neurons such as parvalbumin- and somatostatin-positive neurons.

**GAD65, GAD67 and VGAT expression in transgenic mice**

In GAD67–GFP knock-in mice, a cDNA encoding EGFP followed by SV40 polyadenylation signal was targeted to GAD67 gene by homologous recombination and GAD67 gene was disrupted (Kaneko et al., 2008; Tamamaki et al., 2003). GABA content in the heterozygous GAD67–GFP knock-in mouse brain was reduced during early development compared to their wild-type mice (Tamamaki et al., 2003). Nakayama et al. (2007) showed that developmental elimination of climbing fiber to Purkinje cell synapses was impaired in heterozygous GAD67–GFP knock-in mice. Conditional knockdown of the GAD67 in basket cells caused deficits in axon branching, perisomatic synapse formation around pyramidal neurons and complexity of the innervations fields in the adolescent mouse visual cortex (Chattopadhyaya et al., 2007). These results suggest that the expression level of GAD67 is important for synapse formation. We investigated the expression level of GAD67 as well as GAD65 and VGAT in the heterozygous GAD67–GFP knock-in mouse and the VGAT–Venus transgenic mice by Western blot analysis. As shown in Fig. 3, the GAD67 protein level in the GAD67–GFP knock-in whole brain was 64% of that in the wild-type whole brain. On the other hand, the GAD67 protein levels in three lines of the VGAT–Venus whole brain were similar to that in the wild-type whole brain. The protein expression levels of GAD65 and VGAT in the GAD67–GFP knock-in and three lines of the VGAT–Venus whole brains were also similar to those in the wild-type whole brain. The β-actin protein expression level as a control was comparable among transgenic and wild-type mice. These results indicate that the expression levels of GABA-synthesizing enzymes in all VGAT–Venus mice were distinct in each line. A possible explanation of the different expression level of GABA-synthesizing enzymes may be a positional effect (Bessis et al., 1995). As shown in Fig. 2B, the line #39 mouse contained both 5′- and 3′-arms, while loss of 5′-arm was observed for the other transgenic lines (#04, #29, and #49). These results indicate that the BAC transgene was intact only in line #39, but was eroded at the 5′-end in lines #04, #29, and #49.

![Fig. 3. Western blot analysis of the whole brain to detect the expression levels of proteins related to GABAergic signaling. The expression levels of GAD67, GAD65 and VGAT in the wild-type (lane 1), GAD67–GFP knock-in (lane 2) and three lines of VGAT–Venus mice (lanes 3–5) were compared by Western blot. The expression level of β-actin was used as a control. Equal amounts of total protein were loaded in each lane.](image-url)
transgenic lines examined were almost the same as those in wild-type mice. We have previously generated the VGAT–Venus transgenic rat using the same DNA construct, which was used for the generation of the current VGAT–Venus mice. The GABA content in the VGAT–Venus transgenic rat brain was similar to that in the wild-type rat brain (Uematsu et al., 2008). Taken together, the VGAT–Venus transgenic mice will be a valuable tool for characterizing the functions and morphology of GABAergic neurons during early development.

Fluorescent protein expression

The transgenic mouse expressing fluorescent proteins will enable us to identify fluorescent protein-positive neurons easily and to use them for their morphological and electrophysiological studies. The GAD67–GFP knock-in mouse has been used for various studies on the GABAergic neurons (Tamamaki et al., 2003; Marowsky et al., 2005; Tanaka et al., 2006). The brighter the fluorescent intensity is, the easier the identification of fluorescent cells is. For instance, the bright cells with abundant fluorescent proteins are useful for their long-term imaging with two-photon fluorescence excitation microscopy. Because the high photon flux is required in two-photon fluorescence excitation microscopy, and it leads to irreversible photobleaching of GFP in live cells, which results in disturbance of long-term imaging of the cells (Chen et al., 2002).

We compared the relative amounts of the fluorescent protein in the whole brain from three transgenic lines (#04, #39, and #49) and the GAD67–GFP knock-in mouse by Western blots using anti-GFP antibody (Fig. 4). Anti-GFP antibody recognizes Venus as well as GFP (Uematsu et al., 2008). Western blot detected much more GFP immunoreactivity in the line #39 than in the lines #04 and #49 and GAD67–GFP knock-in mice (Fig. 4A). To determine the relative amounts of the fluorescent protein in the line #39 and GAD67–GFP knock-in mice more accurately, we performed 1.5-fold successive dilutions starting from 7 µg protein of whole brain S1 fraction of the line #39. We loaded various amounts of protein of the line #39 whole brain S1 fraction and 7 µg protein of the GAD67–GFP knock-in mouse whole brain S1 fraction on the SDS-PAGE. The GFP immunoreactivity of 7 µg protein of S1 fraction from the GAD67–GFP knock-in mouse whole brain...
was lower than that of 0.92 μg protein and higher than 0.61 μg protein from the line #39 whole brain (Fig. 4B, C). These data show fluorescent protein level of line #39 was 8–11 times higher than that of the GAD67–GFP knock-in mouse whole brain. The distributions of GABAergic and glycinergic neurons are different in the CNS. GABAergic neurons are widespread in the mammalian CNS, whereas glycinergic neurons are largely restricted to the brainstem and spinal cord (Esclapez et al., 1994; Legendre, 2001). Most inhibitory neurons in the forebrain are GABAergic, but not glycinergic. Thus, in order to compare expression levels of the fluorescent proteins in GABAergic neurons among the GAD67–GFP knock-in and three lines of the VGAT–Venus mice, we measured GFP-immunoreactivities in the forebrain by Western blots. Protein level of the line #39 forebrain was much higher than that of lines #04 and #49 (Fig. 4D). To determine the relative amounts of the fluorescent protein in the line #39 and GAD67–GFP knock-in mouse forebrain, we performed 1.5-fold successive dilutions starting from 8 μg protein of forebrain S1 fraction of the line #39. We loaded various amounts of protein of the line #39 forebrain S1 fraction and 8 μg protein of the GAD67–GFP knock-in mouse forebrain S1 fraction on the SDS–PAGE. The GFP-immunoreactivity of 8 μg protein of S1 fraction from the GAD67–GFP knock-in mouse forebrain was lower than that of 1.58 μg protein and higher than 1.05 μg protein from the line #39 forebrain (Fig. 4E, F). These data show that the fluorescent protein level of the line #39 was 5–7 times higher than that of GAD67–GFP knock-in mouse forebrain.

The fluorescence intensities of the VGAT–Venus transgenic mouse lines (#04, #39, and #49) and GAD67–GFP knock-in mouse were also measured by fluorescence spectroscopy (Fig. 5). First, we compared fluorescence intensities of the S2 fraction prepared from forebrain among three lines of VGAT–Venus mice. The fluorescence intensity of the line #39 at 528 nm was 8.4 and 6.7 times higher than that of the lines #04 and #49, respectively (Fig. 5A, B). These data showed that the line #39 forebrain displayed stronger fluorescent signal than the lines #04 and #49 forebrains. These results confirmed the expression protein level of Venus in each line by Western blot (Fig. 4). Next, we compared Venus fluorescence intensity of the S2 fraction prepared from the VGAT–Venus line #39 forebrain and GFP fluorescence intensity from the GAD67–GFP knock-in mouse forebrain. Fig. 5C shows the emission spectra of S2 fraction from the GAD67–GFP knock-in and the line #39 mouse brain. Excitation of S2 fraction from the GAD67–GFP knock-in and the line #39 mouse forebrain at 473 and 500 nm, respectively resulted in substantial emission, compared to their wild-type littermates. Fig. 5C, D showed that the line #39 forebrain displayed stronger fluorescent signal than the GAD67–GFP knock-in mouse forebrain. These results indicate that the line #39 mouse is preferable to the GAD67–GFP knock-in mouse, when experiments require brighter fluorescent signals.

Distribution of Venus-expressing cells in line #39 mice

Based on the results that the line #39 showed stronger GFP immunoreactivity and brighter fluorescent signal than other transgenic lines and GAD67–GFP knock-in mouse, we focused on the line #39 mouse and analyzed the distribution of Venus in the line #39 mouse brain. First, we compared the detailed expression of Venus mRNA to that of VGAT by in situ hybridization with the alternate consecutive coronal brain sections. As shown in Fig. 6, the regional distribution and intensity of Venus mRNA paralleled very well with that of VGAT in most brain regions (McIntire et al., 1997; Sagné et al., 1997). Venus mRNAs were expressed at a high level in the olfactory neuronal layers, striatum, thalamic reticular nucleus, zona incerta and cerebellar cortex as well as scattered neurons in the cerebral cortex, hippocampus and various brain stem nuclei.

In the hippocampus, however, there was a discrepancy of the expression between Venus and VGAT. In addition to scattered interneurons, Venus mRNA was expressed intensely to moderately in the CA1 pyramidal cells and dentate granule cells that did not express VGAT mRNA. Aberrant Venus mRNA expression in the CA1 pyramidal cells and dentate granule cells may result from a positional effect, in which transcriptional regulation of the transgene is influenced by the chromosomal sequences flanking the point of transgene integration (Bessis et al., 1995).

On the other hand, transgenic Venus mRNA was moderately to weakly expressed in cerebellar Purkinje cells, although endogenous VGAT mRNA was strongly expressed (Fig. 6). In addition, Venus mRNA was weakly expressed in the caudal brainstem, whereas VGAT was more strongly expressed (Fig. 6). These discrepancies could be derived from either its reduced transcription due to a positional effect of the transgene or possible instability of Venus mRNA.

To examine whether Venus transcripts are appropriately translated in inhibitory GABAergic and glycinergic neurons in the VGAT–Venus line #39 mouse, we performed double-immunostaining analysis with antibodies against GFP and GABA or glycine.

In the hippocampal formation, a number of Venus-immunoreactive cells were scattered throughout all layers with cell bodies and proximal dendrites being intensely labeled (Fig. 7A, B, E). In the CA1 region and dentate gyrus, 97.3% (n=75) and 75.0% (n=36) of Venus-positive cells expressed GABA, respectively, while 92.4% (n=79) and 79.4% (n=34) of GABA-immunoreactive cells expressed Venus, respectively (Fig. 7B–G, Table 1). To be noted, although the excitatory neurons such as hippocampal pyramidal and dentate granule cells expressed Venus mRNA at a substantial level (Fig. 6), Venus-immunoreactivity was not observed in their cell bodies and mossy fiber terminals (Fig. 7B–G). We observed a discrepancy between Venus expression levels of mRNA and protein in hippocampal CA1 pyramidal cells and dentate granule cells (Figs. 6, 7), suggesting that its expression is regulated at a posttranscriptional level. The discrepancy could be derived from cell-specific translation inefficiency with
Venus mRNA and/or instability of Venus protein. Such a discrepancy has been reported in the case for acid β-glucosidase (Xu and Grabowski, 1998). When the cDNA for acid β-glucosidase was overexpressed in mammalian cells, there was large discrepancy between acid β-glucosidase expression level of mRNA and protein. Translational inefficiency of acid β-glucosidase mRNA has been suggested in transgenic mammalian cells possibly due to a heat labile cytoplasmic protein. It is noteworthy that Venus protein is specifically present in GABAergic neurons, but neither in CA1 pyramidal cells nor dentate granule cells in VGAT–Venus line #39 mouse (Fig. 7B–G, Table 1). These results indicate that the line #39 mice are also useful for hippocampal studies.

In the cerebral cortex, Venus-immunoreactive cells were scattered throughout the layers I–VI with their cell bodies and neuropils being intensely labeled. Comparison of the Venus immunostaining (Fig. 7H), GABA immunostaining (Fig. 7I) and their superimposition (Fig. 7J) shows that the majority of Venus-immunoreactive cells (95.9%, n=844) expressed GABA, while 93.1% of GABA-immunoreactive cells were positive for Venus (n=870) (Table 1). These results demonstrate the prominent expression of Venus in cerebral GABAergic cells.

The cerebellar cortex contains three types of inhibitory interneurons (basket and stellate cells in the molecular layer and Golgi cells in the granular layer) and one type of inhibitory projection neuron (Purkinje cell). Among these, the cell bodies of the former three inhibitory interneurons were intensely immunoreactive for Venus as revealed by double-immunostaining with anti-GFP and anti-GABA antibodies (Fig. 7K–M, Table 1). In contrast, the Purkinje cells somata were only faintly immunolabeled (Fig. 7K), consistent with weak mRNA expression level in in situ hybridiza-
Fig. 6. Comparison of the expression patterns of Venus and VGAT in the brain from the transgenic line #39 mouse by in situ hybridization analysis. Consecutive coronal sections were hybridized with radioactive probes for either Venus or VGAT. The expression of Venus and VGAT is shown by dark-field photographs and consecutive sections stained with toluidine blue are also shown to identify neuronal nuclei. Note similar spatial expression patterns of Venus and VGAT mRNAs in the brains of the transgenic line #39 mouse. 3, oculomotor nucleus; 7, facial nucleus; 12, hypoglossal nucleus; ac, anterior commissure; Acb, accumbens nucleus; Am, amygdaloid nuclei; Arc, arcuate hypothalamic nucleus; CA1–3, CA1–3 fields of Ammon’s horn; cc, central canal; CG, central gray; CPU, caudate putamen; DC, dorsal cochlear nucleus; DG, dentate gyrus; DH, dorsal horn; Gi, gigantocellular reticular nucleus; Gl, glomerular layer; GrA, granule cell layer of accessory olfactory bulb; IC, inferior colliculus; IGL, internal granular layer; Int, interposed cerebellar nucleus; IO, inferior olive; IP, interpeduncular nucleus; Lat, lateral cerebellar nucleus; LG, lateral geniculate nucleus; LRI, lateral reticular nucleus; LS, lateral septal nuclei; LSO, lateral superior olive; Me5, mesencephalic trigeminal nucleus; MG, medial geniculate nucleus; MH, medial habenular nucleus; Mi, mitral cell layer; Mo5, motor trigeminal nucleus; MV, medial vestibular nucleus; PI, piniform cortex; Ph, pontine nuclei; PrNa, pontine reticular nucleus; R, red nucleus; PrS, principal sensory trigeminal nucleus; PrH, prepositus hypoglossal nucleus; Rt, reticular thalamic nucleus; SC, superior colliculus; SHi, septohippocampal nucleus; SN, substantia nigra; Sol, nucleus of the solitary tract; Sp5, spinal trigeminal nucleus; Tg, posterodorsal tegmental nucleus; Tu, olfactory tubercle; Tz, trapezoid body; VH, ventral horn; VMH, ventromedial hypothalamic nucleus; VN, vomeronasal nerve layer; ZI, zona incerta.
In addition, anti-GFP antibody labeled the cerebellar glomeruli and pinceau, an axon terminal of the basket cell onto the axon hillock of Purkinje cell (Fig. 7K). Numerous immunoreactive neurites were also distributed throughout the molecular layer.

While most of GABAergic neurons are specifically labeled in the GAD67–GFP knock-in mouse (Tamamaki et al., 2003), in VGAT–Venus transgenic mice not only GABAergic neurons but also glycinergic neurons should be labeled, because VGAT is specifically expressed in both GABAergic neurons and glycinergic neurons (Chaudhry et al., 1998; Dumoulin et al., 1999), and Venus transgene was expressed under the control of the VGAT promoter. To evaluate whether Venus is expressed in both GABAergic and glycinergic neurons, we selected the dorsal cochlear nucleus (DCN), which is known to contain both GABAergic and glycinergic neurons (Mugnaini, 1985; Aoki et al., 1988; Kolston et al., 1992; Moore et al., 1996). In the DCN, Venus-immunoreactive cells were distributed in both the superficial and deep layers. Double-immunostaining analysis revealed that a large population of Venus-immunoreactive cells (85.2%, n=535) expressed glycine, while a small population of Venus-immunoreactive cells (9.2%, n=521) expressed GABA. Conversely, almost all glycine-

**Table 1. Colocalization of Venus and GABA or glycine in VGAT–Venus line #39 mouse**

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>% Colocalization</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GABA</td>
<td>Glycine</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>95.9 (n=844)</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA1</td>
<td>97.3 (n=75)</td>
<td></td>
</tr>
<tr>
<td>DG</td>
<td>75.0 (n=36)</td>
<td></td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stellate/basket cells</td>
<td>99.3 (n=304)</td>
<td></td>
</tr>
<tr>
<td>Golgi cells</td>
<td>100 (n=152)</td>
<td></td>
</tr>
<tr>
<td>Dorsal cochlear nucleus</td>
<td>9.2 (n=521)</td>
<td>85.2 (n=535)</td>
</tr>
</tbody>
</table>

Coronal brain sections were double-immunostained with antibodies against GFP and GABA or glycine.

Data are shown as % of Venus-positive neurons that express GABA or glycine.
and GABA-immunoreactive cells expressed Venus (100%, $n=456$ and 98.0%, $n=49$, respectively) (Fig. 8, Table 1). All these findings strongly suggest that Venus is expressed in both GABAergic and glycinergic neurons in the VGAT–Venus line #39 mouse brain.

To compare the fluorescence intensity between GFP-positive cells in the GAD67–GFP knock-in mice and Venus-positive cells in the line #39 mice, the sections including the DCN were obtained by the same fixation procedure and their digital images were printed without any change in exposure time (Fig. 9A, B). Comparison of these images revealed that Venus-positive cells in the line #39 mice were much brighter than GFP-positive cells in the GAD67–GFP knock-in mice (Fig. 9A, B). This raises the possibility that some inhibitory neurons weakly expressing fluorescent proteins may not be detected by fluorescent microscopy. In such a case, immunostaining of GFP-positive neurons with anti-GFP antibody can be helpful to increase the signal-to-noise ratio of GFP (Zeilhofer et al., 2005) and improve detection of the GFP-positive neurons. Therefore, we performed immunostaining of the DCN of both the GAD67–GFP knock-in and line #39 mice with anti-GFP antibody, and compared the distribution of GFP-immunoreactive cells between the GAD67–GFP knock-in and line #39 mice. Even when immunostaining was performed, the different distributions of GFP-immunoreactive cells between the GAD67–GFP knock-in and line #39 mice were observed (Fig. 9C, D). When we compared the distribution of fluorescent and GFP-immunoreactive cells in GAD67–GFP knock-in sections, some weakly GFP-fluorescent cells in the DCN were clearly detected by immunostaining (Fig. 9A, C). In the line #39 mouse, on the other hand, the distribution pattern of Venus-fluorescent cells was very similar to that of GFP-immunostained cells in the DCN (Fig. 9B, D). These results indicate that both GABAergic and glycinergic cells expressing Venus in the line #39

Fig. 8. Comparison of the localization of Venus and GABA or glycine in the cochlear nuclei of the VGAT–Venus line #39 mice. Coronal sections of cochlear nuclei of the VGAT–Venus line #39 mice at P4W were double-immunostained with anti-GFP and anti-GABA (A–F) or anti-glycine (G–L) antibodies. Fluorescent photomicrographs of the dorsal cochlear nucleus (DCN) at a high magnification (D–F, J–L) show the colocalization of Venus with GABA or glycine. VCN, ventral cochlear nucleus. Scale bars=100 μm in A and G; 50 μm in D and J.
mouse can be easily detected by fluorescent microscopy without immunostaining. The advantage that most inhibitory cells show brighter fluorescence in the line #39 mouse is very useful for identification of inhibitory neurons in the CNS, in which inhibitory neurons with heterogeneous morphological and electrophysiological properties are scattered.

CONCLUSION

In this study, we generated a novel transgenic mouse line expressing Venus under the control of VGAT promoter to directly visualize inhibitory neurons. We found a transgenic mouse line that showed bright Venus fluorescence, mimicking endogenous pattern of VGAT expression. By using this transgenic line, we could visualize inhibitory cells with Venus without immunostaining. Venus was present not only in somata but also in neurites. Because it is very difficult to identify inhibitory neurons in living brain preparations, this transgenic line should be useful for electrophysiological recordings of inhibitory cells and visualization of their morphology.

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Fig. 9. The expression of fluorescent proteins in the cochlear nucleus of the GAD67–GFP knock-in and VGAT–Venus line #39 mice. Fluorescent photomicrographs of the cochlear nucleus in the GAD67–GFP knock-in (A, C) and VGAT–Venus line #39 mice (B, D). Sections shown in C and D are identical to those shown in A and B, respectively. A and B show fluorescent views of GFP and Venus, respectively. C and D show Alexa 594 views of immunostaining with anti-GFP antibody. The exposure time to take the photographs was the same in A and B, while in C, the photograph was taken at the double exposure time of that in D. DCN, dorsal cochlear nucleus; VCN, ventral cochlear nucleus. Scale bar = 200 μm.

REFERENCES


Young A, Sun QQ (in press) GABAergic inhibitory interneurons in the posterior piriform cortex of the GAD67-GFP mouse. Cereb Cortex, in press.


