Chemically Specific Circuit Composed of Vesicular Glutamate Transporter 3- and Preprotachykinin B-producing Interneurons in the Rat Neocortex

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The third vesicular glutamate transporter, VGLUT3, is distributed in cell bodies of neocortical neurons and axon terminals mainly in the superficial part of layer II/III of the cerebral cortex. We examined the chemical characteristics of VGLUT3-expressing neurons by immunohistochemistry in the rat neocortex. Since the vast majority of VGLUT3-immunoreactive neurons showed immunoreactivities for GABA, preprotachykinin B (PPTB) and cholecystokinin, VGLUT3-immunoreactive neocortical neurons were considered to constitute a subgroup of GABAergic interneurons. VGLUT3-immunoreactive axon terminals were immunopositive for either vesicular GABA transporter (VGAT) or serotonin. These results together with anterogradetracer injection and chemical lesion experiments in the dorsal and median raphe nuclei revealed that the neocortex contains at least two kinds of VGLUT3-laden axon terminals: one is serotonergic and derived from the raphe nuclei, and the other is GABAergic and intrinsic in the neocortex. Furthermore, many VGLUT3/VGAT-immunoreactive terminals formed axon baskets and made axo- somatic symmetric synapses on neocortical neurons, most of which were immunoreactive for PPTB. VGLUT3-immunopositive axon baskets surrounded about a half of PPTB-positive and almost all VGLUT3-positive neurons. Thus, VGLUT3-expressing GABAergic interneurons form a chemically specific circuit within the PPTB-producing interneuron group and it is likely that glutamate is used within the chemically specific circuit.

Keywords: cerebral cortex, GABA, glutamate, serotonin, vesicular glutamate transporter

Introduction

Glutamate is well known to be the major excitatory neurotransmitter in the mammalian central nervous system. Before exocytotic release, glutamate is transported into the synaptic vesicles by a specific transport system. Recently, vesicular glutamate transporters 1 and 2 (VGLUT1 and VGLUT2), which are responsible for uptake of glutamate into synaptic vesicles, have been identified (Bellocchio et al., 2000; Takamori et al., 2000, 2001; Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001; Varoqui et al., 2002). VGLUT1 and VGLUT2 have been revealed to be located on synaptic vesicles of excitatory axon terminals and to possess very similar biochemical and pharmacological characteristics for glutamate uptake. However, the regional expression patterns of the two proteins are highly complementary and the use of the proteins appears largely segregated to different neuronal populations. VGLUT1 is mainly expressed in telencephalic regions, whereas VGLUT2 is produced principally in diencephalic and lower brainstem regions (Fremeau et al., 2001; Fujiyama et al., 2001; Kaneko and Fujiyama, 2002; Kaneko et al., 2002; Varoqui et al., 2002; Hioki et al., 2003).

More recently, the third vesicular glutamate transporter, VGLUT3, has been isolated from rat, mouse and human brain cDNA libraries (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002; Takamori et al., 2002). VGLUT3 shows >70% amino acid identity with and similar biochemical characteristics to VGLUT1 and VGLUT2. VGLUT3 mRNA is, however, expressed in some restricted brain regions including the hippocampus, striatum and raphe nuclei. Remarkably, VGLUT3 is expressed by GABAergic interneurons in the hippocampus, cholinergic interneurons in the striatum and serotonergic neurons in the raphe nuclei (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002). It thus seems likely that, in contrast to VGLUT1 and VGLUT2, VGLUT3 is not expressed by principal excitatory neurons.

Although VGLUT3 mRNA expression has been reported in the cerebral cortex (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002; Takamori et al., 2002), the chemical characteristics of VGLUT3-expressing neocortical neurons has not been reported yet. In the present study, we produced specific antibodies against VGLUT3 and characterized VGLUT3-expressing neurons immunocytochemically in the rat neocortex.

Materials and Methods

The experiments were conducted in accordance with the rules of animal care by Institute of Laboratory Animals, Faculty of Medicine, Kyoto University. Twenty-six adult male Wistar rats (200–250 g; Japan SLC, Shizuoka, Japan), four female guinea pigs (20 g; Shimizu Exper- imental Materials, Kyoto, Japan) and two female white rabbits (2 kg; Japan SLC) were used in the present study. All efforts were made to minimize animal suffering and the number of animals used.

Production of Antibodies

Peptide CQQRESAFEGEELSPYQNEEDFLSET, which corresponded to C-terminal 25 amino acids (residues 564–588) of rat VGLUT3, was synthesized. The peptide was conjugated with an equal weight of maleimide-activated bovine serum albumin (Pierce, Rockford, IL) through the N-terminal cysteine. Four female guinea pigs and two female white rabbits were immunized by intracutaneous injections of the conjugate (0.5 mg/guinea pig, 2 mg/rabbit) in Freund’s complete adjuvant (Difco, Detroit, MI) and of the same amount in incomplete adjuvant 4 weeks later. The sera were recovered 10–21 days after the second immunization. The guinea pig antibody was purified to crude yglobulin fraction by ammonium sulfate fractionation (50% saturation) and the rabbit antibody was purified by 2-step sodium sulfate fractionation (18 and 14%; Johnstone and Thorpe, 1982). The polyclonal antibodies were further processed by affinity chromatography on a SulfoLink gel (Pierce) coupled with the peptide (2 mg peptide/ml gel). The specific antibodies were eluted from the column with 0.1 M glycine–HCl (pH 2.5).

Western Blotting

Two grams of rat brains were homogenized with 9 vols of 50 mM Tris–HCl (pH 7.4) containing 10 mM EDTA, 5 µg/ml aprotinin (Sigma,
St Louis, MO), 1 µg/ml leupeptin (Nacalai Tesque, Kyoto, Japan), 1 µg/ml pepstatin A (Nacalai Tesque), 0.1 mg/ml phenylmethyl-sulfonyl fluoride (Sigma), 0.1% (w/v) sodium dodecyl sulfate (SDS) and 1% (v/v) TritonX-100. The homogenate was centrifuged at 20,000g for 30 min at 4°C. The supernatant was reduced by heating at 95°C for 10 min with 0.7% (v/v) 2-mercaptoethanol and 2% (w/v) SDS in the presence or absence of 8M urea and electrophoresed in 10% polyacrylamide gel in the presence of 0.1% (w/v) SDS. The electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane (BioRad, Richmond, CA). After blocking with 100% Block-Ace (Dainippon Pharmaceutical, Osaka, Japan) for 60 min, the membranes were incubated overnight at room temperature with 0.5 µg/ml of guinea pig or rabbit antibody against VGLUT3 and then for 1 h with 1/10,000 diluted alkaline phosphatase-conjugated anti-guinea pig IgG) goat antibody or 1/20,000 diluted anti-rabbit IgG) antibody (Chemicon, Temecula, CA). The antibodies were diluted with 5 mM phosphate-buffered 0.9% (w/v) saline (PBS; pH 7.4) containing 10% (v/v) Block-Ace and 0.2% (v/v) Tween 20. The membranes were finally developed with CDP-Star™ detection reagent (Amersham BioSciences, Buckinghamshire, UK). For control experiments, some membranes were incubated with the primary antibodies in the presence of 10,000-fold (in mol) excess amount of the antigen peptide.

**Anterograde Labeling and Chemical Depletion of Serotonergic Neurons in the Dorsal and Median Raphe Nuclei**

Fourteen rats were deeply anesthetized with chloral hydrate (35 mg/100 g body wt). Two milligram of 5,7-dihydroxytryptamine (Sigma) was freshly dissolved in 200 µl of 0.9% (w/v) saline containing 0.1% (w/v) ascorbic acid. Six or eight rats were received 0.5 µl of 10% (w/v) biotinylated dextran amine (BDA, 10,000 MW; Molecular Probes, Eugene, OR) in 5 mM PBS for anterograde labeling or 2 µl of 5,7-dihydroxytryptamine solution for chemical depletion, respectively. The solutions were injected stereotaxically into the dorsal and median raphe nuclei by pressure glass micropipette attached to Picospritzer III (General Valve Corporation, East Hanover, NJ). The rats were allowed to survive for 7–21 days (Reader, 1989; Reiner et al., 2000).

**Immunoperoxidase Staining**

The rats injected with BDA or 5,7-dihydroxytryptamine and four normal rats were deeply anesthetized with chloral hydrate (70 mg/100 g body wt) and perfused transcardially with 200 ml of PBS. The rats were further perfused for 30 min with 200 ml of 3% (w/v) formaldehyde, 0.5% Na2HPO4 (pH 7.0; adjusted with NaOH). The brains were removed, cut into several blocks and post-fixed with the same fixative above for 8 h at 4°C. For immunostaining of GABA and serotonin, 0.1% (w/v) glutaraldehyde was added to the fixative used for the perfusion. After cryoprotection with 30% (w/v) sucrose in PBS, the blocks were cut into 30-µm-thick sections on a freezing microtome.

The sections were incubated overnight with 0.2 µg/ml affinity-purified anti-VGLUT3 antibody in PBS containing 0.2% (v/v) formaldehyde and 1 µg/ml anti-guinea pig IgG) donkey antibody (Jackson, West Grove, PA) or anti-rabbit IgG) donkey antibody (Chemicon). The incubation was carried out at room temperature in PBS containing 0.5% (v/v) Triton X-100, 0.25% (w/v) 3-carrageenan and 1% (v/v) donkey serum (Biotinconjugate) and followed by a rinse with PBS containing 0.3% (v/v) Triton X-100 (Biotinconjugate). The sections were further incubated in 0.1% (v/v) Photo Flo (Kodak, Rochester NY) in PBS-G with 0.8% (w/v) diaminobenzidine–4HCl and 0.001% (v/v) H2O2 in 50 mM Tris–HCl (pH 7.6), mounted onto gelatinized glass slides, dehydrated in ethanol series, cleared in xylene, and coverslipped. For control experiments, the sections were incubated with the primary antibodies in the presence of 10,000-fold (in mol) excess amount of the antigen peptide.

**Double Immunofluorescence Labeling**

The sections, which were fixed as described above, were incubated overnight in PBS-XCD with a mixture of 1 µg/ml anti-VGLUT3 guinea pig antibody and one of the following antibodies: anti-GABA rabbit serum (Sigma), 1:1000; anti-parvalbumin mouse IgG (Sigma), 1:8000 from ascites; anti-calbindin mouse IgG (Sigma), 1:2000 from ascites; anti-calretinin mouse IgG (Chemicon), 1:1000 from ascites; antiserotonin rabbit serum (Peninsula, Belmont, CA), 1:2000; antivasoactive intestinal polypeptide rabbit serum (Peninsula), 1:200; anti-cholecystokinin (CCK) rabbit serum (Incatar, Stillwater, MN), 1:1000; anti-corticotropin-releasing factor rabbit serum (Incatar), 1:2000; anti-neuronal nitric oxide synthase sheep serum (Chemicon), 1:2000; anti-neuropeptide Y rabbit serum (Chemicon), 1:2000; anti-choline acetyltransferase rabbit serum (Chemicon), 1:2000; affinity-purified anti-preproparathykinin A rabbit antibody (Lee et al., 1997), 1 µg/ml; affinity-purified anti-preproparathykinin B (PPTB) rabbit antibody (Kaneko et al., 1998), 1 µg/ml; affinity-purified anti-VGLUT1 rabbit antibody (Hioki et al., 2003), 1 µg/ml; affinity-purified anti-VGLUT2 rabbit antibody (Hioki et al., 2003), 1 µg/ml; anti-vesicular GABA transporter (VGAT) rabbit serum (Chemicon), 1:1000; antiserotonin rabbit serum (Sigma), 1:100; anti-tyrosine hydroxylase rabbit serum (Chemicon), 1:2000; anti-dopamine β-hydroxylase rabbit serum (Chemicon), 1:2000; and anti-vesicular acetylcholine transporter goat serum (Chemicon), 1:1000.

After a rinse with PBS-X, the sections were incubated for 1 h in PBS-XCD with 10 µg/ml biotinylated anti-rabbit, mouse, sheep or goat IgG) donkey antibody (Chemicon) and then for 1 h with 5 µg/ml Alexa488-conjugated anti-guinea pig IgG) goat antibody and 1 µg/ml Alexa594- or Alexa647-conjugated streptavidin (Molecular Probes) in the presence of 10% (v/v) normal rabbit, mouse, sheep or goat serum, respectively. The sections were mounted onto gelatinized glass slides and coveredslipped with 50% (v/v) glycerol and 2.5% (w/v) triethylenediamine (antifading reagent) in PBS. The sections were observed under epifluorescence microscope Axioskop 2 (Zeiss, Oberkochen, Germany) with appropriate filter sets for Alexa488 (excitation, 450–490 nm; emission, 514–565 nm) and Alexa594 (excitation, 530–585 nm; emission, 5615 nm), or under confocal laser-scanning microscope LSM 5 Pascal (Zeiss) with a confocal depth of 1.0 µm, appropriate laser beams and filters for Alexa488 (excitation, 488 nm; emission, 505–530 nm) and Alexa647 (excitation, 635 nm; emission, 6260 nm).

**Immunoelectron Microscopy**

Five rats were deeply anesthetized with chloral hydrate (70 mg/100 g body wt), perfused transcardially with 200 ml of PBS. The rats were further perfused with 200 ml of 0.4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were post-fixed at 4°C for 3 h in 4% (w/v) paraformaldehyde, cut into 50 µm thick frontal sections on a vibratome (Micoslicer DTK-1000; Dosaka, Kyoto, Japan). Subsequently, the sections were incubated in the presence of 0.1% (v/v) Photo Flo (Kodak, Rochester NY) in PBS-G with 0.8% (w/v) diaminobenzidine–4HCl and 0.001% (v/v) H2O2 in 50 mM Tris–HCl (pH 7.6), mounted onto gelatinized glass slides, dehydrated in ethanol series, cleared in xylene, and coverslipped. For control experiments, the sections were incubated with the primary antibodies in the presence of 10,000-fold (in mol) excess amount of the antigen peptide.

In **St Hybridization Histochemistry**

Complementary DNA fragment corresponding to a region of the rat VGLUT3 cDNA (2105–2462 of gb: AJ917985, GenBank) was cloned into pbLueScript II SK (+) (Strategene, La Jolla, CA). Using this plasmid as a template, sense and antisense single-strand RNA probes were synthesized with a digoxigenin labeling kit (Roche Diagnostics,
Mannheim, Germany). Three rats were deeply anesthetized with chloral hydrate (70 mg/100 g body wt) and perfused transcardially with 200 ml of PBS, followed by 200 ml of 2% (w/v) formaldehyde in PB. The brains were removed, cut into several blocks and post-fixed at 4°C for 2 h in the same fixative. After cryoprotection with 50% (w/w) sucrose in PBS, the blocks were cut into 20 µm thick frontal sections on a freezing microtome.

The free-floating sections were washed in PBS for 5 min, equilibrated in 0.1 M triethanolamine for 5 min and acetylated in freshly prepared 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine for 10 min by vigorous shaking. After a rinse with PBS for 5 min three times, the sections were incubated in a prehybridization buffer containing 50% (v/v) formamide, 5× SSC, 5× Denhardt’s solution, 250 µg/ml yeast tRNA and 500 µg/ml salmon sperm DNA for 2 h at room temperature. Then, the sections were hybridized with 500 ng/ml digoxigenin-labeled sense or antisense RNA probe for VGLUT3 in the prehybridization buffer for 12–24 h at 60°C. After a rinse with 5× SSC, the sections were subjected to high stringency washes at 60°C. Subsequently, the sections were incubated at 4°C overnight with 1/2000-diluted alkaline phosphatase-conjugated anti-digoxigenin antibody Fab fragment in a blocking reagent (Roche) 10 min by vigorous shaking. After a rinse with PBS for 5 min three times, the sections were incubated in a prehybridization buffer for 12–24 h at 60°C. After a rinse with 5× SSC, the sections were subjected to high stringency washes at 60°C. Subsequently, the sections were incubated at 4°C overnight with 1/2000-diluted alkaline phosphatase-conjugated anti-digoxigenin antibody Fab fragment in a blocking reagent (Roche) and the bound phosphatase was visualized by reaction for 24–36 h in situ.

Immunoreactivity and In Situ Hybridization Signal for VGLUT3 in the Neocortex

The immunoreactivity of VGLUT3 was widely distributed throughout the neocortex (Fig. 1a). No significant difference in VGLUT3 immunoreactivity was observed between neocortical areas including motor, somatosensory, auditory and visual cortices. VGLUT3 immunoreactivity was found not only in axon terminals but also in some cell bodies of neocortical neurons (Fig. 2a). VGLUT3-immunoreactive neurons were small vertically elongated cells, which were frequently distributed in the superficial part of layer II/III of the cerebral cortex and less frequently in layer VI (Fig. 2b,d). VGLUT3-immunoreactivity in the somatosensory cortex. VGLUT3 immunoreactivity in the neocortex was observed not only in axon terminals but also in cell bodies of certain neocortical neurons. VGLUT3-immunoreactive fibers were dense in the superficial part of layer II/III (a) and VGLUT3-immunoreactive interneuronal cells were also frequently found in layer II/III (b). Some of the VGLUT3-immunoreactive axon terminals formed basket-like structures and surrounded VGLUT3-positive (b) or negative cell bodies (c). Each filled circle represents a VGLUT3 immunoreactive neuronal cell body in a 30-µm-thick section (d). Scale bars = 200 µm (a); 10 µm (b, c); 1 mm (d).
active axon terminals were dense in the superficial part of layer II/III and scattered in the other layers (Fig. 2a). Some VGLUT3-immunoreactive axon terminals formed basket-like structures (Fig. 2b,c). These VGLUT3-positive axon baskets surrounded soma and proximal dendrites of VGLUT3-positive or negative neurons (Fig. 2b,c). VGLUT3-positive axon baskets were distributed frequently in the superficial part of layer II/III and less frequently in the other layers.

We performed double labeling experiment by a combination of immunoperoxidase staining for VGLUT3 protein and in situ hybridization for VGLUT3 mRNA (Fig. 3). More than 96% (61 of 63) of VGLUT3 immunoreactive neurons showed signals for VGLUT3 mRNA in the neocortex. Inversely, 100% (61 of 61) of VGLUT3 mRNA-expressing neurons were immunoreactive for VGLUT3. Sense RNA displayed no signal in the neocortex (not shown). These results indicate that the number of VGLUT3-producing neurons was not underestimated by the present immunohistochemical method.

Precise subcellular localization of VGLUT3 immunoreactivity was examined by electron microscopy with the gold colloid/silver intensification method. VGLUT3-immunoreactive silver grains were found in axon terminals of both asymmetric (Fig. 4a) and symmetric types of synapses (Fig. 4b,c). Asymmetric synapses with VGLUT3-immunoreactive presynaptic elements were found mostly in the superficial layers of the cerebral cortex, whereas symmetric synapses were observed throughout layers II–VI. In the asymmetric synapses, most VGLUT3-immunoreactive grains were found associated with synaptic vesicles (Fig. 4a). However, VGLUT3-immunoreactive grains in the symmetric synapses were associated with not only synaptic vesicles but also other membranous structures, although the silver grains were accumulated in the presynaptic profiles including active zones (Fig. 4b,c). This was partly because the presynaptic elements of the symmetric synapses contained less synaptic vesicles than those of the asymmetric synapses, or might be because VGLUT3 was involved in other functions than synaptic vesicular release. In VGLUT3-positive axon baskets, the axon terminals invariably formed symmetric synapses with cell bodies and proximal dendrites (Fig. 4b,c). No asymmetric synapses were found in the axon baskets, even when the axon baskets were examined with serial ultrathin sections.
VGLUT3-expressing Neurons

More than 95% of VGLUT3-immunoreactive neurons in the neocortex showed GABA immunoreactivity (Fig. 5a–d′) and Table 1), suggesting that VGLUT3-immunoreactive neocortical neurons were GAABAergic interneurons. Inversely, 1.3% of GABA-immunopositive neurons showed immunoreactivity for serotonin (Fig. 5a–d′). Arrowheads point to the colocalization of VGLUT3 and other chemical markers. DR, the dorsal raphe nuclei. Scale bars = 25 μm (a–d′).

VGLUT3-immunoreactive Axon Terminals in the Neocortex

Chemical characteristics of VGLUT3-immunoreactive axon terminals were examined by confocal laser-scanning microscopy. No VGLUT3-immunoreactive axon swellings showed immunoreactivities for VGLUT1 or VGLUT2 (Fig. 6a–b′), suggesting that VGLUT3-immunoreactive axons were not derived from cortical pyramidal cells or thalamic projection neurons (Fujiyama et al., 2001). VGLUT3-immunopositive axon terminals showed immunoreactivity for VGAT, CCK or serotonin (Fig. 6c–e′). When VGAT and serotonin immunoreactivities were co-stained red, most green VGLUT3-positive axon terminals displayed red immunofluorescence (not shown). Thus, VGLUT3-immunoreactive axon terminals in the neocortex were considered to consist of two populations: GAABAergic and serotonergic. Since CCK immunoreactivity is known to be located in GAABAergic axon terminals (Hendry et al., 1983), it is likely that CCK-immunoreactive terminals were included in GAABAergic terminals. However, in contrast to

Table 1

<table>
<thead>
<tr>
<th>Chemical marker</th>
<th>Numbers of neurons in the neocortical regions</th>
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<tr>
<td>Marker+/VGLUT3 + (%)</td>
<td>VGLUT3 +/Marker + (%)</td>
</tr>
<tr>
<td>GABA</td>
<td>166/173 (97.1)</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>0/123 (0.0)</td>
</tr>
<tr>
<td>Calbindin</td>
<td>0/121 (0.0)</td>
</tr>
<tr>
<td>Calretinin</td>
<td>0/111 (0.0)</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>0/104 (0.0)</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>2/24 (1.6)</td>
</tr>
<tr>
<td>Neuronal nitric oxide synthase</td>
<td>0/135 (0.0)</td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
<td>0/126 (0.0)</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>0/105 (0.0)</td>
</tr>
<tr>
<td>Corticotropin-releasing factor</td>
<td>0/124 (0.0)</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td>117/125 (93.6)</td>
</tr>
<tr>
<td>Peptidyltyrosine A</td>
<td>0/107 (0.0)</td>
</tr>
<tr>
<td>Peptidyltyrosine B</td>
<td>171/179 (95.5)</td>
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Six 30 μm thick frontal sections through the frontoparietal cortex, including the motor and somatosensory areas, were examined for each combination of immunoreactivities.

neurons in the frontoparietal cortex. About 95% of VGLUT3-immunoreactive neurons were positive for PPTB, the precursor of neurokinin B, and CCK (Fig. 5b–b′,c–c′) and almost negative for the other chemical markers. About 5% of PPTB-positive neurons and 16% of CCK-positive neurons were immunoreactive for VGLUT3.

Messenger RNA for VGLUT3 has been reported to be positive in the dorsal and median raphe nuclei (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002). Since these raphe nuclei are the main source of serotonergic axon fibers in the neocortex, we studied serotonergic neurons in these nuclei immunocytochemically with the anti-VGLUT3 antibody. In the dorsal and median raphe nuclei, 426 (95.3%) of 447 serotonin-immunoreactive neuronal cell bodies were immunopositive for VGLUT3 and 426 (87.7%) of 486 VGLUT3-immunoreactive neurons were positive for serotonin (Fig. 5d–d′).

Figure 5. Double immunofluorescence staining in the somatosensory cortex and dorsal raphe nucleus. VGLUT3 and other chemical markers were labeled with Alexa488 and Alexa594, respectively. In the somatosensory cortex, VGLUT3-immunoreactive neurons were also immunoreactive for GABA, CCK and PPTB (a–d′). In the dorsal raphe nucleus, VGLUT3-immunoreactive neurons showed immunoreactivity for serotonin (a, d′). Arrowheads point to the colocalization of VGLUT3 and other chemical markers. DR, the dorsal raphe nuclei. Scale bars = 25 μm (a–d′).
VGAT, no CCK immunoreactivity was detected in VGLUT3-positive axon baskets, suggesting that VGLUT3 was loaded by at least two kinds of GABAergic terminals. Immunoreactivity for PPTB or neurokinin B was too weak to be observed in axon terminals by the present immunofluorescence method. No other chemical markers including tyrosine hydroxylase, dopamine-β-hydroxylase, vesicular acetylcholine transporter, calretinin and vasoactive intestinal polypeptide were detected in VGLUT3-immunoreactive axon terminals.

Anterograde Labeling and Chemical Depletion of Serotonergic Neurons

To confirm the origin of axon terminals immunoreactive for VGLUT3 and serotonin, we performed anterograde labeling and chemical lesion experiments. After injection of BDA into the dorsal and median raphe nuclei, some of serotonergic neurons in the nuclei were labeled with BDA (not shown). VGLUT3 immunoreactivity was frequently found in anterogradely labeled axon terminals in layer II/III of the cerebral cortex and less frequently in the other layers (Fig. 6f–f″). After 5,7-dihydroxytryptamine was injected into the dorsal and median raphe nuclei, the nuclei appeared depleted of a large population of serotonergic neurons (Fig. 7b). By this treatment, VGLUT3-immunoreactive axon terminals in the neocortex decreased largely (Fig. 7d), but VGLUT3-positive axon baskets were well preserved (Fig. 7e/f). Furthermore, most preserved VGLUT3-immunoreactive axon terminals were positive for VGAT, but negative for serotonin (not shown).

These findings indicate that VGLUT3-serotonin-immunoreactive axon terminals arise from the dorsal and median raphe nuclei, whereas VGLUT3/VGAT-immunoreactive axon terminals, some of which form the basket-like structures, are intrinsic in the neocortex.

Chemically Specific Connections

Since GABA immunoreactivity was detected in the neurons surrounded by VGLUT3-positive axon baskets (not shown), the surrounded neurons were considered to be GABAergic interneurons. We next examined what kind of GABAergic interneurons received VGLUT3-immunoreactive axon baskets. Interestingly, 219 (89%) of 245 or 72 (31%) of 231 neurons surrounded by VGLUT3-positive axon baskets displayed immunoreactivities for PPTB (Fig. 8a–b″) or CCK (not shown), respectively. Inversely, 219 (51%) of 426 PPTB-immunoreactive neurons and 72 (46%) of 156 CCK-positive neurons were surrounded by VGLUT3-positive axon baskets. VGLUT3-immunopositive neuronal cell bodies, the vast majority of which were immunopositive for PPTB, were invariably surrounded by VGLUT3-positive baskets (Fig. 8a–a″). These VGLUT3-positive neurons constituted 19% (38/198) of neurons surrounded by VGLUT3-positive axon baskets (Fig. 8a–a″) and PPTB-positive but VGLUT3-negative neurons consisted of 70% (138/198) (Fig. 8b–b″). Furthermore, a very few neurons surrounded by VGLUT3-positive axon baskets showed immunoreactivity for parvalbumin, calretinin, calbindin, somatostatin, neuropeptide Y, neuronal nitric oxide synthase, choline acetyltransferase, corticotropin-releasing factor, preprotracchkinin A or vasoactive intestinal polypeptide (0.5–1.5%). This suggests that only a specific subset of GABAergic neocortical interneurons receive inputs of basket-like axons loaded with VGLUT3.

Discussion

VGLUT3 immunoreactivity was observed not only in axon terminals but also in some cell bodies of neocortical neurons. Almost all VGLUT3-expressing neocortical neurons were GABAergic interneurons producing PPTB and cholecystokinin.

Figure 6. Confocal laser-scanning analysis of VGLUT3-immunoreactive axon terminals in the neocortex. VGLUT3 and other chemical markers were visualized green (Alexa488) and red (Alexa647), respectively. VGLUT3-positive profiles were negative for both VGLUT1 and VGLUT2 (a–b″). VGLUT3 immunoreactivity was colocalized in single axon terminals with VGAT, CCK or serotonin (c–e″; arrowheads). In the basket-like structures, VGLUT3-immunoreactive axon terminals were positive for VGAT alone (c–e″). Scale bars = 4 μm (a–f″). After injection of BDA into the dorsal and median raphe nuclei, VGLUT3 immunoreactivity was found in anterogradely labeled axon terminals in the neocortex (f–f″; arrowheads).
The neocortex contains two kinds of VGLUT3-laden axon terminals: one is serotonergic and derived from the raphe nuclei, and the other is GABAergic and intrinsic in the neocortex (Fig. 9b). VGLUT3-laden GABAergic terminals made basket-like structures and the axon baskets terminated mostly on cell bodies of PPTB-expressing interneurons, some of which themselves expressed VGLUT3 (Fig. 9b). Thus, VGLUT3-expressing GABAergic interneurons were considered to form a chemically specific circuit within the PPTB-producing interneuron group in the rat neocortex.

VGLUT3-expressing Neurons in the Neocortex

Neocortical GABAergic interneurons are roughly classified into three subgroups by chemical markers (for reviews, see Kubota et al., 1994; Gonchar and Burkhalter, 1997). The first group is immunopositive for parvalbumin. The second group is distinguished by immunoreactivities for somatostatin, neuropeptide Y and neuronal nitric oxide synthase. Although calbindin-
immunoreactive neurons belong mainly to the second group, calbindin immunoreactivity has been found not only in some parvalbumin-positive neurons but also in pyramidal neurons (Van Brederode et al., 1990; Kubota et al., 1994). The third group is less chemically homogenous, but is incompletely characterized by production of calretinin, vasoactive intestinal polypeptide, corticotropin-releasing factor, choline acetyltransferase and µ-opioid receptor (Demeulemeester et al., 1988; Bayraktar et al., 1997; Taki et al., 2000). In the present study, almost no VGLUT3-expressing neurons were positive for those chemical markers listed for the three groups of interneurons. However, since CCK and PPTB immunoreactivities were observed in neurons of the third interneuron group (Kubota and Kawaguchi, 1997; Kaneko et al., 1998), VGLUT3-expressing neurons were considered to constitute a small subgroup in the third group.

**Co-transmission of Glutamate and Serotonin in the Neocortex**

Serotonergic axon terminals make both asymmetric and symmetric synapses in the rat neocortex (Papadopoulos et al., 1987) and the corticopetal serotonergic axons originate primarily in the dorsal and median raphe nuclei (Azmitia, 1978). In these raphe nuclei, VGLUT3 mRNA was highly expressed (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002) and almost all serotonergic neurons showed signals for VGLUT3 mRNA (Gras et al., 2002). This is supported by the present result that >95% serotonin-immunoreactive neurons were positive for VGLUT3 in these raphe nuclei. Furthermore, anterograde tracer injection and chemical lesion experiments in the dorsal and median raphe nuclei revealed that VGLUT3-serotonin-immunopositive axon terminals in the neocortex are derived from the raphe nuclei.

Some serotonergic neurons have been suggested to be glutamatergic by several lines of evidence. Almost all serotonergic neurons in the dorsal and median raphe nuclei show immuno-reactivity for phosphate-activated glutaminase (Kaneko et al., 1990), which is a major synthetic enzyme of transmitter glutamate in the central nervous system (for reviews, see Kaneko and Mizuno, 1994; Kaneko, 2000). Glutamate immunoreactivity has been also found in serotonergic neurons (Fung and Barnes, 1989; Nicholas et al., 1992). Electrical stimulation of the dorsal raphe nucleus evoked not only serotonin-mediated inhibition with long latency, but glutamate-mediated excitation with short latency in the locus coeruleus (Segal, 1979). Furthermore, mesopontine serotonergic neurons in microcultures produced biphasic responses consisting of fast EPSP and slow IPSP and these fast EPSP and slow IPSP were blocked by glutamate receptor antagonist and serotonin receptor antagonist, respectively (Johnson, 1994; Johnson and Yee, 1995). These findings together with the present observation suggest that glutamate is co-released with serotonin and used for fast excitatory transmission in the neocortex by the corticopetal axons of dorsal and median raphe nuclei neurons.

**Co-release of Glutamate and GABA in the Cerebral Cortex**

The present study revealed that a subgroup of GABAergic interneurons expressed VGLUT3 in the neocortex, supporting the recent finding that some hippocampal interneurons expressed VGLUT3 (Fremeau et al., 2002). Although the co-release of glutamate with GABA has not been reported in the neocortex, Docherty et al. (1987), using an affinity separation technique of cortical synaptosomes, showed that some GABAergic synaptosomes were enriched in glutamate as well as in GABA. These findings strongly suggest that some GABAergic neocortical interneurons release glutamate as a neurotransmitter. The released glutamate may activate ionotropic and/or metabotropic glutamate receptors. If the released glutamate from GABAergic terminals acted on ionotropic glutamate receptors, glutamate would counteract the inhibitory effect of GABA and the net effect on postsynaptic neurons could be difficult to determine. However, co-release of glutamate and GABA and involvement of both ionotropic glutamate and GABA receptors have been reported in granule cells of the hippocampal dentate gyrus, although granule cells give rise to mossy fibers making mainly glutamatergic excitatory synapses on hippocampal CA3 neurons and are thus in inverse situation to GABAergic neurons expressing VGLUT3. Dentate granule cells are known to produce glutamate decarboxylase (GAD), GABA and VGAT (Sandler and Smith, 1991; Cao et al., 1996; Lehmann et al., 1996; Sloviter et al., 1996; Jongen-Relo et al., 1999; Lamas et al., 2001; Ramirez and Gutierrez, 2001) and the expressions of GAD and VGAT have been reported to increase transiently after kindled seizures (Sloviter et al., 1996; Lamas et al., 2001; Ramirez and Gutierrez, 2001). Moreover, Walker et al. (2001) have shown that electrical and chemical stimuli of dentate granule cells simultaneously elicited GABA-mediated fast inhibition in addition to glutamate-induced fast excitation on CA3 pyramidal neurons. Thus, some neural connections may utilize glutamate and
GABA at the same time for fast excitatory and inhibitory transmissions, leaving the possibility that VGLUT3-expressing GABAergic neocortical interneurons exert fast excitatory neurotransmission on the target neurons.

Another possible function of glutamate released by VGLUT3-loaded GABAergic axon terminals in the cerebral cortex is that the released glutamate acts on metabotropic glutamate receptors (mGluRs) and produce modulatory effects. mGluRs are classified into three subclasses: group I (mGluR1, 5), group II (mGluR2, 3) and group III (mGluR4, 7, 8). Group I mGluRs were known to be localized postsynaptically (for a review, see Cartmell and Schoepp, 2000) and reported to potentiate depolarization of postsynaptic neocortical interneurons (Wang et al., 1996). However, since mGluR1 and mGluR5 in the rat cerebral cortex are expressed mostly by somatostatin- or parvalbumin-producing interneurons (Shigemoto et al., 1992; Kermer et al., 1997) that were not surrounded with VGLUT3-laden axon baskets in the present study, it is unlikely that glutamate released from VGLUT3-expressing axons works postsynaptically on those interneurons expressing group I mGluRs. In contrast, group II and group III mGluRs, which were located mainly in presynaptic terminals (for a review, see Cartmell and Schoepp, 2000), were described to suppress GABA release from GABAergic terminals in the neocortex (Schauffhauser et al., 1998). Thus, the release of glutamate may serve as a mechanism for feedback inhibition in axon terminals of some GABAergic neurons.

**Chemically Specific Circuit of PPTB-producing Interneurons**

The present study indicated that VGLUT3 is used within a subgroup of GABAergic cortical interneurons, which were characterized by the production of PPTB and CCK (Fig. 9a, b). About 50% of PPTB-producing interneurons received inputs of VGLUT3-equipped axon baskets and ∼20% of PPTB-producing neurons surrounded by the axon baskets expressed VGLUT3. Inversely, all VGLUT3-expressing neurons were surrounded by VGLUT3-equipped axon baskets. Thus, VGLUT3-expressing interneurons formed a partially mutual network within the PPTB-producing interneuron group (Fig. 9b); VGLUT3-expressing neurons, constituting ∼10% of PPTB-producing neurons, formed a mutual network and ∼40% of PPTB-producing neurons unilaterally received inputs from VGLUT3-expressing neurons. To answer the question of how glutamate released from GABAergic axons of PPTB-producing neurons works within the partially mutual network, we need to further investigate the localization of ionotropic and metabotropic glutamate receptors on PPTB-producing neurons. Thus, it has not yet been answered how the partially mutual network of PPTB-producing neurons processes received information, or how glutamate released from the GABAergic axons modulates the processing. However, the information processed within the mutual network of PPTB-producing neurons may be transferred to layer V pyramidal neurons and have a modulatory effect on cortical output of the pyramidal neurons, since NK3 receptor, a receptor for neurokinin B, are expressed mostly by pyramidal neurons in layer V of the cerebral cortex (Ding et al., 1996; Shughrue et al., 1996).

**Notes**

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