Quantitative Chemical Composition of Cortical GABAergic Neurons Revealed in Transgenic Venus-Expressing Rats

Although neocortical GABAergic (γ-aminobutyric acidergic) interneurons have been the focus of intense study, especially in the rat, a consensus view of the functional diversity and organization of inhibitory cortical neurons has not yet been achieved. To better analyze GABAergic neurons in the rat, we used a bacterial artificial chromosome (BAC) construct and established 2 lines of transgenic rats that coexpress Venus, a yellow fluorescent protein, with the vesicular GABA transporter. The brain GABA content from both transgenic lines was similar to the level found in wild-type rats. In the frontal cortex, Venus was expressed in >95% of GABAergic neurons, most of which also expressed at least one of 6 biochemical markers, including α-actinin-2, which preferentially labeled late-spiking neurogliaform cells. Taking advantage of the fact that Venus expression allows for targeted recording from all classes of nonpyramidal cells, irrespective of their somatic morphologies, we demonstrated that fast-spiking neurons, which were heterogeneous in somatic size as well as vertical dendritic projection, had relatively uniform horizontal dimensions, suggesting a cell type-specific columnar input territory. Our data demonstrate the benefits of VGAT-Venus rats for investigating GABAergic circuits, as well as the feasibility of using BAC technology in rats to label subsets of specific, genetically defined neurons.

Keywords: bacterial artificial chromosome, cortex, interneuron, transgenic rat, Venus, vesicular GABA transporter

Introduction

Inhibitory neurons in the central nervous system (CNS) are diverse in their morphological and physiological characteristics (Somogyi et al. 1998). To explore the functional diversity of cortical interneurons in the rat neocortex, numerous studies have investigated various aspects of these neurons, such as their axonal and dendritic morphologies, chemical expression patterns, firing patterns, and synaptic connectivity (Kawaguchi and Kondo 1997; Gupta et al. 2000; Kawaguchi and Kondo 2002; Markram et al. 2004; Somogyi and Klausberger 2005; Kubota et al. 2007). However, the implications of the data have been hotly debated, and a consensus view on how these neurons can best be functionally classified has not yet been achieved (Nelson 2002; Yuste 2005). Because the expression of several peptides and calcium-binding proteins is correlated with their physiological and morphological characteristics (Kawaguchi and Kubota 1993; Cauli et al. 2000; Wang et al. 2002; Toledo-Rodriguez et al. 2004; Sugino et al. 2006), as well as their differential response to neuromodulators such as acetylcholine and serotonin (Kawaguchi 1997; Porter et al. 1999; Ferezou et al. 2002; Guldledge et al. 2007), their expression can be used to identify functional subclasses of cortical interneurons. However, it has not yet been determined if these chemical markers identify the entire GABAergic cell population in the neocortex. On the other hand, traditional methods of examining nonpyramidal neurons via intracellular recording may sometimes fail to identify GABAergic (γ-aminobutyric acidergic) cell subtypes that are morphologically and/or physiologically similar to pyramidal neurons. Therefore, selective fluorescent labeling of the entire GABAergic cell population in the neocortex would greatly advance the analysis of their cellular and circuit organizations.

To date, transgenic labeling of inhibitory cells in mice has utilized green fluorescent protein (GFP) controlled by the promoter of the biosynthetic enzyme for GABA, glutamate decarboxylase (GAD). In these mice, however, GFP expression was restricted to subsets of GABAergic neurons (Oliva et al. 2000; Chattopadhyaya et al. 2004; Lopez-Bendito et al. 2004; Ma et al. 2006). In GAD67-GFP knock-in mice, the overall GABA content was shown to be reduced following destruction of the endogenous GAD67 gene (Tamamaki et al. 2003). Bacterial artificial chromosome (BAC) technology has gradually come to be regarded as a potent method to identify and manipulate specific neuron types in mice (Heintz 2001; Meyer et al. 2002), but its application in rats has not previously been reported. In this study we have, for the first time, produced transgenic rats that have both fluorescent labeling of nearly all cortical GABAergic cells and normal levels of GABA synthesis, thus facilitating the study of GABAergic neurons in tissue that is otherwise physiologically normal. To do this we used a BAC containing 2 genes: one for VGAT, which is responsible for transporting GABA into synaptic vesicles (Ebihara et al. 2003), and another for Venus, a fluorescent marker much brighter than...
eGFP (Nagai et al. 2002). This manipulation generated 2 lines of VGAT-Venus transgenic rats from the same BAC construct. We show that in at least one of the 2 lines, GABAergic cells in individual areas in the forebrain are selectively labeled with Venus. In the cerebral cortex almost all Venus-expressing cells in both lines were positive for GABA, and the vast majority expressed at least one of 6 chemical markers used to classify cortical interneurons. Venus labeling facilitated the identification of GABAergic cells and made possible targeted recording of the interneurons with morphological features typical of pyramidal cells.

**Materials and Methods**

**Identification of a Suitable BAC Clone**

A BAC clone (#160L22) from a 129SV mouse genomic BAC library (Genome System, St Louis, MO), containing the VGAT gene was identified by PCR as previously described (Ebihara et al. 2003). We determined the nucleotide sequences of the 5' and 3' termini of the DNA inserted into the BAC vector and compared their sequences with the EMBL database (accession number AL663901). The comparison revealed that BAC clone #160L22 contains the entire VGAT gene, with an additional 102 and 25 kb of DNA flanking the 5' end of exon 1 and the 3' end of exon 3, respectively.

**Modification of the BAC Clone**

pCS2-Venus, which contains Venus complementary DNA (cDNA) followed by the SV40 polyadenylation signals, was generously provided by Dr A. Miyawaki (RIKEN, Wako, Japan) (Nagai et al. 2002). pKOV-Kan and pDF25s plasmids were a generous gift from Dr M. D. Lalio (University of Birmingham, Birmingham, UK).

The multiple cloning site following the SV40 polyadenylation signals of pCS2-Venus was changed to a NotI–NcoI–ApaI site by ligation of a synthetic linker to pCS2-Venus digested with NotI and ApaI, and the resultant plasmid was named pCS2-Venus-N. A 3.6-kb EcoRI fragment containing the 5' flanking region, exon 1 and part of intron 1 of the mouse VGAT (mVGAT) gene was subcloned into pBluescript II (Stratagene, LaJolla, CA) from a BAC clone encoding the mVGAT gene (Ebihara et al. 2003), and the resultant plasmid was named pBS-VGAT-E1. A 1.0-kb NcoI fragment containing the Venus cDNA followed by the SV40 polyadenylation signals (poly-A) was excised from pCS2-Venus-N and inserted into pBS-VGAT-E1 digested with NcoI in order to place the Venus cDNA in frame into the ATG translational initiation codon of exon 1 of the VGAT gene. This plasmid was named pBS-VGAT-E1-Venus. The multiple cloning site of pBluescript II was changed to a SaI–EcoRI–Nhel–BanHI site by ligation of a synthetic linker to pBluescriptII digested with SaI and BanHI and the resultant plasmid was named pBS-SENBa. A 3.3-kb EcoRI–Nhel fragment was excised from pBS-VGAT-E1-Venus and inserted into pBS-SENBa, and the resultant plasmid was named pBS-VE-NEN-Venus. The cloning site of pKOV-Kan was digested to pKOV-Kan digested with SaI and BamHI, and the resultant plasmid was named pKOV-SENBa. A 3.3-kb EcoRI–Nhel fragment was excised from pBS-VGAT-EN-Venus and inserted into pKOV-SENBa, and the resultant clone was named pKOV-VGAT-Venus. The shuttle vector pKOV-VGAT-Venus contains the Venus cDNA and SV40 poly-A flanked by 2 homologous regions: one is genomic DNA homologous to the 5' end of exon 1 of the VGAT gene and the other is genomic DNA homologous to 914 bp (NheI–NcoI) upstream of the translation initiation site of exon 1 of the VGAT gene.

BAC recombinations were performed according to the methods described by Lalio et al. (2001). Venus-modified BAC clones were confirmed by PCR, pulse field gel electrophoresis, and Southern blotting.

**Generation of BAC Transgenic Rats**

For pronuclear injections, BAC DNA was purified with a NucleoBond BAC100 kit ( Macherey-Nagel, Düren, Germany) and digested with NotI to release the VGAT gene, which consisted of the entire insert of genomic DNA and part of the vector DNA from the BAC backbone. The 133-kb linearized BAC DNA fragment was purified using pulse field gel electrophoresis, dissolved at a concentration of 3 ng/μL in 10 mM TrisCl and 0.1 mM ethylenediaminetetraacetic acid, and then stored at 4 °C. The purified and stored BAC DNA was injected into fertilized eggs of Crlj:Wistar rats (Charles River Japan, Inc., Kanagawa, Japan) according to the procedure described by Takahashi et al. (1999).

Among 318 oocytes injected, 182 could be transferred into pseudo-pregnant female rats. From them, 25 rats were born. Two transgenic founders were identified from 21 pups by PCR for the presence of Venus. Primers used for the PCR were Venus-F: 5’-ATGTGAGG-AAGGCCGAGGACGCTGT-3’ and Venus-R: 5’-TTACTTGTAAGCTGTCATGCCCAG-3’. The lines derived from these 2 founders were expanded for further analysis. Venus expression in both lines of newborn rat brains was detected by fluorescence microscopy.

**Measurement of GABA Content**

Samples from rat brains were homogenized with 0.1 M perchloric acid to extract amino acids and precipitate proteins. The homogenates were centrifuged at 6000 × g for 15 min at 4 °C and then the supernatant was neutralized with 0.1 M sodium carbonate. After addition of sodium carbonate, the samples were filtered by using Ultrafree-MC (Millipore Corp., Bedford, MA). Amino acids in the samples were derivatized with o-phthalaldehyde/2-mercaptoethanol reagent, and GABA concentrations were measured by using high performance liquid chromatography (BAS, Tokyo, Japan) and fluorescence detection. Protein concentrations were determined using bichinchoninic acid protein assay reagent (Pierce, Rockford, IL), with bovine serum albumin (BSA) as a standard.

**Antibodies**

Venus was immunohistochemically detected using a rabbit anti-Venus antibody against eGFP (1: 2000; kind gift from Dr Nobuaki Tamamaki, Kumamoto University). In the cortex of transgenic rats, Venus fluorescence almost always colocalized with the immunofluorescence by its antibody. For GABA immunohistochemistry, a mouse monoclonal antibody was used (1:500; Chemicon [Temecula, CA] MAB316).

Parvalbumin was visualized by a mouse monoclonal antibody (Sigma [Saint Louis, MO] P-3171; 1:2000) and a rabbit antisera (Swant, Bellinzona, Switzerland PV-28; 1:4000). Somatostatin was immunohistochecmically detected by a rabbit antisera (antisomatostatin 28; Dr Robert Benoit, S 309; 1:4000) or a rat monoclonal antibody against somatostatin (Chemicon, MAB354; 1:250). Calretinin was detected by a mouse monoclonal antibody (Chemicon, MAB1568; 1:4000) or a rabbit antisera (Swant, 7699/4; 1:2000, Diasorin, Stillwater, MN). Cholecystokinin (CCK) immunoreactivity was detected by a monoclonal antibody raised against CCK/Gastrin (#28.2.2 MoAb, CURE/UCLA/DCC Antibody/RIA Core; 1:4000) or a rabbit antisera against CCK-8 (Sigma, C-2581; 1:1000). These 2 antibodies against the same antigens stained the same populations of cells in the rat frontal cortex. Vasoactive intestinal polypeptide (VIP) was immunohistochemically found by a rabbit antisera (Diasorin, 2077; 1:2000). Calbindin immunoreaction was done by a rabbit antisera (anticalbindin D; Dr Piers C. Emson; 1:2000).

The expression pattern of α2-antin-2 was investigated using a rabbit specific antisera against α2-antin-2 (1:40; kind gift from Dr Alan H. Beggs, Children’s Hospital Boston; 1:2000) (Wyszyński et al. 1998) and a mouse monoclonal antibody against α2-antin-2 and -3 (EA53; Sigma, A7811; 1:2000). Because α2-antin-3 is not expressed in the brain, the EA53 antibody should be specific for α2-antin-2 in rat brain (Wyszyński et al. 1998). This was confirmed using double immunofluorescence showing the rabbit antisera and the monoclonal antibody against α2-antin-2 stain the same population of cells in the rat cortex.

**Immunohistochemistry in Perfusion-Fixed Brains**

After anesthesia with an overdose of Nembutal, transgenic and wild-type Wistar rats (28–32 days postnatal) were perfused through the heart with 10 mL of a solution of 250 mM sucrose, 5 mM MgCl2 in 0.02 M phosphate buffer (pH 7.4) (PB), followed by 200 mL of fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M PB. For GABA immunohistochemistry, the glutaraldehyde concentration was increased to 0.5%. The brains were then removed and postfixed in the same fixative for 2 h.

For the immunoperoxidase reaction, the brains were cut at thickness of 50 μm by a vibrating slicer, to the frontal, parasagittal, or oblique horizontal (parallel to the rhinal fissure) sections. Sections were incubated with 1% H2O2 in PB to suppress intrinsic peroxidase activity. Sections were
incubated overnight in the primary antisera in 0.5 M Tris-HCl buffered saline (pH 7.6) (TBS) containing 10% normal goat serum (NGS). 2% BSA, and 0.5% TritonX-100 (TX). After washes with TBS, the sections were incubated with biotinylated secondary antibody (Vector Laboratories [Burlingame, CA] 1: 100) in TBS containing 10% NGS, 2% BSA and 0.5% TX. After additional washes, they were incubated in ABC complex in TBS (1: 100; Vector). After incubation with 0.5 M Tris-HCl buffer (TB), the sections were reacted with 3.3-diaminobenzidine tetrahydrochloride (DAB) and H2O2. After washing in PB, the sections were put on gelatin-coated slides and dried. After further treatment with 0.1% OsO4 in PB, they were dehydrated and covedipped with Entellan.

Colocalization between Venus and a substance or pairs of substances was investigated using immunofluorescence with cryostat sections. Fixed brains were put in 15% sucrose in PB overnight followed by 30% sucrose in PB overnight before sectioning on a cryostat. The sections were put on a cryostat and sectioned at thickness of 2 μm for GABA immunohistochemistry and 4 μm for other chemical markers.

For combined single immunofluorescence and Venus observation, sections were washed in TBS and then incubated with a primary antibody in TBS containing 10% NGS, 2% BSA, and 0.5% TX overnight. After washes with TBS, the sections were incubated with an Alexa 594-conjugated secondary antibody (1:200; Molecular Probes, Eugene, OR). For experiments using double immunofluorescence for α-actinin-2 and other markers in wild-type rats, sections were incubated with mouse and rabbit primary antibodies, followed by a mixture of Alexa 488- and Alexa 594-conjugated secondary antibodies. After washing in TBS the sections were mounted on gelatin-coated glass slides and dried. The sections were coveredipped with 50% glycérin in TBS and observed by epifluorescence. Cross-reactivity of the secondary antiserum used for dual immunofluorescence was tested by incubating control sections first with a single primary antisera or monocolonal antibody and then with a secondary antibody appropriate for the different primary applied for double staining.

The following enzims were used for fluorescence observation: excitation, 485–515 nm; emission, 525–555 nm for Venus; excitation, 454–580 nm; emission, 610 nm for Alexa 594; excitation, 360–370 nm; emission, 420–460 nm for Alexa 430. Peak absorption and emission of Venus are 515 and 528 nm, respectively (Nagai et al. 2002).

Single- and dual-labeled fluorescent cells were counted directly under a fluorescent microscope or from photomicrographs of sections containing the medial agranular and anterior cingulate cortex. Percentages were calculated from the number of dual-labeled cells (Venus plus GABA) divided by the total population of single-labeled cells (Venus or GABA).

**Slice Preparation and Whole-Cell Recording**

Oblique horizontal sections with 300 μm thickness were cut along the line of rhinal fissure from rat frontal cortex (19–23 days postnatal), immersed in a buffered solution (NaCl, 124.0; KCl, 3.0; CaCl2, 2.4; MgCl2, 1.2, NaHCO3, 26.0; NaH2PO4, 1.0; glucose, 10.0; in mM) aerated with a mixture of 95% O2 and 5% CO2. Cells in the frontal cortex (medial agranular and anterior cingulate cortex) were visualized using a 40× water immersion objective, and recorded in whole-cell current-clamp mode at 32 °C. The electrode solution for the current-clamp recording consisted of potassium gluconate 130, NaCl 1.8 MgCl2 1.8, adenosine triphosphate 2.8, guanosine triphosphate 0.3, and (2-hydroxyethyl)-1-piperazineethanesulfonic acid 10, and bicarbonate 20. The pH of the solution was adjusted to 7.3 with KOH. The osmolarity was adjusted to 290 mOsm. The recorded Venus-expressing cells were observed by direct visual inspection, combined with Nomarski optics, in fluorescence microscope (BX50WI, Olympus, Tokyo, Japan). Current-clamp recordings were made in a fast current-clamp mode of EPC9/dual (HEKA, Lambrecht/Pfalz, Germany; WaveMetrics, Lake Oswego, OR). Electrophysiological data were analyzed by IGOR Pro (WaveMetrics). Cellular input resistance was determined from the membrane response to small hyperpolarizing current injections (≤100 pA, 0.5 s duration). Spike widths at half amplitude were measured from spikes elicited by brief (10 ms) depolarizing current injections, whereas the presence of low-threshold (LTS) or burst-spiking behavior was assessed using just-threshold current injection from a starting potential of −75 to −85 mV.

**Physiological Identification of Nontypical Cells**

Cortical nontypical cells are physiologically heterogeneous and have been classified according to their patterns of action potential generation in response to depolarizing current pulses, and have been divided into 3 broad subclasses: fast-spiking (FS) cells, late-spiking (LS) cells, and non-FS neurons (Kawaguchi and Kubota 1997; Kawaguchi and Kondo 2002; Karube et al. 2004). FS cells are characterized by nonadapting, repetitive spike discharges at a threshold frequency of 40–150 Hz. At just-threshold current levels, FS cell discharges consist of either single spikes at the beginning of the current pulse or irregular bursts of high frequency spike generation with constant spike intervals interspersed. LS cells are characterized by a slow ramp depolarization and delayed action potential firing following just-threshold current injections. Non-FS cells are more heterogeneous in their electrophysiological characteristics than are FS and LS neurons. Some common non-FS cells are LTS neurons (also known as burst-spiking nonpyramidal [BNSP] neurons), regular-spiking nonpyramidal (RSNP) cells, and irregularly spiking neurons, which we have classified here as either BNSP or RSNP cells according to the presence of an initial LTS-like hump (Kawaguchi and Kubota 1996).

**Immunohistochemical and Morphological Analysis of Recorded Cells**

Tissue slices containing biocytin-loaded cells were fixed by immersion in 4% paraformaldehyde and 0.2% picric acid overnight, followed by a freeze-thawing procedure in sucrose-containing PB using liquid nitrogen twice. Slices were resectioned to a thickness of 50 μm, and were processed for fluorescence immunohistochemistry. Slices were incubated with a primary antibody in TBS containing 2% BSA, 10% NGS, and 0.1% TX. The primary antibody was antiparvalbumin (mouse; 1:500), antissomatostatin (rat; 1:250), or anti-α-actinin-2 (mouse monoclonal; 1:100,000). After washing in TBS, they were incubated in a Alexa 594-conjugated secondary antibody in TBS containing BSA, NGS, and 0.1% TX for 2 h, followed by incubation with Alexa 350 streptavidin (1:4000, S-11249, Molecular Probes) in TBS for 40 min. After examination for fluorescence, the slices were incubated with ABC complex in TBS containing 0.04% TX, and reacted with DAB and H2O2 in TB. They were then postfixed in 1% OsO4 in PB for 20 min, dehydrated, and flat-embedded on glass slides in Epon. Somata, axons, and dendrites were reconstructed 3-dimensionally using the NeuroLucida system (MicroBrightField, Williston, VT). The proportion of boutons in apposition with other somata was calculated from more than 200 randomly sampled boutons.

Data are given as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used for confirmation of significant differences among subtypes in individual parameters, followed by post hoc Tukey test for the group comparison.

**Results**

**Generation of BAC Transgenic Rats**

A BAC construct harboring a modified VGAT allele (Fig. 1A) was made as described in the Methods. The modified BAC construct included 102 kb upstream of the VGAT gene, and 25 kb downstream. As the VGAT gene itself is only 5 kb (Ebihara et al. 2003), all of the VGAT gene’s regulatory regions are likely included within the BAC. Although eGFP is a well-known in vivo marker, we used Venus, a derivative of yellow fluorescent protein (YFP), in place of eGFP because of its enhanced fluorescence (~20-fold brighter than eGFP). Nagai et al. 2002).

Two founder rat lines carrying the VGAT-Venus transgenes were identified by PCR analysis and maintained on a Wistar background. Both lines (lines A and B) transmitted the transgene to their progeny, and lines A and B were named VGAT-Venus-A and VGAT-Venus-B, respectively. Both lines of transgenic rats were characterized for transgene expression, and Venus expression could be observed at postnatal days 1–2 through the skin and skull by fluorescence microscopy.

Both lines of VGAT-Venus transgenic rats exhibit normal growth and reproductive behavior. To confirm the normal production of GABA, we compared the GABA content to total
protein in the cerebrum and cerebellum in VGAT-Venus transgenic rats and their Venus-negative littermates (taking the means of wild-type rats as 100%). VGAT-Venus-A expressing brains had a GABA content (n = 7; 103.3 ± 11.3% in cerebrum; 101.3 ± 14.4 in cerebellum) similar to that found in wild-type littermates (n = 7; 100.0 ± 8.4% in cerebrum and 100.0 ± 11.4 in cerebellum). VGAT-Venus-B brains also contained similar GABA content (n = 6; 103.1 ± 10.1% in cerebrum; 96.7 ± 12.2% in cerebellum) to that of their wild-type littermates (n = 6; 100.0 ± 10.3% in cerebrum and 100.0 ± 15.9 in cerebellum). In addition to having normal GABA, the brain structures of both lines showed no abnormalities at the macroscopic level (Fig. 2), and in sections from fixed brains we observed many fluorescent cells. Fluorescence was evident not only in somata, but also in dendrites and axonal boutons (Fig. 1B-D).

**Venus Expression Patterns**

To confirm whether Venus-expressing cells are also GABAergic in the neocortex, Venus and GABA immunofluorescence were compared in the frontal cortex (Fig. 1B-D). About 98% of Venus-positive cells in line A and ~95% of Venus-positive cells in line B animals were also positive for GABA (Table 1). Conversely, in line A, almost all GABA cells displayed Venus fluorescence (Fig. 1B), indicating GABA and Venus cells comprise almost completely overlapping populations of neurons in the frontal cortex (Table 1). On the other hand, in the B line, GABA-positive cells in deeper cortical layers were mostly positive for Venus, but some GABAergic cells in the superficial layers, especially layer I, did not express Venus (Fig. 1C). This indicates that Venus-positive cells in the frontal cortex of VGAT-Venus-B rats are GABAergic, but that not all GABAergic cells express Venus, especially in layer I (Table 1).

Colocalization of Venus and GABA immunofluorescence was also investigated in the cerebellar cortex of VGAT-Venus-A animals. In the granule cell layer, a few large, putative Golgi cells, had Venus fluorescence that completely overlapped with GABA immunofluorescence (38 cells positive for both Venus and GABA; Fig. 1D). Venus cells in the molecular layer also greatly overlapped with GABA-positive cells (484 Venus cells among 492 GABA cells, 98.4%). In contrast, GABAergic Purkinje cells showed only weak Venus fluorescence. GABA immunofluorescence was also weak at their somata (Fig. 1D). These data indicate that GABAergic interneurons and Venus cells show a high degree of overlap in the cerebral and cerebellar cortices of transgenic rats, especially in the A line, which had an almost complete overlap of GABA-positive and Venus-positive neurons.

We next compared the Venus and GABA expression patterns in the 2 VGAT-Venus lines, and in wild-type rats, in various brain regions using specific antibodies and DAB staining (Figs 2 and 3).

**Figure 1.** Generation of BAC transgenic rats with Venus fluorescence in VGAT (vesicular GABA transporter)-expressing neurons. (A) Schematic representation of the structure of BAC #160L22 containing the VGAT gene (top; wild-type BAC), the recombination cassette (middle), and the modified BAC (bottom). Venus cDNA followed by a poly-A signal was placed into exon 1 of the VGAT gene in frame with the translational initiation codon, ATG. (B-D) Colocalization of Venus fluorescence and GABA immunoreactivity in the cerebral and cerebellar cortex of VGAT-Venus transgenic rats. (B) Venus-positive cells (left) almost completely overlapped with the GABA-positive population (middle) in layer I/II of the frontal cortex in the "A" line of VGAT-Venus (VGAT-Venus-A) rats. The 2 fluorescence images are merged at the right. (C) Venus-positive cells (left) mostly overlapped with the GABA-positive population (middle) in the "B" line of VGAT-Venus (VGAT-Venus-B) rats. However, a few cells were positive for GABA but not for Venus (arrow). The 2 fluorescence images are merged at the right. (D) Venus-positive cells (left) in the molecular and granular layer of the cerebellar cortex almost completely overlapped with the GABA-positive population (middle) in VGAT-Venus-A rats, whereas Purkinje cells displayed little Venus fluorescence. GABA immunofluorescence was also weak in Purkinje somata. Abbreviations: mol, molecular layer; Pur, Purkinje cell layer; gra, granule cell layer.

**Figure 2.** Distribution patterns of Venus in VGAT-Venus rats. Venus was visualized in parasagittal brain slices using the antibody and peroxidase DAB reaction. (A) Venus expression in a VGAT-Venus-A rat. (B) Venus expression in a VGAT-Venus-B rat. In the forebrain, Venus distribution was similar to that of GABA, except in the hippocampal CA1 region in VGAT-Venus-B rats and thalamus in VGAT-Venus-A rats.
Neocortex
As was found in the frontal cortex, described above, other cortical regions in both animal lines had Venus-positive cells in all layers. In A line animals, Venus cells in layers I–VI were positive for GABA. In addition to the expected GABA-positive neurons, layer VI of nonfrontal cortical regions also had some neurons that were faintly labeled with Venus but negative for GABA. In the B line, Venus-expressing cells in layers I–VI were positive for GABA. In addition, in B line animals, a few pyramidal cells in the ventral cortex showed weak Venus fluorescence but were negative for GABA.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Relation of Venus fluorescent cells and GABA immunopositive cells in frontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VGAT-Venus-A</td>
</tr>
<tr>
<td></td>
<td>Venus/GABA</td>
</tr>
<tr>
<td>Layer</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>98.6 ± 1.4 (148)</td>
</tr>
<tr>
<td>II/III</td>
<td>97.3 ± 3.6 (1367)</td>
</tr>
<tr>
<td>V</td>
<td>97.7 ± 2.5 (1075)</td>
</tr>
<tr>
<td>VI</td>
<td>98.0 ± 2.1 (1172)</td>
</tr>
<tr>
<td>Total</td>
<td>97.6 ± 2.8 (3782)</td>
</tr>
</tbody>
</table>

Note: Data are percentages and mean ± SD of 3 animals. (), total numbers of GABA or Venus cells of 3 VGAT-Venus-A or -B rats.

Hippocampus
In VGAT-Venus-A rats, nonpyramidal cells were labeled with Venus, but no Venus was detected in pyramidal cells in the CA1–3 regions, or in granule cells in the dentate gyrus (Fig. 3A), indicating that Venus cells overlap with GABAergic neurons. In VGAT-Venus-B rats, nonpyramidal cells were selectively labeled with Venus in CA2, CA3, and dentate gyrus, but in CA1 both pyramidal and nonpyramidal cells showed fluorescence at their somata and dendrites (Fig. 3B).

Figure 3. Comparative distribution of Venus-positive cells in 2 lines of VGAT-Venus rats and GABA-positive cells in wild-type rats. Venus expression was more clearly seen in somata than was GABA immunoreactivity. (A) Dentate gyrus and CA3 region of the hippocampus: nonpyramidal cells, but not granule or pyramidal cells, expressed Venus in both animal lines. (B) CA1 regions of the hippocampus: only nonpyramidal cells expressed Venus in the VGAT-Venus-A rat, but pyramidal cells also expressed Venus in the VGAT-Venus-B rat. (C) Thalamus: reticular nucleus cells expressed Venus in both lines of VGAT-Venus rats, but Venus was found in other nucleus in the VGAT-Venus-A rats. (D) Olfactory bulb: periglomerular and granule cells were stained in both lines. In the external plexiform layer, dendrites of granule cells were stained. Abbreviations: mol, stratum moleculare; gr, stratum granulare; pyr, stratum pyramidale; ori, stratum oriens; rad, stratum radiatum; Rt, reticular nucleus; VL, ventrolateral nucleus; onl, olfactory nerve layer; glo, glomerular layer; epl, external plexiform layer; mit, mitral cell layer; gra, granular layer.
Venus expression was found in the GABAergic reticular nucleus of both lines (Fig. 3C). Further, the lateral geniculate nuclei in both lines contained Venus-positive cells that likely correspond to GABAergic interneurons. In VGAT-Venus-A rats, Venus-positive cells were also found in projection neurons (likely glutamatergic) in other thalamic nuclei such as the ventrolateral, ventroposterior, and laterodorsal nuclei. Thalamic projection cells were not labeled with Venus in VGAT-Venus-B rats.

**Olfactory Bulb**

Excitatory mitral and tufted cells did not express Venus, whereas granule and periglomerular cells, considered to be GABAergic, were labeled with Venus (Fig. 3D). In the granule and inner plexiform layers, a few Venus-positive large cells existed, perhaps corresponding to so-called "short axon cells." The external plexiform layer also had a few large Venus-positive cells that may also belong to the short axon cell group or to other unidentified subtypes. No differences were found in the distribution patterns of Venus-positive cells between the 2 lines.

**Basal Ganglia Nuclei**

Venus was expressed in GABAergic cells in the basal ganglia of both VGAT-Venus-A and -B animals. In the striatum, most cells expressed Venus, indicating labeling of GABAergic projection cells. Qualitatively, most parvalbumin, somatostatin, and calretinin cells were fluorescently labeled in VGAT-Venus-A rats, but large cells positive for cholera acetyletransferase were devoid of Venus. Parvalbumin cells showed stronger fluorescence than other types. In both the globus pallidus and entopeduncular nucleus, large multipolar cells were labeled with Venus, corresponding to GABAergic principal cells. The subthalamicus showed no Venus fluorescence, whereas many Venus cells were found in zona incerta. In the substantia nigra, the pars reticulata had many Venus cells, corresponding to GABAergic output cells, whereas no Venus cells were localized in the dopaminergic neurons of the pars compacta.

**Venus in Cortical GABA Cell Subtypes**

To determine whether various GABAergic cell subtypes were labeled with Venus, we investigated colocalization patterns of Venus with the chemical markers for cortical GABAergic cells: parvalbumin, somatostatin, VIP, CCK (Fig. 4A–D). Further, we examined the colocalization of Venus and α-actinin-2, an actin-binding protein expressed by some GABAergic neurons in the hippocampus (Ratzliff and Soltész 2001). In VGAT-Venus-A rats, α-actinin-2-positive cells in all layers also expressed Venus (100% in all layers; Table 2), suggesting that all α-actinin-2-positive cells are GABAergic (Fig. 4F). Calretinin is also expressed in GABAergic neurons (Kubota et al. 1994), but we found calretinin immunofluorescence to be variable in intensity. Most cells with strong immunofluorescence also had Venus fluorescence, but some cells with weaker labeling were devoid of Venus in VGAT-Venus-A rats (Fig. 4E), suggesting that some non-GABAergic cells may express calretinin (Melchitzky et al. 2005). Therefore, Venus expression was quantitatively analyzed for colocalization with biochemical markers other than calretinin (Table 2). However, we also calculated the proportion of cells expressing all of the above biochemical markers, including calretinin, among the total Venus-positive population (Table 3).

In VGAT-Venus-A rats, cells positive for parvalbumin, somatostatin, VIP, CCK, or α-actinin-2 were almost always positive for Venus (Table 2). This result is consistent with the finding that Venus cells overlap fully with GABAergic cells in the VGAT-Venus-A line. In VGAT-Venus-B rats, the vast majority of parvalbumin and somatostatin cells were also positive for Venus, but a few VIP and CCK cells, and many α-actinin-2 cells were negative for Venus (Table 2). These data suggest that GABAergic cells negative for Venus in VGAT-Venus-B correspond to subpopulations of VIP, CCK, and especially α-actinin-2 cells.

**Composition of Cortical GABAergic Population**

Because the Venus-positive neuron population overlapped completely with GABA immunoreactivity in VGAT-Venus-A rats, we were able to measure the proportion of each chemical subtype in the total GABAergic (Venus-positive) population in 3 rats. This method, using Venus-expressing tissue, was found to be both easier and more reliable than doing double immunostaining with antibodies to both GABA and a second chemical marker (Fig. 4A–F). The mean values obtained from 3 rats are shown in Table 3 and Figure 4G. Parvalbumin-positive cells were not found in layer 1 neurons expressing Venus, but made up 36.4% of Venus-positive cells in layer II/III, 52.7% of cells in layer V, and 38.7% of cells in layer VI. Somatostatin-positive cells were not found in layer I Venus-positive cells, but made up 17.4% of cells in layer II/III, 28.6% of cells in layer V, and 29.0% of cells in layer VI. VIP-positive cells made up 3.3% of layer 1 Venus-positive cells, 24.0% of cells in layer II/III, 9.6% of cells in layer V, and 8.0% of cells in layer VI. Calretinin cells comprised 35.8% of Venus cells in layer I, 21.0% of cells in layer II/III, 11.1% of cells in layer V, and 11.4% of cells in layer VI. CCK was found in just 0.9% of cells in layer I, 8.9% of cells in layer II/III, 2.9% of cells in layer V, and 3.0% of cells in layer VI. Finally, α-actinin-2-positive cells comprised 66.5% of Venus-positive cells in layer I, 9.8% of cells in layer II/III, 1.5% of cells in layer V, and 6.2% of cells in layer VI.

To estimate the proportion of all GABAergic cells expressing the above 6 chemical markers, their colocalization patterns should be known. Parvalbumin and somatostatin cells were completely independent groups and were also devoid of the other 3 chemical markers (Kubota et al. 1994; Gonchar and Burghalder 1997; Kubota and Kawaguchi 1997). Coexistence of calretinin and CCK was found in a few cells. Among VIP-positive cells, calretinin was expressed in 33% in layer II/III, 71% in layer V, and 54% in layer VI. Among VIP-positive cells, CCK was expressed in 32% in layer II/III, 21% in layer V, and 19% in layer VI (Kubota and Kawaguchi 1997 and related unpublished observations). Results of the α-actinin-2 colocalization with the other chemical markers in wild-type rats are available in the Appendix below (Fig. 8, Table 5). Parvalbumin-positive and somatostatin-positive cells did not express α-actinin-2, and only a very few VIP-positive and CCK-positive neurons were positive for α-actinin-2. Some α-actinin-2-positive cells showed calretinin immunoreactivity, especially in layer 1 (14.8%), layer V (18.8%) and layer VI (16.2%). Assuming the colocalization of α-actinin-2 and calretinin in addition to the previously revealed relationships, these 6 chemical markers cover more than 90% of the total GABAergic populations in layers I, II/III, and V, and nearly 90% of cells in VI (Fig. 4G).

**Functional Characteristics of Venus Cells**

To determine whether Venus-expressing cells have differentiated normally into GABAergic cell subtypes, Venus-positive cells
in the cortex were examined for their physiological, morphological, and chemical characteristics (Kawaguchi and Kondo 2002). In layer V of the frontal cortex of VGAT-Venus-A rats, we made whole-cell recordings from FS, RSNP, and BSNP cells. Among 12 recorded FS cells in layer V, 11 cells showed parvalbumin immunoreactivity (Fig. 5A), of which 9 cells were identified as basket cells with similar morphology to cells in the wild-type animals (Fig. 5C2, D). The remaining 3 FS cells were not morphologically identified because of poor axonal staining. Seven RSNP cells in layer V were immunoreactive for somatostatin, of which 5 cells were Martinotti cells with ascending axonal arbors similar to those found in wild-type rats. Of 8 somatostatin-positive BSNP cells, 6 had morphologies consistent with Martinotti cells (Fig. 5C1, T). Parvalbumin-positive FS cells had more negative resting potentials, smaller input resistances, and shorter spike widths than did somatostatin-positive cells (Table 4), and somatostatin-positive BSNP cells had larger input resistances than RSNP cells (Kawaguchi and Kubota 1996).

To confirm the differentiation of VIP cells, we identified 5 Venus-expressing VIP-positive cells in layer II/III (Fig. 6A), among which 2 cells well stained showed the descending axonal arbors morphologically. Physiologically, they included cells with either strong firing adaptation or irregular spiking (Fig. 6B, C), and were classified as either RSNP or BSNP. Thus, we found no differences in the physiological, chemical, and morphological characteristics between wild-type and VGAT-Venus rat. These data suggest normal functional differentiation of cortical GABAergic cells in VGAT-Venus rats.

Although most GABAergic cell subtypes can be labeled with calcium-binding proteins and neuropeptides, specific chemical markers have not previously been identified in neocortical...
neurogliaform cells. In hippocampus, α-actinin-2 is expressed not only in dendrites of pyramidal cells, but also in somata of interneurons (Wyszyński et al. 1998; Ratzliff and Soltesz 2001), including neurogliaform cells (Price et al. 2005). We investigated whether LS cells coincide with the α-actinin-2–positive cell population in frontal cortex. Immunofluorescence for α-actinin-2 was investigated in LS cells whose somata were well stained (n = 6 in layer II/III and n = 2 in layer V). Five LS cells were positive for α-actinin-2 in layer II/III and both positive in layer V (Fig. 5B,C). LS neurogliaform cells positive for α-actinin-2 had more hyperpolarized resting potentials than somatostatin cells, longer spike widths than parvalbumin FS cells, and lower input resistances than somatostatin BSNP cells (Table 4).

To further reveal the functional differentiation between parvalbumin, somatostatin, and α-actinin-2 cells, we compared their firing characteristics quantitatively. Parvalbumin FS cells required more current to induce spikes than was required in α-actinin-2 LS cells or somatostatin BSNP cells (Fig. 5E). When depolarized just above action potential threshold, parvalbumin and α-actinin-2 cells fired spikes at relatively constant intervals, whereas somatostatin cells showed spike-frequency adaptation. Parvalbumin-positive FS, somatostatin-positive RSNP, and α-actinin-2-positive LS cells increased their firing rate linearly with larger amplitude current injections (up to 500 pA) (Fig. 5F). The slope of firing frequency versus injected current (duration, 0.5 s) was 270 Hz/nA in parvalbumin FS cells (correlation coefficient [c.c.] = 0.65), 163 Hz/nA in somatostatin RSNP cells (c.c. = 0.92), and 34 Hz/nA in α-actinin-2 LS cells (c.c. = 0.65), indicating significant differences in the output characteristics of these neurons.

### Table 2

Colocalization of chemical markers for GABAergic nonpyramidal cells with Venus in frontal cortex of VGAT-Venus rats

<table>
<thead>
<tr>
<th>Layer</th>
<th>PV</th>
<th>SOM</th>
<th>VIP</th>
<th>CCK</th>
<th>αAc2</th>
</tr>
</thead>
<tbody>
<tr>
<td>VGAT-Venus-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>No cells</td>
<td>No cells</td>
<td>100% (6)</td>
<td>100% (104)</td>
<td>100% (104)</td>
</tr>
<tr>
<td>II/III</td>
<td>99.8% (470)</td>
<td>98.6% (142)</td>
<td>98.3% (359)</td>
<td>100% (165)</td>
<td>100% (154)</td>
</tr>
<tr>
<td>V</td>
<td>100% (370)</td>
<td>96.2% (132)</td>
<td>98.8% (85)</td>
<td>100% (28)</td>
<td>100% (15)</td>
</tr>
<tr>
<td>VI</td>
<td>99.7% (306)</td>
<td>98.1% (211)</td>
<td>97.6% (85)</td>
<td>97.7% (43)</td>
<td>100% (52)</td>
</tr>
<tr>
<td>Total</td>
<td>99.8% (1154)</td>
<td>97.7% (485)</td>
<td>98.3% (535)</td>
<td>99.6% (240)</td>
<td>100% (325)</td>
</tr>
<tr>
<td>VGAT-Venus-B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>No cells</td>
<td>No cells</td>
<td>28.6% (7)</td>
<td>0.0% (1)</td>
<td>47.0% (134)</td>
</tr>
<tr>
<td>II/III</td>
<td>99.7% (622)</td>
<td>98.2% (336)</td>
<td>98.5% (226)</td>
<td>88.9% (235)</td>
<td>59.6% (136)</td>
</tr>
<tr>
<td>V</td>
<td>99.9% (731)</td>
<td>98.8% (248)</td>
<td>95.0% (60)</td>
<td>82.9% (41)</td>
<td>85.7% (14)</td>
</tr>
<tr>
<td>VI</td>
<td>99.7% (751)</td>
<td>98.4% (189)</td>
<td>95.1% (61)</td>
<td>94.8% (58)</td>
<td>74.5% (47)</td>
</tr>
<tr>
<td>Total</td>
<td>99.8% (2104)</td>
<td>98.4% (773)</td>
<td>98.5% (354)</td>
<td>98.0% (335)</td>
<td>57.7% (331)</td>
</tr>
</tbody>
</table>

Note: Venus cells/cells positive for a chemical marker. Data are combined from 3 animals in A line, 2 animals in B line. ( ), number of cells positive for individual chemical markers (denominator). PV, parvalbumin, SOM, somatostatin; αAc2, α-actinin-2.

### Table 3

Mean percentages of cells positive for individual chemical markers among Venus cells of frontal cortex in 3 VGAT-Venus-A rats

<table>
<thead>
<tr>
<th>Layer</th>
<th>PV</th>
<th>SOM</th>
<th>VIP</th>
<th>CCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>No cells (118)</td>
<td>No cells (63)</td>
<td>3.3 ± 2.2 (121)</td>
<td>35.2 ± 10.1 (78)</td>
</tr>
<tr>
<td>II/III</td>
<td>36.4 ± 2.9 (1284)</td>
<td>17.4 ± 1.4 (628)</td>
<td>24.0 ± 2.5 (1473)</td>
<td>21.0 ± 2.1 (1236)</td>
</tr>
<tr>
<td>V</td>
<td>52.7 ± 3.9 (726)</td>
<td>28.6 ± 1.9 (458)</td>
<td>9.6 ± 3.5 (857)</td>
<td>11.1 ± 3.1 (576)</td>
</tr>
<tr>
<td>VI</td>
<td>38.7 ± 4.8 (801)</td>
<td>23.0 ± 0.4 (713)</td>
<td>8.0 ± 0.3 (1048)</td>
<td>11.4 ± 4.4 (907)</td>
</tr>
<tr>
<td>Total</td>
<td>39.3 ± 1.4 (2929)</td>
<td>23.4 ± 1.3 (2054)</td>
<td>15.0 ± 0.7 (1499)</td>
<td>16.1 ± 2.7 (2797)</td>
</tr>
</tbody>
</table>

Note: Mean ± SD of 3 VGAT-Venus-A rats. PV, parvalbumin; SOM, somatostatin; CR, calretinin; αAc2, α-actinin-2. ( ), total numbers of Venus cells of 3 VGAT-Venus-A rats.

FS Basket Cells Heterogeneous in their Somatic Size and Dendritic Territory

When recording in wild-type rats, we identified cortical GABAergic nonpyramidal cells on the basis of their somatic shape and size. Layer V contains a few large nonpyramidal cells with somatic sizes comparable with pyramidal cells, or having pyramid-shaped somata (Marin-Padilla 1969; Jones 1975; DeFelipe et al. 1986). This has made discriminating these large nonpyramidal cells difficult. Although their number is small, large interneurons may play an important role in cortical function (Hensch 2005). Recording from slices of VGAT-Venus rat, we found large FS cells that were of similar size or shape to pyramidal cells (Fig. 7A). The maximum cross-sectional areas of layer V FS cells recorded from Venus rats were 220 ± 67 μm² (maximum = 356 μm²).

To assess further the differentiation of Venus-expressing GABAergic neurons, we examined the dendritic morphology and synaptic innervation patterns of neurons exhibiting physiological and chemical correlations. The number of dendrites issuing from the soma (primary dendrites) was 6.2 ± 1.6 (n = 9; range, 4–9) in parvalbumin-positive FS basket cells, 6.0 ± 1.8 (n = 6; range, 4–9) in α-actinin-2–positive LS neurogliaform cells, and 3.2 ± 0.8 (n = 11; range, 2–4) in somatostatin-positive Martinotti cells (which were significantly fewer than in the above FS and LS cells, P < 0.01).

In a previous study, we used electron microscopy to confirm that about 80% of visually identified (via light microscopy) nonpyramidal neuron axonal boutons apposed to soma make synaptic junctions onto them (Karube et al. 2004). Here we also used Nomarski optics to identify appositions of intracellularly stained axonal boutons onto unstained postsynaptic somata (Fig. 5D). For 3 nonpyramidal cell subtypes (FS, Martinotti, and LS cells), we measured the number of stained boutons apposed to other soma as a proportion of the total number of boutons observed. Boutons arising from parvalbumin-positive FS basket cells and somatostatin-positive Martinotti cells targeted somata in 22.3 ± 4.3% and 2.5 ± 1.9%, respectively (P < 0.01; Fig. 5G). The proportion of somatic apposition in boutons from α-actinin-2–positive LS neurogliaform cells was 14.3 ± 6.4% (larger than the above somatostatin Martinotti cells, P < 0.01; smaller than the above parvalbumin FS cells, P < 0.05) (Fig. 5G), and in addition showed more variability within its group than other subtypes. A comparison of these results with those of wild-type rats is available in the Appendix (Fig. 9), and confirms that the proportion of soma-targeting boutons is similar in wild-type and VGAT-Venus rats. These observations suggest that α-actinin-2–positive LS neurogliaform cells are unique compared with the other cell subtypes in the aspects of physiology, dendritic elongation and innervation targets.
somewhat larger than those recorded in slices from wild-type rats ($131 \pm 34 \ \mu m^2$, maximum $= 202 \ \mu m^2$, $n = 14$) (Kawaguchi et al. 2006), and included cells with similar size to layer V pyramidal cells in the same cortical regions (pyramidal cells projecting to the contralateral striatum, $224 \pm 60 \ \mu m^2$, maximum $= 396 \ \mu m^2$, $n = 19$; pyramidal cells projecting to the pons, $262 \pm 45 \ \mu m^2$, maximum $= 360 \ \mu m^2$, $n = 9$) (Morishima and Kawaguchi 2006). The use of VGAT-Venus rats
allowed us to record nonpyramidal cells in an unbiased way irrespective of the soma size and shape.

Although the distribution of somatic size in FS cells was continuous from smaller to larger neurons (Fig. 7B), we looked for correlation between physiological properties and somatic size in 15 FS cells whose somata were stained with biocytin (maximum of the cross-sectional somatic area: 214 ± 65 μm²; range, 128–311 μm²). We did not find distinct differences in firing patterns between smaller and larger FS cells, but there was a weak correlation between input resistance and somatic size (c.c. = 0.32, P = 0.25; 103 ± 44 MΩ in 5 smaller cells and 69 ± 6 MΩ in 5 larger cells). The morphology of dendritic arborizations was investigated in FS basket cells whose dendrites and axons were sufficiently stained. The somatic size of FS cells correlated with the maximum vertical elongation of dendrites (c.c. = 0.55) (Fig. 7A, B), and not with the maximum horizontal distance (c.c. = 0.12) (Fig. 7C). Larger FS cells extended dendrites more widely in a vertical direction. Maximum vertical dendritic elongation was independent of somatic depth within layer V (c.c. = 0.06). This suggests that chemically and physiologically homogeneous FS cells are heterogeneous in their dendritic vertical dimensions, but have relatively uniform horizontal (columnar) dendritic dimensions (Fig. 7C). Findings were similar when observations were repeated in wild-type rats, as shown in the Appendix (Fig. 10). These data suggest that individual nonpyramidal cell subtypes, defined by combination of the spike firing, chemical expression, and axonal arborization patterns, show type-specific horizontal dendritic extensions.

Discussion

By applying BAC technology to rats, we generated, for the first time, transgenic rats with selective Venus fluorescence in GABAergic neurons. In certain brain areas, non-GABAergic neurons were labeled with Venus, but these areas were rarely overlapping in our 2 transgenic lines. Thus, at least one transgenic line can be used for selective identification of GABA cells in most areas of the forebrain. In the neocortex, we utilized the selective expression of Venus in GABAergic neurons to dissociate cortical interneurons into subtypes based on their chemical, physiological, and morphological characteristics. This is the first time that quantitative assessment of cortical interneurons using the above 6 chemical markers has been applied simultaneously to the full GABAergic cell population in the neocortex.

Selective Fluorescence Labeling of Forebrain GABA Cells in BAC Transgenic Rats

The biosynthetic enzyme for GABA, GAD, has 2 isoforms, GAD65 and GAD67. In transgenic mice produced using one of the 2 GAD promoters, only subpopulations of GABAergic cells are labeled with GFP (Oliva et al. 2000; Chattopadhyaya et al. 2004; Lopez-Bendito et al. 2004; Ma et al. 2006; Xu et al. 2006). In GAD67-GFP knock-in mice, most GABA cells are fluorescently labeled, but cells expressing only GAD65 may not be labeled. Furthermore, in GAD67-GFP knock-in mice, the GABA content is decreased because of the deletion of one GAD67 allele (Tamamaki et al. 2003). This reduction of GABA content may affect circuit formation, especially during development (Hensch 2005). Because the gene length of VGAT (~45 kb) is much shorter than those of GAD67 (~45 kb) and GAD65 (~80 kb) (Yanagawa et al. 1997; Makinae et al. 2000; Ebihara et al. 2003), we were able to construct a BAC clone containing the entire structure of VGAT, including regulatory sequences near the gene itself, to selectively label GABAergic neurons in rats. GABAergic neurons are widespread in the mammalian CNS, whereas glycineric neurons are largely restricted to the spinal cord and brainstem (Esclapez et al. 1994; Legendre 2001). VGAT is the vesicular transporter not only for GABA but also for glycine (Sagme et al. 1997; Chaudhry et al. 1998). Therefore, it remains to be investigated whether both GABAergic and glycineric cells are labeled with Venus in the spinal cord and brainstem.

We found Venus expression in non-GABAergic cells in a few regions, such as the relay nuclei of the thalamus in VGAT-Venus-A rats and the CA1 region of the hippocampus in VGAT-Venus-B rats. Even in these regions, however, the other line showed correct expression of Venus. Therefore, one of these 2 lines

Table 4

Electrophysiological properties of nonpyramidal cell subtypes in frontal cortex of VGAT-Venus rats

<table>
<thead>
<tr>
<th>Cell type</th>
<th>N</th>
<th>Resting potential (mV)</th>
<th>Input resistance (M)</th>
<th>Spike half-width (ms)</th>
<th>Threshold current* (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV-FS cells (layer V)</td>
<td>11</td>
<td>~71 ± 3</td>
<td>73 ± 15</td>
<td>0.41 ± 0.07</td>
<td>378 ± 88</td>
</tr>
<tr>
<td>SOM-RSNP cells (layer V)</td>
<td>7</td>
<td>~62 ± 3</td>
<td>170 ± 40</td>
<td>0.57 ± 0.04</td>
<td>71 ± 34</td>
</tr>
<tr>
<td>SOM-BSNP cells (layer V)</td>
<td>8</td>
<td>~60 ± 4</td>
<td>417 ± 138</td>
<td>0.6 ± 0.12</td>
<td>38 ± 33</td>
</tr>
<tr>
<td>αAc2-LS cells (layer II/III)</td>
<td>10</td>
<td>~68 ± 2</td>
<td>126 ± 26</td>
<td>0.69 ± 0.06</td>
<td>178 ± 39</td>
</tr>
<tr>
<td>αAc2-SOM cells (layer II/III)</td>
<td>6*</td>
<td>~69 ± 2</td>
<td>209 ± 20</td>
<td>0.57 ± 0.04</td>
<td>178 ± 39</td>
</tr>
<tr>
<td>ANOVA/post hoc test (Tukey test, P &lt; 0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Data are mean ± SD. N, number of cells; PV, parvalbumin; SOM, somatostatin; αAc2, α-actinin-2.

*Depolarizing current pulse of 0.5 s to induce spikes.

Downloaded from https://academic.oup.com/cercor/article-abstract/18/2/315/334705 by guest on 01 January 2019
should be selected according to the investigated nucleus, region, or layer.

In the neocortex there were also subtle differences in Venus expression between the 2 lines. In VGAT-Venus-A rats, almost all GABAergic cells expressed Venus, but some layer VI pyramidal cells were weakly labeled in nonfrontal cortical areas. In VGAT-Venus-B rats, Venus fluorescence was not observed in layer VI pyramidal cells, and there was also failure to label all GABAergic cells. Nonfluorescent GABA cells in the B line belonged to subpopulations of VIP, CCK, calretinin, or $\alpha$-actinin-2 expressing cells. Parvalbumin- and somatostatin-positive cells were always positive for Venus. The differential expression of Venus among GABAergic subtypes in the B line may reflect differences in the developmental origins of GABAergic neurons. Parvalbumin- and somatostatin-positive cells originate within the medial ganglionic eminence, whereas calretinin-positive cells derive from the caudal ganglionic eminence (Xu et al. 2004). The site of generation within the ganglionic eminence and its timing are critical for interneuron subtype differentiation (Butt et al. 2005). Aberrant Venus expression in some non-GABAergic cells, and the lack of Venus in specific subpopulations of GABAergic 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).

**Cortical GABA Cell Type Organization**

Most GABAergic cell types identified so far can be classified based on their differential expression of a few calcium-binding proteins and neuropeptides, although individual expression patterns often include multiple morphological subtypes. LS neurogliaform cells, however, have not yet been chemically characterized. In the hippocampus, the actin-binding protein, $\alpha$-actinin-2, is expressed not only in the dendrites of pyramidal cells, but also in the somata of some interneurons (Wyszynski et al. 1998; Ratzliff and Soltesz 2001), including neurogliaform cells (Price et al. 2005). Using VGAT-Venus-A rats, we found that $\alpha$-actinin-2 is expressed in a subpopulation of GABAergic cells of the frontal cortex that includes LS neurogliaform cells in both layers II/III and V. $\alpha$-Actinin-2 LS neurogliaform cells had an intermediate current threshold for spike induction that fell between the values for parvalbumin-expressing FS and somatostatin-positive cells. The slope of firing frequency against the injected current was less sharp in $\alpha$-actinin-2 LS neurogliaform cells than in parvalbumin and somatostatin cells, and LS cells fired action potentials at relatively constant intervals, similar to FS cells, but at a much lower frequency. GABAergic non-pyramidal cells could generally be divided into basket and nonbasket cells based on their innervation preference for somatic domains. Innervation patterns of $\alpha$-actinin-2--expressing LS neurogliaform cells, however, were more variable in their tendency to choose somatic targets (Figs 5G and 9). These physiological, chemical, and morphological characteristics suggest that cortical neurogliaform cells are a functionally distinct subgroup of cortical GABAergic cells (Tamás et al. 2003; Price et al. 2005).

The functional diversity of cortical GABAergic interneurons in the cortex remains to be investigated (Nelson 2002; Yuste 2005). Because several chemical markers are differentially

---

**Figure 7.** Large variability in the somatic size of layer V FS basket cells recorded from VGAT-Venus rats. (A) Dendritic reconstructions of 6 FS basket cells (a-f) in layer V sorted (left to right) according to increasing somatic cross-sectional area. Insets, somatic shapes. (B) Distribution of somatic cross-sectional areas. Each letter corresponds to the reconstructed cell shown in (A). (C) Relationship of dendritic arbors to the somatic cross-sectional areas. Horizontal and vertical maximum dendritic distances are calculated as the distances from somatic origin to the tip of the longest dendrite in horizontal and vertical directions, respectively. The vertical, but not horizontal, maximum distance was correlated with somatic size.
correspond to cortical layers. Actinin-2-positive cells showed calretinin immunofluorescence. Roman numerals indicate the relationship of actinin-2 and VIP cells. (A) Dual photomicrographs from the same microscopic field, showing the relationship of actinin-2-positive (αAc2) and parvalbumin-positive cells in wild-type rats. (B) Relationship of actinin-2 and VIP cells. (C) Relationship of actinin-2 and calretinin cells. Some actinin-2-positive cells showed calretinin immunofluorescence. Roman numerals correspond to cortical layers.

Figure 8. In the cortex, actinin-2-positive (αAc2) cells were separate from parvalbumin (PV) and VIP populations, but partially overlapped with calretinin cells. (A) Dual photomicrographs from the same microscopic field, showing the relationship of actinin-2- and parvalbumin-positive cells in wild-type rats. (B) Relationship of actinin-2 and VIP cells. (C) Relationship of actinin-2 and calretinin cells. Some actinin-2-positive cells showed calretinin immunofluorescence. Roman numerals correspond to cortical layers.

Table 5

<table>
<thead>
<tr>
<th>Layer</th>
<th>PV</th>
<th>SOM</th>
<th>CR</th>
<th>VIP</th>
<th>CCK</th>
<th>CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-VI</td>
<td>0% (75)</td>
<td>0% (74)</td>
<td>14.8% (81)</td>
<td>1.4% (74)</td>
<td>0% (78)</td>
<td>0% (33)</td>
</tr>
<tr>
<td>I-VI</td>
<td>0% (81)</td>
<td>0% (101)</td>
<td>3.0% (101)</td>
<td>1.2% (82)</td>
<td>0% (97)</td>
<td>0% (48)</td>
</tr>
<tr>
<td>V</td>
<td>0% (17)</td>
<td>0% (20)</td>
<td>18.8% (16)</td>
<td>0% (19)</td>
<td>0% (14)</td>
<td>0% (12)</td>
</tr>
<tr>
<td>VI</td>
<td>0% (41)</td>
<td>0% (49)</td>
<td>16.2% (37)</td>
<td>0% (53)</td>
<td>2.4% (42)</td>
<td>0% (34)</td>
</tr>
<tr>
<td>Total</td>
<td>0% (214)</td>
<td>0% (244)</td>
<td>10.2% (225)</td>
<td>0.9% (226)</td>
<td>0.4% (231)</td>
<td>0.4% (121)</td>
</tr>
</tbody>
</table>

Note: Cells positive for a chemical marker/α-actinin-2 cells. PV, parvalbumin; SOM, somatostatin; CR, calretinin; CB, calbindin. Data are combined from 3 animals. ||, number of cells positive for α-actinin-2 (denominator).

found large FS basket cells that were of similar size or shape to pyramidal cells, as well as much smaller neurons, and their vertical dendritic extent varied with somatic size. These data indicate basket cells having similar chemical and physiological characteristics can be further differentiated based on dendritic domains, even within the same layer. Because of the robust Venus fluorescence in our VGAT-Venus animals, we can now identify and record from neocortical nonpyramidal cells in an unbiased manner, irrespective of somatic size or shape.

Utility of VGAT-Venus Transgenic Rats

Largely as a result of technical advances such as gene targeting (knockout and knock-in) technology in ES cells, the mouse will continue to be a convenient mammalian model, especially for genetic and developmental studies. In contrast, the rat has for many years provided the most appropriate physiological and neurobiological model. The rat is useful for a whole range of physiological, surgical, and neurobiological manipulations that would be much more difficult in the mouse because of its smaller size (Phang 1993). Examples of such manipulations include the measurement of blood pressure, multiple automated blood sampling, and the introduction of probes into specific areas of the brain for electrophysiological recordings, cellular ablation, and drug delivery. In addition, the rat has been the preferred model for behavioral studies because rats are superior to mice in learning and generally less aggressive during handling. Furthermore, it should be possible to record Venus-positive cortical cells by direct visual guidance of 2-photon microscopy (Margrie et al. 2003). Given that cortical GABAergic neurons play a central role in generating appropriate behavioral output (Fuchs et al. 2007), VGAT-Venus rats will be an important tool for in vivo recording from cortical GABAergic neurons together with single cell fluorescence imaging.

In conclusion, the BAC technology described here permits specific fluorescent labeling of GABAergic neurons in the intact brain. This suggests possible genetic manipulation of GABAergic interneurons in the rat by driving Cre recombinase expression using the VGAT BAC construct. We expect VGAT-Venus transgenic rats to be highly useful for a multitude of in vitro or in vivo investigations of the structure and function of GABAergic neurons, both in basic research and in clinically oriented studies.

Appendix

We also investigated the following 3 characteristics in nonpyramidal cells of wild-type Wistar rats: 1) the colocalization pattern of α-actinin-2 with the other chemical markers at the somata to calculate the proportion of GABAergic cells expressing the chemical markers in VGAT-Venus rats; 2) the proportion of boutons from the nonpyramidal cells making somatic appositions in order to confirm the similarity of the somatic innervation patterns between the VGAT-Venus and wild-type
rats; and 3) the horizontal spread of dendrites, which, from the data of FS basket cells obtained from the VGAT-Venus rat appeared specific for each subtype.

To determine the frequency for colocalization of α-actinin-2 with the other chemical markers, we investigated the relationship of their immunofluorescences in the frontal cortex (Fig. 8). Parvalbumin-positive and somatostatin-positive cells did not express α-actinin-2, and only a very few VIP-positive and CCK-positive neurons were positive for α-actinin-2 (Table 5). However, some α-actinin-2-positive cells showed calretinin immunoreactivity, especially in layer 1 (14.8%).

Figure 9. Somatic innervation patterns in wild-type rats. (A) Comparisons of the proportion of axonal boutons making appositions on other somata between nonpyramidal cell subtypes in wild-type rats. Basket and nonbasket classes were mostly differentiated. (B) Reconstructions of nonpyramidal cell subtypes. Somata and dendrites are shown in black, and axons in red. Ten subtypes were investigated in layer II/III: 18 FS basket cells, 2 FS chandelier cells, 7 LS neurogliaform cells, 6 somatostatin Martinotti cells, a somatostatin wide arbor cell, 11 CCK large basket cells, 7 small basket cells positive for VIP, CCK, or CRF, 4 descending basket cells positive for VIP or CRF, 7 double bouquet cells positive for VIP, CRF, or calretinin, and 1 calretinin horizontal/descending arbor cell. In layer V, 6 subtypes were investigated: 16 FS basket cells, 2 FS cells without basket terminals, 2 LS neurogliaform cells, 5 somatostatin Martinotti cells, 3 double bouquet cells positive for VIP, and a VIP arcade cell.
layer V (18.8%), and layer VI (16.2%) (Table 5). Another calcium-binding protein, calbindin was not found in α-actinin-2 cells.

We compared the proportion of boutons apposed with target somata for 12 subtypes of nonpyramidal cells in wild-type rats, including previously recorded and stained cells by Karube et al. (2004), and also newly reconstructed cells (Fig. 9; 64 cells of 10 subtypes in layer II/III and 29 cells of 6 subtypes in layer V). These measurements suggest that, whereas nonpyramidal cell subtypes are different in their preferences for axonal innervation of somata, more than half of GABAergic boutons contact domains other than soma, even in basket cells. The somatic apposition proportions of LS neurogliaform cells were found to be more variable in wild-type rats.

We compared the horizontal spread of dendrites among the 6 GABAergic cell subtypes previously described by Kawaguchi et al. (2006) (Fig. 10). The mean horizontal length of dendrites (as measured from soma to uncut endings) of the layer V FS basket cell was 117 ± 68 µm (249 true endings from 14 cells) in Venus rats, and 109 ± 66 µm (215 from 15 cells) in tissue from wild-type rats (Fig. 10A). Horizontal dendritic distances of layer V somatostatin Martinotti cells were similar, being 109 ± 55 µm (154 from 8 cells). In layer II/III, dendrites of CCK large basket cells had a longer horizontal reach than dendrites in LS neurogliaform, double bouquet, non-FS small basket, FS basket, and somatostatin Martinotti cells (P < 0.01; Fig. 10B). Horizontal dendritic distances of layer V somatostatin Martinotti cells were similar (206 ± 50 µm, 8 cells). In layer II/III, CCK large basket cells had a larger maximum horizontal spread than LS neurogliaform, non-FS small basket, double bouquet, and FS basket cells (P < 0.01; Fig. 10C).

These observations from wild-type rats, combined with those from VGAT-Venus rats, support the normal differentiation of cortical GABAergic cells in VGAT-Venus rats, and demonstrate that α-actinin-2 is a useful chemical marker for a distinct cortical interneuron subtype. Further, they suggest a cortical interneurons maintain an inherent, cell type-specific horizontal dendritic territory.

Notes

The authors thank Ms M. Saito and Mr Y. Ito for technical assistance, and Drs A. Gulledge, Y. Kubota and M. Morishima for discussion and comments. We also thank staffs in Center for Experimental Animals, National Institute for Physiological Sciences (NIPS). We are grateful to Dr A. Miyawaki for providing pCS2-Venus, to Dr M. D. Lalioti for pKOV-Kan and pDF25 plasmids, to Dr A.H. Beggs for an antiserum against α-actinin-2, and to Dr N. Tamamaki for a rabbit antiserum against eGFP. We are also grateful to Dr T. Kosaka for helpful discussions about Venus expression patterns. This work was supported by Grant-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and on Psychiatric and Neurological Diseases and Mental Health from the Ministry of Health, Labor and Welfare of Japan, and also supported by The Uehara Memorial Foundation and Brain Science Foundation. This paper is dedicated to the late Ms Kazuko Kawaguchi who had supported Y.K. continuously.

Conflict of Interest: None declared.

Address correspondence to Yasuo Kawaguchi, Division of Cerebral Circuitry, National Institute for Physiological Sciences, Myodaiji, Okazaki 444-8585, Japan. Email: yasuo@nips.ac.jp, or to Yuchio Yanagawa, Department of Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan. Email: yanagawa@med.gunma-u.ac.jp.

References


