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Title

Expression of Gap Junction Protein Connexin36 in Multiple Subtypes of GABAergic Neurons in Adult Rat Somatosensory Cortex

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Abbreviated Title: Cx36 expression in GABAergic neurons in S1 cortex

Abstract

To characterize Connexin36 (Cx36)-expressing neurons of the adult rat somatosensory cortex, we examined fluorescence signals for Cx36 mRNA in three non-overlapping subpopulations of GABAergic interneurons, which showed immunoreactivity for 1) parvalbumin (PV); 2) somatostatin (SOM); and 3) either calretinin (CR), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK) or choline acetyltransferase (ChAT). About 80% of PV-, 52% of SOM-, 37% of CR/VIP/CCK/ChAT-immunoreactive cells displayed Cx36 signals across all cortical layers, and inversely 64%, 25% and 9% of Cx36-expressing neurons were positive for PV, SOM or CR/VIP/CCK/ChAT, respectively. Notably, although almost all Cx36-expressing neurons in layer (L) 4, L5 and L6 were positive for one of these markers, a substantial proportion of those in L1 (90%) and L2/3 (10%) were negative for the markers tested, suggesting that other types of neurons might express Cx36. We further investigated the colocalization of Cx36 mRNA and α -actinin2 immunoreactivity, as a marker for late-spiking GABAergic neurons, by using mirror-image sections. Surprisingly, more than 77% of α -actinin2-positive cells displayed Cx36 signals in L1–L3, and about 50% and 13% of Cx36-expressing neurons were positive for α -actinin2 in L1 and L2/3, respectively. These findings suggest that all the subtypes of GABAergic interneurons might form gap junctions in the neocortex.

Keywords: double labeling; immunofluorescence; *in situ* hybridization; interneuron; neocortex.

Introduction

Gap junctions, intercellular transmembrane channels, permit direct passage of ions and molecules smaller than 1000 Da between adjacent cells, and thereby metabolic and electrical activities of the contacting cells are coupled through gap junction channels. Each channel consists of two hemichannels, each of which is composed of six connexin (Cx) or pannexin protein subunits. Connexins and pannexins are encoded by a multigene family with at least 20 and 3 members, respectively, in mice and humans (for review, see Söhl et al. 2005) and, in the case of connexins, commonly named by their predicted molecular weights in kDa (e.g., Cx36). Of this large gene family, only a few members are expressed by neurons in the adult mammalian cortex: Cx30.2, Cx36, Cx45, pannexin 1 and pannexin 2 (for review, see Söhl et al. 2005; Kreuzberg et al. 2008). It has been reported that Cx36 and Cx30.2 are expressed in interneurons, whereas Cx45 and pannexins are principally expressed in pyramidal neurons in the neocortex (Belluardo et al. 2000; Deans et al. 2001; Bruzzone et al. 2003; Maxeiner et al. 2003; Degen et al. 2004; Vogt et al. 2005; Kreuzberg et al. 2008).

Electrophysiological studies have shown that the electrical coupling/synapse of neurons through gap junctions is mostly found between cortical GABAergic interneurons in the adult (Galarreta and Hestrin 2002; Meyer et al. 2002) and juvenile rodent neocortex (Galarreta and Hestrin 1999; Gibson et al. 1999, 2005; Tamás et al. 2000; Blatow et al. 2003; Chu et al. 2003; Galarreta et al. 2004; Simon et al. 2005; Mancilla et al. 2007). Since the electrical coupling is not

found between pyramidal neurons in those neocortex (Galarreta and Hestrin 1999), Cx45 and pannexins are unlikely to serve as gap junction proteins in the adult cortex. Furthermore, pannexins have recently been reported to have various non-junctional functions including release mechanism of ATP (for review, see MacVicar and Thompson 2010; D'hondt et al. 2011). Thus, both Cx36 and Cx30.2 might be important for electrical coupling of cortical neurons. Actually, electrical coupling between interneurons in the neocortex and hippocampus was nearly absent or profoundly reduced in Cx36-deficient mice (Deans et al. 2001; Hormuzdi et al. 2001), suggesting that Cx36 is the most important protein for the electrical coupling. Moreover, the depletion of Cx36 gene largely decreases gamma frequency (30–80 Hz) oscillation (Hormuzdi et al. 2001; Buhl et al. 2003), which is believed to be dependent on the electrical coupling between interneurons, whereas Cx30.2 gene disruption does not reduce gamma oscillation in the hippocampus (Kreuzberg et al. 2008). This confirmed that Cx36, but not Cx30.2, is a key protein for cortical interneurons involved in electrical coupling and gamma oscillation.

It is well established by immunohistochemical characteristics for calcium-binding proteins, neuropeptides and neurotransmitters that cortical GABAergic interneurons are divided into several distinct subgroups in the rat neocortex: (1) parvalbumin (PV)-immunoreactive (ir) cells; (2) somatostatin (SOM)-ir cells; (3) calretinin (CR)- and/or vasoactive intestinal polypeptide (VIP)-ir cells, partially positive for cholecystokinin (CCK) or choline acetyltransferase (ChAT) (Eckenstein and Baughman 1984; Kosaka et al. 1987; Rogers 1992; Chédotal et al. 1994 ; Kubota et al. 1994;

Bayraktar et al. 1997; Gonchar and Burkhalter 1997; Kawaguchi and Kubota 1996; Kubota and Kawaguchi 1997; Taki et al. 2000; Kawaguchi and Kondo 2002; Uematsu et al. 2008). Although PV-, SOM-, and CR/VIP/CCK/ChAT-ir subgroups are well segregated from one another, CR, VIP, CCK and ChAT immunoreactivities are more or less colocalized with one another in single neurons except the combination of CCK and ChAT. Furthermore, the frequency of each group of GABAergic neurons in the rat neocortex is different among cortical layers (Kubota et al. 1994; Gonchar and Burkhalter 1997; Uematsu et al. 2008). Thus, for the better understanding of the fundamental roles played by Cx36-based neuronal gap junction in the mature cortical circuitry, it is necessary to identify the types, numbers and laminar distribution of Cx36-expressing neurons in the adult neocortex. It has so far been reported that Cx36 is expressed in about 50–99% of PV-ir neurons in the adult rodent neocortex (Belluardo et al. 2000; Priest et al. 2001; Helbig et al. 2010). In addition, a significant number of SOM-ir neurons express Cx36 in the juvenile mouse neocortex (Deans et al. 2001). However, no information on Cx36 expression is available for the other subgroup(s) of cortical interneurons.

To address this issue, we examined the colocalization of Cx36 mRNA signals and immunoreactivity for GABAergic subgroup markers (PV, SOM, CR, VIP, CCK or ChAT) in each layer of the primary somatosensory (S1) cortex of adult rat by combining fluorescence *in situ* hybridization (FISH) histochemistry and immunofluorescence staining. Since a GABAergic subgroup expressing Cx36 but negative for all the markers tested was found in layer (L) 1 and L2/3,

we further investigated the immunoreactivity for α -actinin2, which was recently reported to be a marker for neocortical neurogliaform cells (Uematsu et al. 2008), by using the method of mirror-image sections.

Materials and Methods

The experiments were conducted in accordance with the Committee for Animal Care and Use and that for Recombinant DNA Study in Kyoto University. Nineteen adult male Wistar rats (200–250 g; Japan SLC, Hamamatsu, Japan) were used in the present study. All efforts were made to minimize animal suffering and the number of animals used.

Tissue Preparation

The rats were deeply anaesthetized with chloral hydrate (70 mg/100 g body weight) and perfused transcardially with 200 mL of 5 mM sodium phosphate (pH 7.4)-buffered 0.9% (w/v) saline (PBS), followed by 300 mL of 4% (w/v) formaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4). The brains were removed, cut into several blocks and postfixed with the same fixative for 3 days at 4°C. After cryoprotection with 30% (w/v) sucrose in diethylpyrocarbonate (DEPC)-treated PBS at 4°C overnight, the blocks were cut into 20- μ m-thick frontal sections on a freezing microtome.

In Situ Hybridization Histochemistry for Bright Field Microscopy

Complementary DNA fragment corresponding to a region of Cx36 (probe 1 for nucleotides 109–1166, probe 2 for 519–1050, probe 3 for 109–679, probe 4 for 519–1489 and probe 5 for 109–1489 of GenBank accession number Y16898.1), glutamate decarboxylase 67 kDa isoform (GAD67; Tamamaki et al. 2003) or vesicular glutamate transporter 1 (VGLUT1; Nakamura et al.

2007) was cloned into pBluescript II SK (+) vector (Stratagene, La Jolla, CA). Sense and antisense single-strand riboprobes were synthesized with a digoxigenin (DIG) or fluorescein isothiocyanate (FITC) RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

The following hybridization procedure was carried out as reported previously (Hioki et al. 2010) with some modifications. Briefly, free-floating sections were hybridized for 16–20 h at 60°C with 1 µg/mL DIG-labeled sense or antisense riboprobes in a hybridization buffer. After washes and ribonuclease A (RNase A) treatment, the sections were incubated overnight with 1:1,000-diluted alkaline phosphatase (AP)-conjugated anti-DIG sheep antibody (11-093-274-910; Roche Diagnostics), and then reacted with 0.375 mg/mL nitroblue tetrazolium and 0.188 mg/mL 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP; Roche Diagnostics) for several hours. Sense probes detected no signal higher than the background.

To detect the signals for Cx36 mRNA sensitively, we applied biotinylated tyramine (BT)-glucose oxidase (GO) amplification method (Furuta et al. 2009; Kuramoto et al. 2009; Ge et al. 2010). Briefly, after hybridization with DIG-labeled Cx36 riboprobe, the sections were incubated with 1:4,000-diluted peroxidase-conjugated anti-DIG sheep antibody (11-207-733-910; Roche Diagnostics). Subsequently, the sections were reacted with a mixture containing 31 µM BT, 3 µg/mL of GO, 2 mg/mL of beta-D-glucose and 2% bovine serum albumin in 0.1 M PB for 30 min. The sections were further incubated with 1:8,000-diluted AP-conjugated streptavidin (02516-71; nacalai tesque) for 2 h, and finally reacted with NBT/BCIP.

Double Fluorescence In Situ Hybridization Histochemistry

For double fluorescence *in situ* hybridization histochemistry (double FISH), sections were hybridized with a mixture of DIG-labeled Cx36 riboprobe and FITC-labeled riboprobe for VGLUT1 or GAD67. After washes and RNase treatment, the hybridized sections were incubated overnight with 1:4,000-diluted peroxidase-conjugated anti-DIG sheep antibody, treated with BT-GO reaction mixture, and incubated with AP-conjugated streptavidin. The sections were further incubated overnight with 1:2,000-diluted peroxidase-conjugated anti-FITC sheep antibody (11-426-346-910; Roche Diagnostics), treated with 1:100-diluted tyramide signal amplification (TSA)-Plus dinitrophenyl (DNP) kit (NEL747A; PerkinElmer, Wellesley, MA) for 30 min, and incubated with 10 µg/ml AlexaFluor488-conjugated anti-DNP rabbit antibody (A-11097; Invitrogen, Eugene, OR) for 2 h, to visualize the signals for VGLUT1 or GAD67 mRNA. Subsequently, the sections were reacted with 2-hydroxy-3-naphtoic acid-2'-phenylamide phosphate (HNPP) Fluorescence Detection kit (HNPP/FastRed TR; 11-758-888-001; Roche Diagnostics) for several hours, to detect the signals for Cx36 mRNA. When the anti-DIG or anti-FITC antibody was omitted for control experiments, no fluorescence signals for the omitted antibody were detected.

Double Labeling with Fluorescence In Situ Hybridization and Immunofluorescence

The sections hybridized with DIG-labeled Cx36 riboprobe were incubated overnight with a mixture of 1:4,000-diluted peroxidase-conjugated anti-DIG sheep antibody and one of the following antibodies: mouse monoclonal antibody against neuron-specific nuclear protein (NeuN; MAB377; Millipore, Temecula, CA; 1:1,000); mouse monoclonal antibody against parvalbumin (PV; P-3088; Sigma; 1:8,000); rabbit polyclonal antibody against somatostatin (SOM; T-4103; Peninsula Laboratories, San Carlos, CA; 1:2,000); rabbit polyclonal antibody against calretinin (CR; C-7479; Sigma; 1:1,000); rabbit polyclonal antibody against vasoactive intestinal polypeptide (VIP; 20077; ImmunoStar, Hudson, WI; 1:1,000); goat polyclonal antibody against choline acetyltransferase (ChAT; AB144P; Millipore; 1:500); rabbit polyclonal antibody to cholecystokinin (CCK; 20078; ImmunoStar; 1:500); or a mixture of the antibodies against CR, VIP, CCK or ChAT. The sections were then incubated for 2 h with 10 µg/mL AlexaFluor488-conjugated antibody against mouse IgG (A-11029; Invitrogen), rabbit IgG (A-11034; Invitrogen) and/or goat IgG (A-11055; Invitrogen). After washes, the sections were treated with BT-GO reaction mixture, and then incubated with AP-conjugated streptavidin. The sections were finally reacted with HNPP/FastRed TR.

Mirror Image Section

To explore the colocalization of Cx36 mRNA signals and α -actinin2 immunoreactivity, we had to circumvent the difficulty of the double labeling with FISH for Cx36 mRNA and

immunofluorescence staining for α -actinin2 by applying the method of mirror-image sections. Double labeling for Cx36 mRNA and NeuN immunoreactivity was performed as described above for the first section of a pair of consecutive mirror-image sections, whereas immunofluorescence staining for α -actinin2 and propidium iodide (PI; 29037-76; nacalai tesque) counterstaining were carried out for the second section. Immunofluorescence labeling for α -actinin2 was performed as follows: the sections were incubated overnight with 1:8,000-diluted mouse monoclonal antibody against α -actinin2 (A7811; Sigma). After washes, the sections were incubated for 1 h with 10 μ g/mL peroxidase-conjugated donkey anti-mouse IgG (AP192P; Millipore), and then reacted with 1:2,000-diluted BT-GO mixture. The sections were finally incubated for 2 h with 5 μ g/ml AlexaFluor488-conjugated streptavidin (S11223; Invitrogen).

Image Acquisition and Cell Counting

The sections stained with NBT/BCIP were mounted onto aminopropyltriethoxysilane (APS)-coated glass slides, dehydrated, cleared with xylene and coverslipped. The micrographs were taken by QICAM FAST digital monochrome camera (QImaging, Surrey, BC, Canada). Fluorescent sections were mounted onto APS-coated glass slides and coverslipped with CC/Mount (K002; Diagnostic BioSystems, Pleasanton, CA) or 50% (v/v) glycerol and 2.5% (w/v) triethylenediamine in PBS. The fluorescent-labeled sections were observed under confocal laser-scanning microscope LSM5 PASCAL (Carl Zeiss, Oberkochen, Germany) with appropriate filter sets for AlexaFluor488

(excitation, 488 nm; emission, 505–530 nm), FastRed and PI (excitation, 543 nm; emission, ≥ 560 nm), using a 40 \times objective lens (Plan-NEOFLUAR, NA = 0.75, Carl Zeiss). Digital images were modified ($\pm 20\%$ contrast and brightness enhancement) in software Canvas X (ACD Systems, Saanichton, BC, Canada) and saved as TIFF files. Neocortical areas and cortical layers were determined by NeuN immunoreactivity or DAPI nuclear staining.

To count the number of cells showing a clear nucleus, we selected at least three sections containing the S1 cortex from three rats. Cell counting was performed in rectangular field (160 $\mu\text{m} \times 1670 \mu\text{m}$) covering the entire layers of the S1 cortex, under the epifluorescence microscope Axiophot (Carl Zeiss) with the appropriate filter sets for AlexaFluor488 (excitation, 450–490 nm; emission, 514–565 nm), FastRed (excitation, 530–585 nm; emission, ≥ 615 nm).

Results

In Situ Hybridization Histochemistry for Cx36 mRNA

We first tested five kinds of DIG-labeled cRNA probes for Cx36 by using *in situ* hybridization (ISH) on tissue of the adult rat S1 cortex (Fig. 1a). The antisense probes detected weak signals (probes 1, 4 and 5; Fig. 1b,e,f) or almost no signals (probes 2 and 3; Fig. 1c,d). To amplify the signals, we then applied the BT-GO method to ISH. This method is based on tyramide-amplification technology and effective in intensification of the signals (for detail, see Kuramoto et al. 2009). The intensity of Cx36 mRNA signals was dramatically improved by using the BT-GO method, especially in probes 1, 4, and 5 (Fig. 1b'–f'). Although these three probes displayed the same signal distributions in the rat forebrain, the signals with probe 5 were the most intense and clear (Fig. 1f'). All the sense probes displayed no signals in the present method (data not shown).

With the antisense probe 5, weak to intense signals for Cx36 mRNA were observed in many regions of the adult rat brain such as the neostriatum, hippocampus, neocortex, hypothalamus, reticular thalamic nucleus and thalamus (Fig. 2a), as reported previously (Condorelli et al. 1998, 2003; Belluardo et al. 2000). In the neocortex including the S1 cortex, scattered cells expressing Cx36 mRNA were detected throughout all layers. Weak and moderate signals for Cx36 mRNA were observed in layer (L) 1 and L2–L6, respectively (Fig. 2b,c,d). In the following experiments, we employed the BT-GO method with probe 5, to visualize Cx36 mRNA signals efficiently.

Specific Expression of Cx36 in GABAergic Neurons

To survey the neuronal expression of Cx36, we performed double labeling experiment by combining FISH for Cx36 mRNA and immunofluorescence for NeuN in the S1 cortex (Fig. 3a–a”). Across cortical layers, almost all the Cx36-expressing cells were immunopositive for NeuN, indicating that Cx36 expression is restricted to neurons of the neocortex (Table 1). Inversely, Cx36-expressing neurons constituted about 10% of NeuN-ir cells in entire layers. This proportion varied much among layers: highest in L1 (32.6%) and lowest in L6 (6.1%; Table 1).

By double FISH for Cx36 and GAD67 or VGLUT1 mRNAs, we then investigated the expression of Cx36 in GABAergic or glutamatergic neurons in the S1 cortex (Fig. 3b–c”; Table 1). Across all layers, Cx36 was expressed in about half (54.5%) of GAD67-producing GABAergic neurons. As to L1, 33% of GAD67-producing neurons showed the signals for Cx36 mRNA, which proportion is very similar to the percentage of NeuN-ir neurons expressing Cx36 mRNA (see above). This, together with the absence of VGLUT1 mRNA in L1 (data not shown), supports the general notion that neurons in L1 of the neocortex are exclusively GABAergic interneurons (for review, see Markram et al. 2004). From L2/3 to L6, 52.8–66.3% of GAD67-expressing neurons showed the signals for Cx36 mRNA. Inversely, almost all (97.7%) of Cx36-expressing neurons were positive for GAD67 mRNA signals across all layers, especially in L1 (100%). By contrast, very few VGLUT1-producing neurons (0.2%) showed Cx36 mRNA signals in L2–L6, and conversely a small number (1.3%) of Cx36-expressing neurons were positive for VGLUT1 mRNA

signals. These results indicate that Cx36 was expressed almost specifically in GABAergic neurons of the adult rat neocortex.

Distribution and Proportion of GABAergic Neuron Subpopulations in the S1 Cortex

Cortical GABAergic neurons can be divided into three groups by chemical markers: (1) PV-ir cells; (2) SOM-ir cells; (3) CR-, VIP-, CCK- and/or ChAT-ir cells. These three groups constituted distinct subgroups, and we found that none of them overlapped with each other in the adult rat S1 cortex (data not shown). The distribution and proportion of those cells in GABAergic neurons were explored by combining FISH for GAD67 mRNA and immunofluorescence for PV, SOM, or CR/VIP/CCK/ChAT (a combination of CR, VIP, CCK and ChAT; Supplementary Fig. 1). Across entire layers, PV-, SOM- or CR/VIP/CCK/ChAT-ir cells accounted for 40.7%, 23.1% or 17.7% of GAD67-producing neurons, respectively (Supplementary Table 1). The three subgroups collectively covered 87.9%, 97.9%, 88.2% and 82.5% of the total GABAergic populations in L2/3, L4, L5 and L6, respectively. In L1, however, PV- or SOM-ir cells were not found and CR/VIP/CCK/ChAT-ir cells comprised only 21.6% of GAD67-producing neurons. This suggests that other types of neurons constitute the majority of GABAergic neurons in L1.

Expression of Cx36 mRNA in PV- or SOM-Containing Neurons

Since PV- and SOM-containing cells constitute two main distinct groups of cortical GABAergic neurons, we first examined the colocalization of Cx36 mRNA signals with PV or SOM immunoreactivity in the S1 cortex (Fig. 4a–b”; Table 2). About 80% of PV-ir cells showed the signals for Cx36 mRNA across L2–L6. Inversely, 55.8%, 77.0%, 63.9% and 65.7% of Cx36-expressing neurons were immunoreactive for PV in L2/3, L4, L5 and L6, respectively. About 52% of SOM-ir cells displayed the signals for Cx36 mRNA across L2–L6. Conversely, 17.0%, 24.4%, 29.4% and 30.3% of Cx36-expressing neurons were positive for SOM in L2/3, L4, L5 and L6, respectively.

Notably, we found that about 64% and 25% of Cx36-expressing neurons in entire layers were immunoreactive for PV and SOM, respectively, indicating that a substantial proportion (11%) of Cx36-expressing neurons are negative for PV or SOM, especially in L1 (100%) and L2/3 (27%; Table 2).

Expression of Cx36 mRNA in the Third Group of GABAergic Neurons

We then explored the expression of Cx36 mRNA in the third group of GABAergic neurons immunoreactive for CR, VIP, CCK and/or ChAT in the S1 cortex (Fig. 4c–f”; Table 3). Cx36 was expressed in 24.2% of CR-, 8.9% of VIP-, 31.9% of CCK- and 21.6% of ChAT-ir neurons across all layers. Inversely, 5.3%, 4.4%, 2.1% or 2.1% of Cx36-expressing neurons were

immunopositive for CR, VIP, CCK or ChAT, respectively, across all layers. In L2/3, the colocalization frequency was slightly higher than the other layers in VIP-, CCK- or ChAT-ir cells. In L1, although the colocalization of Cx36 mRNA signals and CCK immunoreactivity was observed, no colocalization was detected in CR-, VIP- or ChAT-ir cells.

Since it is well known that CR, VIP, CCK and ChAT immunoreactivities are frequently colocalized with each other as described in the Introduction, we also examined the colocalization of Cx36 mRNA signals and CR/VIP/CCK/ChAT immunoreactivity in the S1 cortex (Fig. 4g–g”; Table 3). In entire layers, 36.8% of CR/VIP/CCK/ChAT-ir cells showed the signals for Cx36 mRNA. Inversely, 9.2% of Cx36-expressing neurons were immunoreactive for CR/VIP/CCK/ChAT across all layers. In L2/3, a large population (17.6%) of Cx36-expressing neurons were immunoreactive for CR/VIP/CCK/ChAT as compared with the other layers (5.9–7.4%).

Together, more than 90% and nearly 10% of Cx36-expressing neurons in L1 and L2/3, respectively, were not labeled by any of the above six markers, although almost all of the Cx36-expressing neurons were immunopositive for PV, SOM or CR/VIP/CCK/ChAT in L4–6 (Tables 2 and 3; Fig. 5). This leaves the possibility that other types of GABAergic neurons, which are not included in any of the major three subgroups, may express Cx36 in L1–3.

Expression of Cx36 mRNA in Alpha-Actinin2-Containing Interneurons

It was recently reported that α -actinin2 is one of the chemical markers for cell bodies of late-spiking neurogliaform cells in the hippocampus (Price et al. 2005) and neocortex (Uematsu et al. 2008), although α -actinin2 immunoreactivity was also located in the dendrites of pyramidal cells (Wyszynski et al. 1998). The chemical, morphological, and physiological characteristics suggest that late-spiking neurogliaform cells constitute a distinct subgroup of cortical GABAergic neurons from the three groups examined above (Tamás et al. 2003; Price et al. 2005; Uematsu et al. 2008).

To determine whether Cx36 is expressed in α -actinin2-containing GABAergic neurons, we investigated the colocalization of Cx36 mRNA signals with α -actinin2 immunoreactivity in L1 and L2/3, where a substantial proportion of Cx36-expressing neurons were negative for all the other markers tested (see above). Neurons coexpressing Cx36 mRNA and α -actinin2 were identified using pairs of consecutive mirror-image sections (Fig. 6; Table 4). In preliminary studies, we tried direct double labeling methods to detect the coexpression of α -actinin2 and Cx36. However, α -actinin2 immunoreactivity was completely lost after ISH probably because of heat denaturation of the antigenic sites. Although the double FISH method was tried to detect α -actinin2 mRNA signals simultaneously with Cx36 signals as applied to discern GAD67 or VGLUT1 signals (Fig. 3), α -actinin2 mRNA signals were almost undetectable even with the present sensitive technique. Thus, we had to develop the complicated mirror-image section method. About 50% and 13% of Cx36-expressing neurons were immunoreactive for α -actinin2 in L1 and L2/3, respectively.

Inversely, 84.4% and 77.6% of α -actinin2-ir cells showed the signals for Cx36 mRNA in L1 and L2/3, respectively. This result suggests that most of α -actinin2-containing neurogliaform cells might form gap junctions with Cx36 channels at least in the superficial layers of the adult rat neocortex.

We further examined whether or not α -actinin2 constituted a GABAergic neuron group segregated from the other three groups in the S1 cortex by using a double immunofluorescence technique (Supplementary Fig. 2; Supplementary Table 2). It was first shown that all α -actinin2-ir neurons showed immunoreactivity for GABA, constituting 62% and 12% of GABAergic neurons in L1 and L2/3, respectively. However, no PV or SOM immunoreactivity was observed in α -actinin2-ir neurons, and CR/VIP/CCK/ChAT immunoreactivity was found in only 1.7% of α -actinin2-ir neurons. A very recent work also confirmed that the α -actinin2-ir neuron group was segregated completely from PV-ir and SOM-ir groups and mostly from CR/VIP/CCK-ir group in the rat frontal cortex (Kubota et al. 2011).

Discussion

In the present study, we investigated the neurochemical profiles and laminar distribution of Cx36-expressing neurons in the adult rat S1 cortex by combining immunofluorescence method with the highly sensitive FISH technique, the specificity of which was discussed in the Supplementary Material (see Supplementary Comment). Across all neocortical layers, Cx36 was expressed almost exclusively by GABAergic neurons. Cx36 expression was detected in 55% of GAD67-expressing GABAergic neurons, including 80% of PV-ir neurons, 52% of SOM-ir neurons and 37% of CR/VIP/CCK/ChAT-ir neurons. Inversely, 64%, 25%, or 9% of Cx36-expressing neurons were positive for PV, SOM or CR/VIP/CCK/ChAT, respectively. Notably, although, in L4–6, almost all Cx36-expressing neurons were positive for PV, SOM or CR/VIP/CCK/ChAT, a substantial proportion of Cx36-expressing neurons in L1 (90%) and L2/3 (10%) were negative for those chemical markers. It was further shown that a significant number of Cx36-expressing neurons in L1 (50%) and L2/3 (12%) were positive for α -actinin2, and conversely that more than 77% of α -actinin2-ir neurons were positive for Cx36 mRNA signals in L1–3. Thus, the present study provides the anatomical evidence that at least four subgroups of GABAergic interneurons expressing PV, SOM, CR/VIP/CCK/ChAT or α -actinin2 may apply Cx36 to form gap junctions within neocortical local circuits of the adult rat.

Cx36 Expression in PV- or SOM-Containing GABAergic Interneurons

Gap junctions have been electron-microscopically identified between PV-ir dendrites in the mammalian neocortex (Tamás et al. 2000; Fukuda and Kosaka 2003; Fukuda et al. 2006), and Cx36 immunoreactivity was detected at the gap junctions (Fukuda et al. 2006). The studies on the Cx36 expression of PV-containing neurons in the mammalian neocortex have supported those electron-microscopic observations (Belluardo et al. 2000; Deans et al. 2001; Priest et al. 2001; Degen et al. 2004; Helbig et al. 2010). From these findings, it is expected that PV-containing cortical interneurons, which are well known to have fast-spiking characteristics in the neocortex (for review, see Burkhalter 2008), show electrical coupling with one another. Actually, two neighboring fast-spiking neurons have been reported to exhibit electrical coupling at the rate of 57–66% in the rat neocortex (Galarreta and Hestrin 1999; Gibson et al. 1999, 2005; Beierlein et al. 2000). When the expression probability of Cx36 is p in PV/fast-spiking interneurons, the maximum probability of electrical coupling formation between two neighboring PV/fast-spiking neurons is p^2 . Since the maximum probability p^2 was more than 0.57–0.66 in the previous reports, p should be 0.75–0.81 and over. The present results of “ $p = 0.8$ ” is, therefore, well compatible with the electrical coupling efficiency reported between PV/fast-spiking interneurons.

In addition to PV-containing neurons, only SOM-producing cortical interneurons have so far been reported to express Cx36 gene. Deans et al. (2001) reported that 27% of SOM-ir neurons expressed Cx36 in L4 of the juvenile mouse S1 cortex by using the beta-galactosidase reaction of

lacZ reporter gene, which was inserted into the Cx36 gene. However, in the adult mouse cortex, Cx36 expression was reported to be almost restricted to PV-ir neurons by using the same knock-in technique (Degen et al. 2004). These findings were very different from the present finding that large percentage (48.0–65.4%) of SOM-ir neurons showed Cx36 mRNA signals in the adult rat S1 cortex. Because the lacZ knock-in technique is generally thought to be more sensitive than the ISH technique (Hormuzdi et al. 2004), the species and/or age difference is likely to be the main cause of this difference.

The SOM-producing GABAergic interneuron group is known to include many Martinotti cells, which characteristically send ascending axon fibers to L1 of the neocortex (Kawaguchi and Kubota 1996, 1998; Goldberg et al. 2004; Karube et al. 2004). This together with the present results suggests that Martinotti cells form gap junction networks like fast-spiking cells at least in the rat cerebral cortex. Actually, electrically coupled cortical interneurons appeared to contain some Martinotti cells in the rat cortex (Fig. 2a in Venance et al. 2000) and, in a recent report, a subset of SOM-producing Martinotti cells formed electrically coupled network even in the mouse cortex (Fanselow et al. 2008).

Cx36 Expression in CR-, VIP-, CCK- or ChAT-Containing GABAergic Interneurons

The present study revealed that, although Cx36 expression was almost limited to GABAergic interneurons in the neocortex, a substantial number of Cx36-expressing interneurons were negative

for PV or SOM (Fig. 5), suggesting the presence of other subgroups of interneurons that were connected by electrical coupling. Cx36 was expressed in 24% of CR-ir, 9% of VIP-ir, 32% of CCK-ir, 22% of ChAT-ir cortical neurons (Table 3). Although these expression rates were lower than those of PV-ir and SOM-ir interneuron groups (80 and 52%, respectively), these results suggest that the electrical coupling mechanism with Cx36 might be ubiquitously utilized by cortical interneurons.

In L2/3 of the rat S1 cortex, some non-pyramidal neurons of regular-spiking type were reported to communicate with one another by electrical coupling (Szabadics et al. 2001), and some CR-containing, VIP-producing, CCK-producing and cholinergic neurons showed regular-spiking characteristics (Kawaguchi Kubota 1996, 1998; von Engelhardt et al. 2007). Thus, these L2/3 interneurons may be electrically coupled using Cx36, although regular-spiking characteristics were observed in many L2/3 SOM-producing neurons of the neocortex (Kawaguchi and Kubota 1996, 1998). This speculation is partly supported by a recent report that CR-expressing cortical neurons frequently formed electrical coupling with one another or with multipolar bursting interneurons (Caputi et al. 2009).

Irregular-spiking neurons expressing cannabinoid receptor-1 in L2/3 of the neocortex were also reported to form electrical coupling with one another at a high rate of 90% (Galarreta et al. 2004). Cannabinoid receptors were found in 33–80% of CCK-ir neurons and 3% of VIP-ir neurons in L2/3 of the S1 cortex (Marsicano and Lutz 1999; Bodor et al. 2005), but not in CR-expressing neurons

(Marsicano and Lutz 1999). As irregular-spiking characteristics were observed in many VIP-expressing neurons, but not in CCK-expressing neurons (Cauli et al. 1997; Uematsu et al. 2008), VIP-ir neurons expressing Cx36 are candidates for irregular-spiking neurons with electrical coupling.

Cx36 Expression in Alpha-Actinin2-Containing Neurogliaform Cells

It is rather well established that most late-spiking interneurons are neurogliaform cells in the rat neocortex (Kawaguchi 1995; Tamás et al. 2003; Karube et al. 2004; Simon et al. 2005; Povysheva et al. 2007; Uematsu et al. 2008), although a few late-spiking cells had a shape of basket cells (Karube et al. 2004). Late-spiking cells have been reported to form electrical coupling with one another at a high rate in L1 (83%; Chu et al. 2003) and in L2/3 (50%; Simon et al. 2005), and with other types of interneurons at a lower rate, such as fast-spiking and regular-spiking interneurons in L2/3 of the rat neocortex (20%; Simon et al. 2005). Recently, it was reported that many late-spiking neurogliaform cells were immunoreactive for α -actinin2 in L2/3 and L5 of the rat neocortex (88%; Uematsu et al. 2008). Thus, to our knowledge, the present result on the frequent colocalization of Cx36 mRNA signals in α -actinin2-ir cells (80%; Table 4) is the first line of evidence revealing that Cx36 protein may participate in the electrical coupling of neurogliaform cells.

Functional Implications of Neuronal Gap Junctions in the Cortical Circuitry

The present results indicate that, although Cx36 is restrictively expressed in GABAergic interneurons in the cerebral cortex, all the four GABAergic subgroups utilize Cx36 presumably for electrical coupling. The disruption of Cx36 gene, followed by the disappearance of electrical coupling between interneurons (Deans et al. 2001), is known to change responsiveness of the cortical and hippocampal network, and result in a form of memory impairment (Frisch et al. 2005):

- 1) A large decrease in gamma frequency oscillation was observed in the cortical and hippocampal slices (Hormuzdi et al. 2001; Maier et al. 2002) and in the hippocampus *in vivo* (Buhl et al. 2003);
- 2) Sharp membrane fluctuation of the membrane potential, which was induced by the application of metabotropic glutamate receptor agonist, in some interneurons was abolished in the neocortical slices (Deans et al. 2001).
- 3) Spontaneous sharp wave-burst discharges were produced in the hippocampal slices (Pais et al. 2003); and
- 4) Very fast oscillations (> 200 Hz) evoked by microstimulation were strongly enhanced in the cerebral cortex *in vivo* (Butovas et al. 2006).

Thus, electrical coupling with Cx36 is an important factor for the normal responsiveness of the cortical and hippocampal circuitry, as theoretically assumed (Sherman and Rinzel 1992; Chow and Kopell 2000; Traub et al. 2001; Pfeuty et al. 2003). However, since the present study showed that all the four GABAergic subgroups expressed Cx36 mRNA, it is unclear which subgroup is responsible for those disturbances in the cortical or hippocampal circuit of Cx36-disrupted rodents. To answer this question, we need a new technique, such as the conditional knock-out method.

Cortical interneurons have been revealed to frequently form homologous electrical coupling with the interneurons belonging to the same subgroup (Galarreta and Hestrin 1999; Gibson et al. 1999, 2005; Beierlein et al. 2000; Deans et al. 2001; Chu et al. 2003). However, since Cx36 hemichannels produced in a subgroup of interneurons can form homotypic gap junctions with Cx36 hemichannels expressed in a different subgroup of interneurons (for review, see Söhl et al. 2005) and since all the four interneuron subgroups in the neocortex have been shown to more or less express Cx36 in the present study, it is expected that heterologous Cx36 electrical coupling is formed between different subgroups of cortical interneurons. Thus, it is an interesting question what kind of molecular mechanisms allow such mostly selective coupling within a interneuron subgroup. There could be participation of subgroup-specific homophilic adhesion molecules in the formation of homologous electrical coupling.

However, a recent work showed that neocortical neurogliaform interneurons in L2/3 frequently established the heterologous electrical coupling with several types of interneurons such as fast-spiking basket cells and regular-spiking non-pyramidal cells, although less frequently than the homologous coupling (Simon et al. 2005). A more recent study further revealed that CR-expressing multipolar cells in L2/3 preferred multipolar bursting cells, which was known to produce PV and calbindin (Blatow et al. 2003), to themselves as a counter part of the electrical coupling (Caputi et al. 2009). In addition, electron-microscopic studies showed the gap junctions formed between PV-positive and PV-negative dendrites in the neocortex of the adult rat (Fukuda

and Kosaka 2003) and cat (Fukuda et al. 2006). Thus, Cx36 expression in all the four GABAergic subgroups and the relative abundance of CR/VIP/CCK/ChAT- and α -actinin2-ir neurons in L1–3 (Supplementary Tables 1; Kubota et al. 2011) may support the frequent occurrence of heterologous electrical coupling between L1–3 interneurons, which may have a different role from homologous electrical coupling in cortical circuitry.

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Table 1. Colocalization of Cx36 mRNA with NeuN-ir, GAD67 mRNA or VGLUT1 mRNA in the S1 cortex.

Layer	NeuN		GAD67		VGLUT1	
	Cx36/NeuN	NeuN/Cx36	Cx36/GAD67	GAD67/Cx36	Cx36/VGLUT1	VGLUT1/Cx36
1	32.6 ± 1.8 ^a	100.0 ± 0.0	33.1 ± 3.3	100.0 ± 0.0	— ^c	0
	(67/207) ^b	(67/67)	(93/291)	(93/93)	—	(0/40)
2/3	12.8 ± 0.8	99.6 ± 0.6	54.1 ± 2.5	97.3 ± 0.9	0.33 ± 0.03	1.85 ± 0.31
	(240/1883)	(240/241)	(507/941)	(507/521)	(7/2140)	(7/376)
4	8.5 ± 0.6	100.0 ± 0.0	66.3 ± 3.5	99.7 ± 0.1	0.05 ± 0.03	0.17 ± 0.30
	(173/2022)	(173/173)	(494/748)	(494/495)	(1/2266)	(1/341)
5	15.8 ± 0.6	99.2 ± 0.7	52.7 ± 0.4	97.4 ± 1.3	0.23 ± 0.06	1.19 ± 0.44
	(232/1472)	(232/234)	(562/1065)	(562/577)	(4/1662)	(4/330)
6	6.1 ± 0.2	99.6 ± 0.7	55.4 ± 6.7	96.2 ± 0.4	0.24 ± 0.04	1.88 ± 0.15
	(215/3523)	(215/216)	(526/961)	(526/547)	(8/3323)	(8/422)
Total	10.2 ± 0.2	99.6 ± 0.2	54.5 ± 1.4	97.7 ± 0.6	0.21 ± 0.02	1.30 ± 0.12
	(927/9107)	(927/931)	(2179/4006)	(2179/2233)	(20/9391)	(20/1509)

^aData are given as mean ± SD of the percentages in three rats.

^bThe denominator and numerator in parentheses are the total number of cells showing immunoreactivity for individual chemical marker or expressing Cx36 mRNA and that of Cx36-expressing neurons positive for individual marker, respectively, in three rats.

^cVGLUT1-expressing cells were not found in L1.

Table 2. Colocalization of Cx36 mRNA with PV-ir or SOM-ir in the S1 cortex.

Layer	PV		SOM	
	Cx36/PV	PV/Cx36	Cx36/SOM	SOM/Cx36
1	— ^a	0	—	0
	—	(0/69)	—	(0/73)
2/3	84.3 ± 10.4 ^b	55.8 ± 9.1	48.0 ± 10.9	17.0 ± 1.3
	(501/591) ^c	(501/923)	(170/381)	(170/991)
4	81.0 ± 8.8	77.0 ± 6.7	65.4 ± 14.3	24.4 ± 6.6
	(672/822)	(672/886)	(198/314)	(198/868)
5	73.3 ± 3.1	63.9 ± 10.1	51.0 ± 7.8	29.4 ± 3.5
	(628/857)	(628/998)	(332/660)	(332/1150)
6	83.9 ± 5.4	65.7 ± 8.5	50.0 ± 1.1	30.3 ± 3.4
	(591/703)	(591/914)	(278/557)	(278/915)
Total	80.2 ± 6.5	64.2 ± 8.3	52.1 ± 5.0	24.9 ± 1.7
	(2392/2973)	(2392/3790)	(978/1912)	(978/3997)

^aPV- or SOM-positive cells were not found in L1.

^bData are given as mean ± SD of the percentages in three rats.

^cThe denominator and numerator in parentheses are the total number of cells showing immunoreactivity for individual chemical marker or expressing Cx36 mRNA and that of Cx36-expressing neurons positive for individual marker, respectively, in three rats.

Table 3. Colocalization of Cx36 mRNA with CR-, VIP-, CCK- or ChAT-ir in the S1 cortex.

Layer	CR		VIP		CCK		ChAT		CR/VIP/CCK/ChAT	
	Cx36	CR	Cx36	VIP	Cx36	CCK	Cx36	ChAT	Cx36/	(CR/VIP/CCK/ChAT)
	/CR	/Cx36	/VIP	/Cx36	/CCK	/Cx36	/ChAT	/Cx36	(CR/VIP/CCK/ChAT)	/Cx36
1	0 ^a	0	0	0	41.1 ± 8.3	6.1 ± 1.9	0	0	3.6 ± 4.8	7.4 ± 1.1
	(0/15) ^b	(0/64)	(0/11)	(0/59)	(5/12)	(5/83)	(0/7)	(0/68)	(5/17)	(5/70)
2/3	25.8 ± 11.1	6.7 ± 2.8	15.8 ± 0.9	13.8 ± 1.5	54.9 ± 8.9	5.4 ± 0.9	33.8 ± 4.7	5.3 ± 1.3	5.9 ± 7.1	17.6 ± 2.8
	(65/232)	(65/906)	(90/571)	(90/652)	(56/102)	(56/1039)	(46/138)	(46/896)	(75/165)	(75/425)
4	23.9 ± 5.8	3.6 ± 1.7	4.5 ± 2.1	1.5 ± 0.5	7.5 ± 6.6	0.2 ± 0.2	12.6 ± 2.7	0.8 ± 0.1	30.2 ± 5.3	5.9 ± 1.7
	(28/112)	(28/765)	(11/251)	(11/725)	(2/21)	(2/858)	(7/57)	(7/889)	(19/64)	(19/337)
5	21.4 ± 2.6	5.2 ± 1.8	4.8 ± 1.2	2.0 ± 1.2	13.8 ± 4.8	0.7 ± 0.3	17.6 ± 1.9	1.7 ± 0.3	29.8 ± 3.4	6.4 ± 0.7
	(53/240)	(53/990)	(12/250)	(12/682)	(7/48)	(7/943)	(16/91)	(16/967)	(27/92)	(27/428)
6	27.3 ± 0.1	5.8 ± 0.5	2.2 ± 0.7	0.9 ± 0.1	20.2 ± 5.1	1.8 ± 0.1	13.4 ± 1.2	1.0 ± 0.3	33.9 ± 3.8	6.3 ± 0.5
	(50/183)	(50/879)	(6/257)	(6/703)	(15/75)	(15/809)	(9/68)	(9/894)	(26/80)	(26/406)
Total	24.2 ± 4.7	5.3 ± 1.6	8.9 ± 0.1	4.4 ± 1.3	31.9 ± 5.2	2.1 ± 0.1	21.6 ± 0.8	2.1 ± 0.3	36.8 ± 2.4	9.2 ± 1.1
	(196/782)	(196/3604)	(119/1340)	(119/2821)	(85/251)	(85/3732)	(78/361)	(78/3714)	(152/418)	(152/1666)

^aData are given as mean ± SD of the percentages in three rats.

^bThe denominator and numerator in parentheses are the total number of cells showing immunoreactivity for individual chemical marker or expressing Cx36 mRNA and that of Cx36-expressing neurons positive for individual marker, respectively, in three rats.

Table 4. Colocalization of Cx36 mRNA with α -actinin2-ir in L1 or L2/3 of the S1 cortex.

Layer	Cx36/ α -actinin2	α -actinin2/Cx36
1	84.4 \pm 6.5 ^a (25/30) ^b	48.9 \pm 5.0 (25/52)
2/3	77.6 \pm 4.2 (32/41)	12.7 \pm 1.4 (32/246)

^aData are given as mean \pm SD of the percentages in three rats.

^bThe denominator and numerator in parentheses are the total number of cells showing α -actinin2 immunoreactivity or expressing Cx36 mRNA and that of Cx36-expressing neurons positive for α -actinin2, respectively, in three rats.

Figure legends

Figure 1. Optimization of *in situ* hybridization histochemistry for Cx36 mRNA. (a) Schematic representation of Cx36 mRNA and riboprobes examined in the present study. Five kinds of riboprobes were synthesized with a digoxigenin RNA labeling kit. (b–f) Five antisense probes (P1–P5) were tested in L2–5 of the adult rat neocortex with or without the BT-GO method, and the signals were visualized with NBT/BCIP. Without the BT-GO method, the probes detected only a few weak signals (b,e,f) or almost no signals (c,d). In contrast, the signals were remarkably improved with the BT-GO method (b'–f'), especially in probe 5 (f'). Sense probes detected no signals higher than the background. CDS, coding sequence; UTR, untranslated region. Scale bar = 50 μm .

Figure 2. Distribution of Cx36 mRNA visualized by using the BT-GO method with NBT/BCIP in the coronal section of the adult rat forebrain. (a) Low-magnification microphotograph showing Cx36 mRNA signals in the various brain regions: CPu, caudate-putamen; CTX, cerebral cortex; Hip, hippocampus; Hy, hypothalamus; RT, reticular thalamic nucleus; TH, thalamus. (b) Cx36 mRNA signals in the S1 cortex. (c,d) Higher-magnification micrographs illustrating the signals for Cx36 mRNA in L1 (c) and L2/3 (d). Scale bars = 1 mm in a; 500 μm in b; 50 μm in d (applies to c,d).

Figure 3. Double labeling for Cx36 mRNA and NeuN immunoreactivity, GAD67 mRNA or VGLUT1 mRNA in the S1 cortex. (*a-a''*) Cx36 mRNA and NeuN immunoreactivity were visualized with FastRed (magenta) and AlexaFluor488 (green), respectively. Almost all of the Cx36-expressing neurons were immunoreactive for NeuN (arrowheads in *a-a''*). (*b-c''*) Cx36 and GAD67 or VGLUT1 mRNAs were visualized with FastRed (magenta) and AlexaFluor488 (green), respectively. The colocalization of the signals for Cx36 and GAD67 mRNAs was frequently observed (arrowheads in *b-b''*) across all layers, and only a few Cx36-expressing neurons were negative for GAD67 mRNA signals (arrows in *b-b''*). In contrast, almost no colocalization of Cx36 and VGLUT1 mRNA signals was observed (*c-c''*). Scale bar = 20 μ m.

Figure 4. Chemical characterization of Cx36-expressing neurons in the S1 cortex. (*a-g''*) Cx36 mRNA and immunoreactivity for chemical markers were visualized with FastRed (magenta) and AlexaFluor488 (green), respectively. The signals for Cx36 mRNA were colocalized with the immunoreactivity for PV (*a-a''*), SOM (*b-b''*), CR (*c-c''*), VIP (*d-d''*), CCK (*e-e''*), ChAT (*f-f''*) or CR/VIP/ChAT/CCK (*g-g''*) in the S1 cortex. Arrowheads in *a-g''* indicate the colocalizations. CR/VIP/ChAT/CCK, immunostained with a combination of CR, VIP, ChAT and CCK antibodies. Scale bar = 20 μ m.

Figure 5. A summary diagram showing relative population sizes of PV-, SOM-, CR-, VIP-, CCK-, ChAT-producing and Cx36-expressing neocortical neuron groups and their overlaps in each layer of the S1 cortex.

Figure 6. Colocalization of Cx36 mRNA signals and α -actinin2 immunoreactivity by using mirror-image sections in the S1 cortex. (a) Schematic representation of mirror-image sections. The mirror-image sections were obtained from two consecutive sections. The first section with upper cut surface was used for observation of the double fluorescence for Cx36 mRNA signals and NeuN immunoreactivity (*b-b''*), whereas the second section with lower cut surface was used to perform immunofluorescence for α -actinin2 and counterstaining with propidium iodide (PI; *c-c''*). Cx36 mRNA and NeuN immunoreactivity were visualized with FastRed (magenta) and AlexaFluor488 (green), respectively. Alpha-actinin2 immunoreactivity was visualized with AlexaFluor488 (green), and PI counterstaining was shown in magenta. After the images of mirror-image sections were captured with a confocal laser-scanning microscopy, the colocalization of Cx36 mRNA signals and α -actinin2 immunoreactivity was analyzed with reference of NeuN and PI labeling for identity of cells. Arrows in *b''* and *c''* indicate double-positive cells showing NeuN immunoreactivity and PI staining but not labeled with Cx36 mRNA or α -actinin2 immunoreactivity. Arrowheads in *b-c''* indicate Cx36-expressing and α -actinin2-positive cells in NeuN-positive and PI-labeled cells. Scale bar = 20 μ m.

Cx36 mRNA

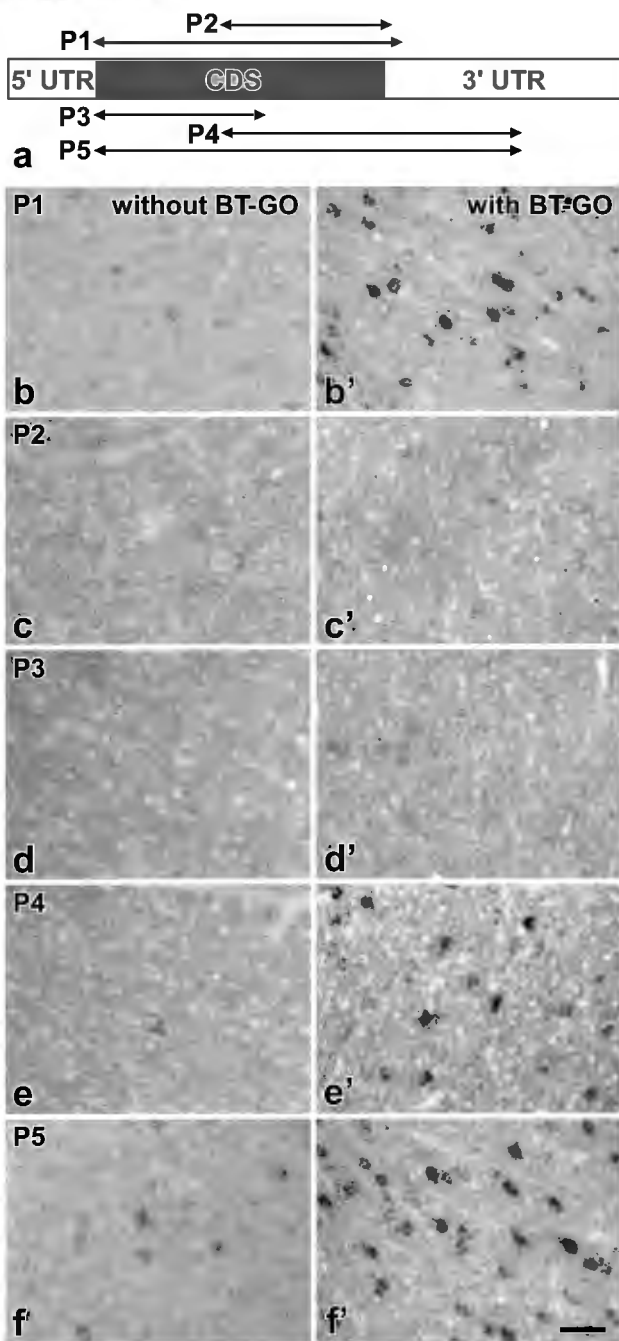


Fig. 1
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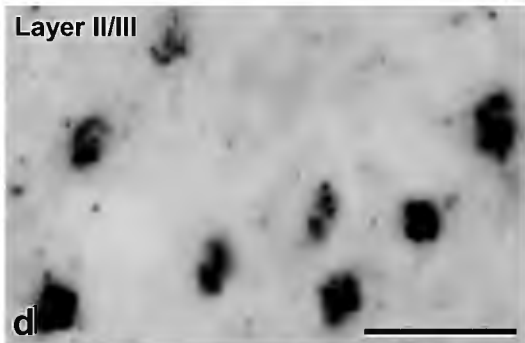
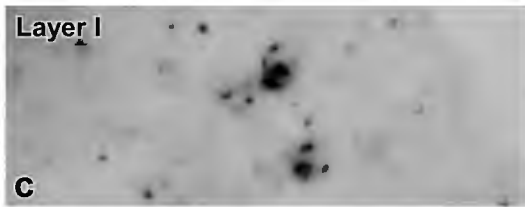
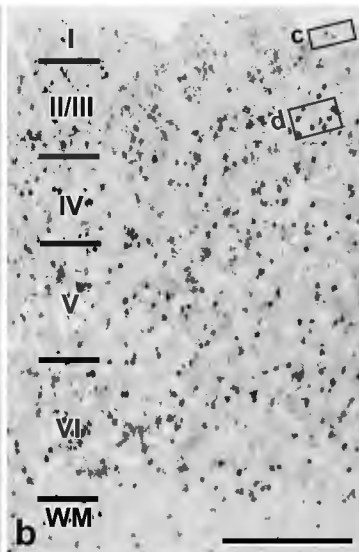
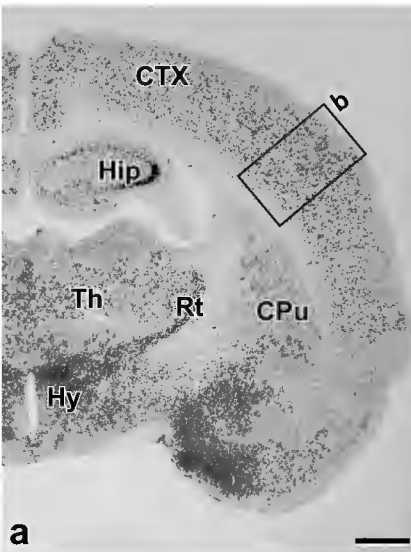


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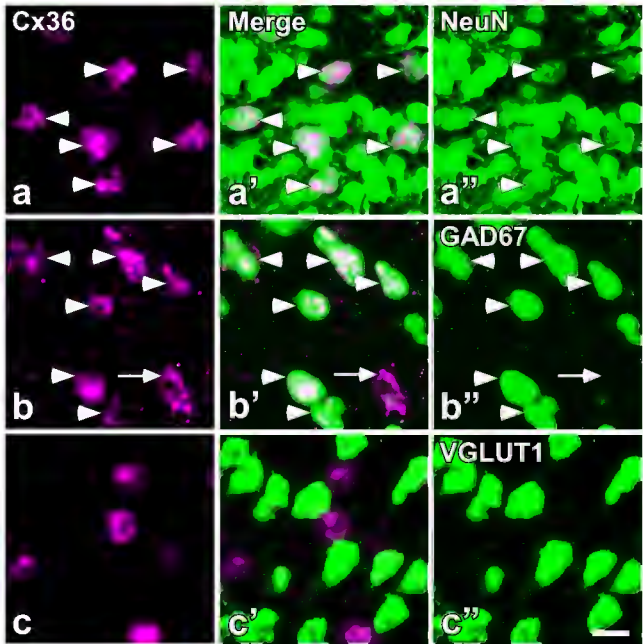


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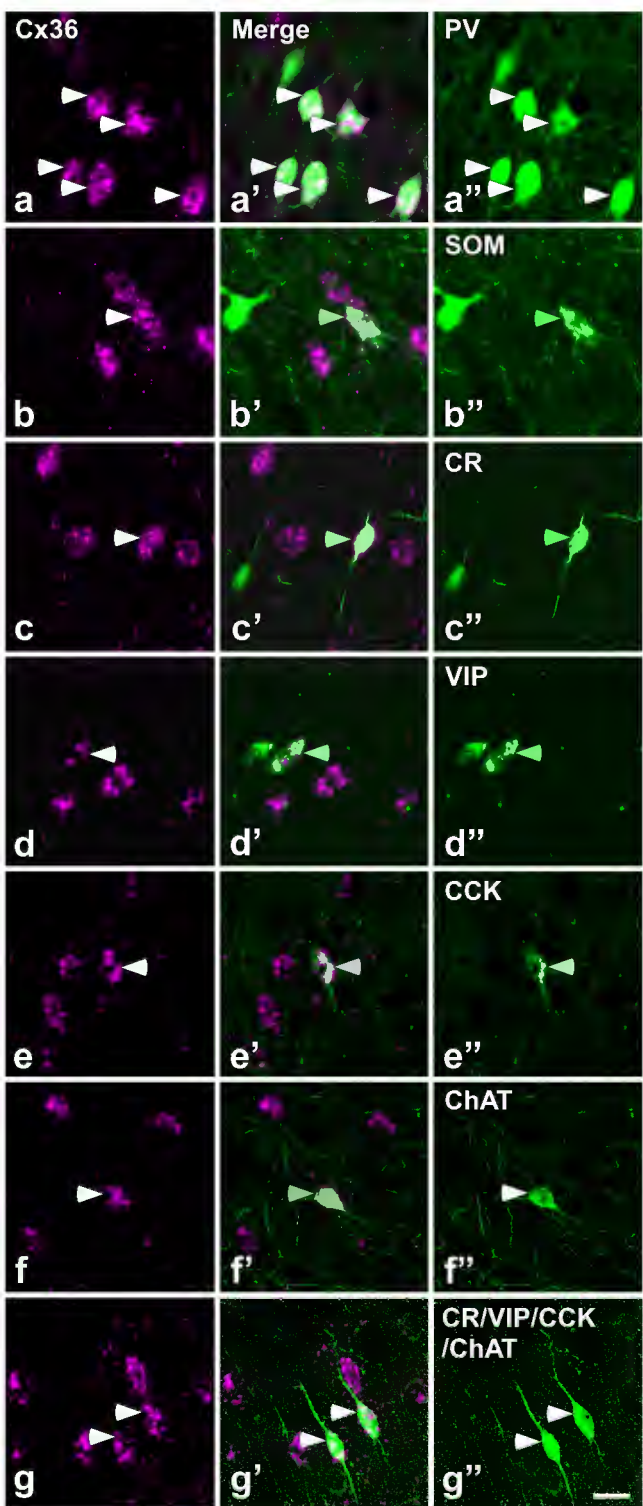


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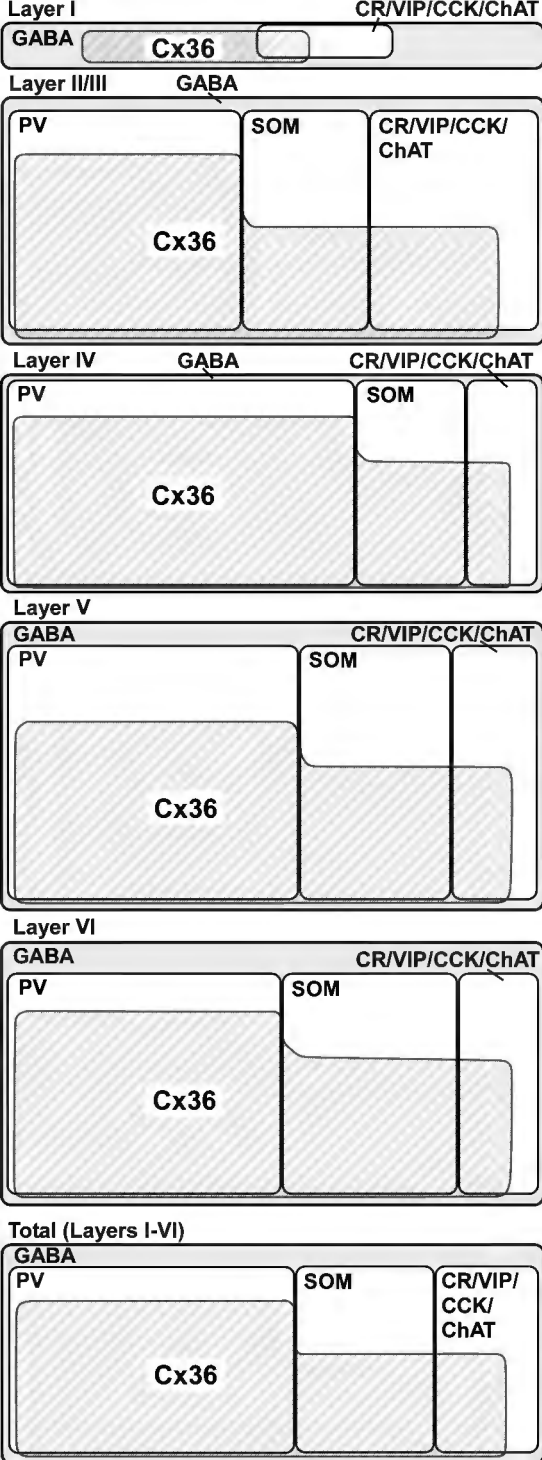
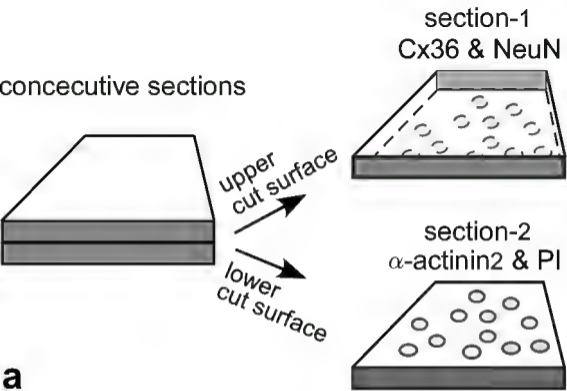


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a

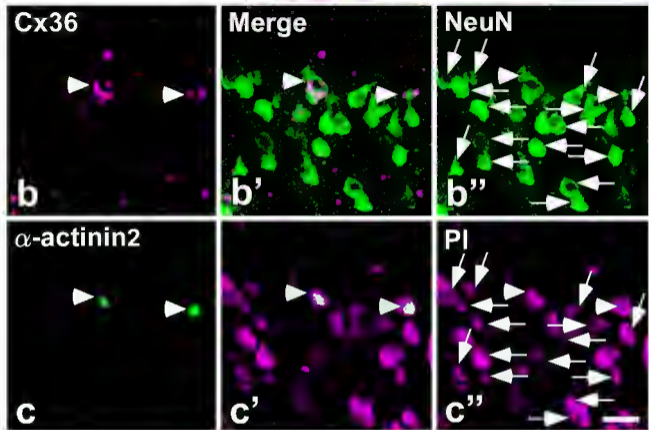


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Supplementary Materials

Supplementary Table 1. Laminar distributions of three distinct subpopulations of

GABAergic neurons in the S1 cortex.

Layer	PV	SOM	CR/VIP/CCK/ChAT
1	0 ^a (0/67) ^b	0 (0/71)	21.6 ± 1.5 (15/69)
2/3	38.6 ± 4.0 (160/414)	20.8 ± 0.1 (123/589)	28.5 ± 3.9 (106/369)
4	65.0 ± 2.6 (279/429)	19.7 ± 1.6 (102/511)	13.2 ± 0.8 (36/273)
5	48.3 ± 6.5 (256/524)	25.7 ± 0.8 (180/698)	14.2 ± 3.5 (59/420)
6	42.8 ± 4.6 (201/470)	27.2 ± 1.0 (188/691)	12.5 ± 2.0 (42/329)
Total	47.1 ± 0.4 (896/1904)	23.1 ± 0.6 (593/2560)	17.7 ± 0.4 (258/1460)

^aData are given as mean ± SD of the percentages in three rats.

^bThe denominator and numerator in parentheses are the total number of cells expressing GAD67 mRNA and that of neurons positive for individual chemical marker, respectively, in three rats.

Supplementary Table 2. Colocalization of α -actinin2 immunoreactivity with GABA-, PV-, SOM- or CR/VIP/CCK/ChAT immunoreactivity in the S1 cortex.

Layer	GABA		PV		SOM		CR/VIP/CCK/ChAT	
	α -actinin2	GABA	α -actinin2	PV	α -actinin2	SOM	α -actinin2/	(CR/VIP/CCK/ChAT)
	/GABA	/ α -actinin2	/PV	/ α -actinin2	/SOM	/ α -actinin2	(CR/VIP/CCK/ChAT)	/ α -actinin2
1	62.1 \pm 2.7 ^a (99/160) ^b	100.0 \pm 0.0 (99/99)	— ^c —	0 (0/70)	— —	0 (0/75)	0 (0/28)	0 (0/107)
2/3	12.4 \pm 0.7 (134/1088)	100.0 \pm 0.0 (134/134)	0 (0/270)	0 (0/105)	0 (0/144)	0 (0/108)	4.7 \pm 1.1 (9/190)	5.6 \pm 1.5 (9/158)
4	2.3 \pm 0.6 (21/901)	100.0 \pm 0.0 (21/21)	0 (0/353)	0 (0/17)	0 (0/93)	0 (0/16)	0 (0/74)	0 (0/20)
5	3.8 \pm 0.8 (37/976)	100.0 \pm 0.0 (37/37)	0 (0/404)	0 (0/31)	0 (0/194)	0 (0/37)	0 (0/114)	0 (0/39)
6	8.1 \pm 0.2 (83/1032)	100.0 \pm 0.0 (83/83)	0 (0/365)	0 (0/48)	0 (0/172)	0 (0/57)	0 (0/111)	0 (0/81)
Total	9.0 \pm 0.6 (374/4157)	100.0 \pm 0.0 (374/374)	0 (0/1392)	0 (0/271)	0 (0/603)	0 (0/293)	1.7 \pm 0.4 (9/517)	2.2 \pm 0.6 (9/405)

^aData are given as mean \pm SD of the percentages in three rats.

^bThe denominator and numerator in parentheses are the total number of cells showing immunoreactivity for GABA, PV, SOM, CR/VIP/CCK/ChAT or α -actinin2 and that of α -actinin2-ir neurons positive for GABA, PV, SOM or CR/VIP/CCK/ChAT, respectively, in three rats.

^cPV- or SOM-positive cells were not found in layer I.

Supplementary Figure 1. Double labeling for GAD67 mRNA and immunoreactivity of various chemical markers in the S1 cortex. The sections hybridized with DIG-labeled GAD67 riboprobe were incubated overnight with a mixture of 1:1,000-diluted AP-conjugated anti-DIG sheep antibody and one of the following antibodies: mouse monoclonal antibody against PV; rabbit polyclonal antibody against SOM; a mixture of the antibodies against CR, VIP, CCK or ChAT. The sections were incubated with AlexaFluor488-conjugated antibody against mouse, rabbit and/or goat IgG, and then reacted with HNPP/FastRed TR. GAD67 mRNA and immunoreactivity for chemical markers were visualized with FastRed (magenta) and AlexaFluor488 (green), respectively. Almost all of the PV- (*a-a''*), SOM- (*b-b''*) or CR/VIP/CCK/ChAT-ir cells (*c-c''*) displayed the signals for GAD67 mRNA (arrowheads in *a-c''*). Scale bar = 50 μ m.

Supplementary Figure 2. Double immunofluorescence labeling for α -actinin2 and GABA, PV, SOM or CR/VIP/CCK/ChAT in the S1 cortex. Sections were prepared as described in the method, except that 0.1% (w/v) glutaraldehyde was added to the fixative for perfusion. The sections were incubated overnight with a mixture of 1:8,000-diluted mouse monoclonal antibody against α -actinin2 and one of the following antibodies: rabbit polyclonal antibody against GABA (A2052; Sigma; 1:1,000); rabbit polyclonal antibody against PV (PC255L; Calbiochem; 1:2,000); the rabbit antibody against SOM; a mixture of the rabbit or goat antibodies against CR, VIP, CCK or ChAT. After incubation with

AlexaFluor488-conjugated antibody against rabbit and/or goat IgG, the sections were incubated with peroxidase-conjugated donkey anti-mouse IgG in the presence of 10% (v/v) normal rabbit and/or goat serum. Subsequently, the sections were reacted with 1:2,000-diluted BT-GO mixture, and finally incubated with AlexaFluor594-conjugated streptavidin (S11223; Invitrogen). Alpha-actinin2 and the other epitopes were visualized with AlexaFluor594 (magenta) and AlexaFluor488 (green), respectively. Almost all the α -actinin2-ir cells were immunopositive for GABA (*a-a''*), but negative for PV (*b-b''*), SOM (*c-c''*) and CR/VIP/CCK/ChAT (*d-d''*). Arrowheads in *a-a''* indicate the colocalizations.

Scale bar = 50 μ m.

Supplementary Comment

Technical Consideration on the Specificity of the Present Method

Although only weak signals for Cx36 mRNA were obtained by the conventional ISH procedure, the sensitive BT-GO amplification method allowed (Furuta et al. 2009; Kuramoto et al. 2009; Ge et al. 2010) us to clearly visualize the signals (Fig. 1). Although ISH experiments using non-radioactive riboprobes were sometimes prone to cross-hybridization artifacts, the signals were considered to be specific to Cx36 mRNA in the present results by the following reasons: 1) The distributions of the signals with probes 1, 4 and 5 were very similar to each other and to that of Cx36 mRNA signals reported previously (Condorelli et al. 1998, 2003; Belluardo et al. 1999, 2000; Parenti et al. 2000; Degen et al. 2004) in many rodent brain regions including the inferior olive, cerebellum, mesencephalon, hypothalamus, thalamic reticular nucleus, striatum, hippocampus, cerebral cortex and olfactory bulb; 2) The signals were almost specific to neurons throughout the brain and mostly selective to GABAergic neurons in the cortical regions. If this method detected the cross-hybridization signals for other connexins, the signals might have been found on glial cells because of the other connexins principally expressed in glial cells (Nagy et al. 2001, 2003; Altevogt and Paul 2004; Li et al. 2004); 3) The signals were resistant to RNase A treatment and high stringency washes; 4) No signals were detected with the control sense probes.

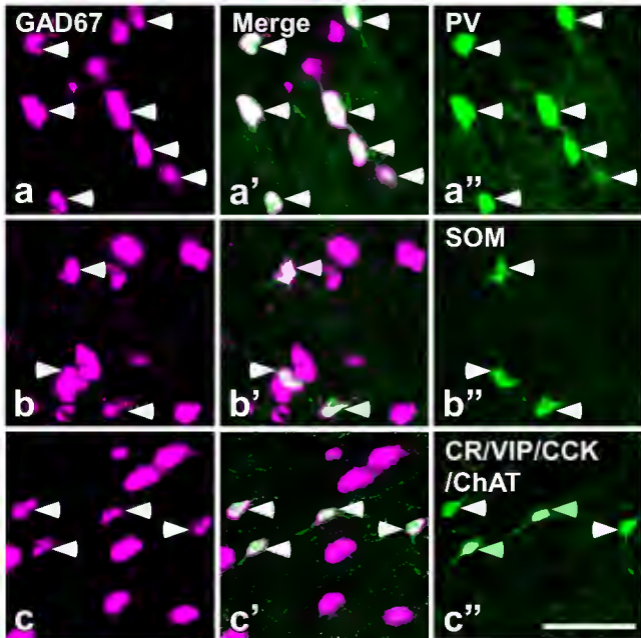
The present quantitative data was mostly in agreement with the results reported in the previous studies. We found that Cx36 was expressed in approximately 6.1–15.8% of

NeuN-ir neurons in L2–6, which was compatible with the previous report that 3.2–11.2% of NeuN-ir neurons in L2–6 showed Cx36 mRNA signals in the rat neocortex (Belluardo et al. 2000). Furthermore, 50–99% of PV-ir neurons have been reported to be positive for Cx36 in the mouse (Deans et al. 2001; Priest et al. 2001; Helbig et al. 2010) and rat neocortex (Belluardo et al. 2000). These previous findings were in accordance with the present result showing that 80.2% of PV-ir neurons were positive for the riboprobe for Cx36 mRNA. Deans et al. (2001) reported that 26% of Cx36-expressing neurons were positive for SOM immunoreactivity in the juvenile mouse neocortex, and the present result displayed that 25% of Cx36-expressing neurons were SOM-positive in the adult rat cortex. Thus, these quantitative results support that the present ISH method was specific in visualization of Cx36 mRNA in the rat brain.

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Supplementary Figure 1
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