Coexpression of VGLUT1 and VGLUT2 in Trigeminothalamic Projection Neurons in the Principal Sensory Trigeminal Nucleus of the Rat

Shun-Nan Ge, Yun-Fei Ma, Hiroyuki Hioki, Yan-Yan Wei, Takeshi Kaneko, Noboru Mizuno, Guo-Dong Gao, and Jin-Lian Li

1Department of Anatomy, Histology and Embryology and K.K. Leung Brain Research Centre, The Fourth Military Medical University, Xi’an 710032, People’s Republic of China
2Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi’an 710038, People’s Republic of China
3Department of Morphological Brain Science, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan

ABSTRACT

VGLUT1 and VGLUT2 have been reported to show complementary distributions in most brain regions and have been assumed to define distinct functional elements. In the present study, we first investigated the expression of VGLUT1 and VGLUT2 in the trigeminal sensory nuclear complex of the rat by dual-fluorescence in situ hybridization. Although VGLUT1 and/or VGLUT2 mRNA signals were detected in all the nuclei, colocalization was found only in the principal sensory trigeminal nucleus (Vp). About 64% of glutamatergic Vp neurons coexpressed VGLUT1 and VGLUT2, and the others expressed either VGLUT1 or VGLUT2, indicating that Vp neurons might be divided into three groups. We then injected retrograde tracer into the thalamic regions, including the posteromedial ventral nucleus (VPM) and posterior nuclei (Po), and observed that the majority of both VGLUT1- and VGLUT2-expressing Vp neurons were retrogradely labeled with the tracer. We further performed anterograde labeling of Vp neurons and observed immunoreactivities for anterograde tracer, VGLUT1, and VGLUT2 in the VPM and Po. Most anterogradely labeled axon terminals showed immunoreactivities for both VGLUT1 and VGLUT2 in the VPM and made asymmetric synapses with dendritic profiles of VPM neurons. On the other hand, in the Po, only a few axon terminals were labeled with anterograde tracer, and they were positive only for VGLUT2. The results indicated that Vp neurons expressing VGLUT1 and VGLUT2 project to the VPM, but not to the Po, although the functional differences of three distinct populations of Vp neurons, VGLUT1-, VGLUT2-, and VGLUT1/VGLUT2-expressing ones, remain unsettled. J. Comp. Neurol. 518:3149–3168, 2010.

INDEXING TERMS: vesicular glutamate transporter; posteromedial ventral thalamic nucleus; posterior thalamic nuclei; fluorescence in situ hybridization; anterograde labeling; retrograde labeling

It has been well known that the trigeminal sensory nuclear complex consists of the mesencephalic trigeminal nucleus (Vmes), principal (or main) sensory trigeminal nucleus (Vp), and nucleus of the spinal tract of the trigeminal nerve (spinal trigeminal nuclear complex of Olszewski, 1950); the spinal trigeminal nuclear complex has usually been divided into oral (Vo), interpolar (Vi), and caudal (Vc) parts. The Vmes and Vp have been verified to receive impulses primarily from proprioceptors and low-threshold mechanoreceptors in the cranioorofacial regions, respectively. On the other hand, nociceptive afferents have been considered to terminate primarily in the spinal trigeminal nuclear complex, especially in the Vc, although the Vp has also been reported to contain a number of cells responding to nociceptive stimuli in the cat (Wall and Taub, 1962; Eisenman et al., 1963, 1964; Greenwood, 1973).

Grant sponsor: National Program of Basic Research of China; Grant number: G2006CB500808 (to J.-L.L.); Grant sponsor: The Ministry of Education, Culture, Sports, Science and Technology (MEXT); Grant number: 20700315 (to H.H.); Grant number: 17022020 (to T.K.); Grant number: 21650083 (to T.K.); Grant sponsor: Fujiwara Memorial Foundation (to H.H.).

*CORRESPONDENCE TO: Dr. Jin-Lian Li, Department of Anatomy, Histology and Embryology and K.K. Leung Brain Research Centre, The Fourth Military Medical University, Xi’an 710032, People’s Republic of China. E-mail: jinlian@fmmu.edu.cn; or Guo-Dong Gao, Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi’an 710038, People’s Republic of China. E-mail: gguodong@fmmu.edu.cn

Received December 16, 2009; Revised February 19, 2010; Accepted March 26, 2010

DOI 10.1002/cne.22389

Published online April 14, 2010 in Wiley InterScience (www.interscience.wiley.com)
1973; Khayyat et al., 1975; Sessle and Greenwood, 1976; Azerad et al., 1982). Thus, the Vp has been assumed to be homologous to the dorsal column nuclei and to be concerned primarily with the transmission of tactile sensibility. In fact, it has often been indicated physiologically and morphologically that information from low-threshold mechanoreceptors in the cranio-orofacial regions are conveyed to the somatosensory thalamus via the Vp (for review see Darian-Smith 1966, 1973; Brodal, 1981; Waite, 2004).

Most excitatory neurons in the central nervous system (CNS) of mammals have been considered to use glutamate as neurotransmitter (for review see Kaneko and Mizuno, 1988; Kaneko et al., 1989, 2002; Broman, 1994; Broman et al., 2000; Kaneko and Fujiyama, 2002). In accordance with this assumption, the existence of the vesicular glutamate transporters (VGLUTs), proteins that are responsible for glutamate uptake into synaptic vesicles and its release at the synapses, has been verified in axon terminals of glutamatergic neurons. These neurons are widely and abundantly distributed throughout the CNS, and the two major isoforms of VGLUTs, VGLUT1 and VGLUT2, have been considered as specific biomarkers for glutamatergic neurons (Takamori et al., 2000, 2001, 2006; Fremeau et al., 2001, 2004a,b; Fujiyama et al., 2001; Kaneko and Fujiyama, 2002; Kaneko et al., 2002).

In our previous studies with in situ hybridization (ISH) histochemistry, the Vp of the rat has been observed to contain neurons expressing both VGLUT1 and VGLUT2 mRNA (Furuta et al., 2008; Pang et al., 2009). The posteromedial ventral nucleus (VPM) and posterior nucleus (Po) of the thalamus, the two main targets of trigeminothalamic fibers arising from the Vp of the rat (Smith, 1973; Fukushima and Kerr, 1979; Kemplay and Webster, 1989; Chiaia et al., 1991a, Bennett-Clarke et al., 1992), have also been known to contain a large number of axon terminals immunoreactive for VGLUT1 or VGLUT2 (Hisano et al., 2001; Sakata-Haga et al., 2001; Kaneko et al., 2002; Barroso-Chinea et al., 2007, 2008). However, coexpression of VGLUT1 and VGLUT2 has also been indicated in some populations of axon terminals or neuronal cell bodies (Sakata-Haga et al., 2001; Hisano et al., 2002; Hioki et al., 2003; Li et al., 2003; Todd et al., 2003; Boulland et al., 2004; Billups, 2005; Danik et al., 2005; De Gois et al., 2005; Nakamura et al., 2005; Herzog et al., 2006; Lizguz-Lecznar and Skangiel-Kramska, 2007; Barroso-Chinea et al., 2007, 2008).

Thus, the main objective of the present study is to examine whether Vp neurons projecting to the VPM and Po express both VGLUT1 and VGLUT2 by use of a variety of cytochemical and morphological procedures. 1) Vp neurons were assessed for expression of VGLUT1 and/or VGLUT2 mRNA by use of dual-fluorescence ISH (dual FISH) histochemistry; 2) trigeminothalamic Vp neurons were examined for VGLUT1 or VGLUT2 mRNA by injecting a retrograde tracer, Fluorogold (FG), into the VPM and Po, combined with the FISH histochemistry; and 3) axon terminals in the VPM and Po were examined for VGLUT1 and/or VGLUT2 by injecting an anterograde tracer, cholera toxin B subunit (CTb), into the Vp, combined with triple-immunofluorescence histochemistry. The results were confirmed by electron microscopy.

**MATERIALS AND METHODS**

**Animals and primary antibodies**

All procedures of the present experiments were conducted in accordance with the committees for Animal Care and Use at the Fourth Military Medical University (Xi’an, People’s Republic of China) and at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (Kyoto, Japan). Fifteen adult male Sprague-Dawley rats (250–300 g; China SH, Xi’an, People’s Republic of China; Japan SLC, Shizuoka, Japan) were used in the present study. All efforts were made to minimize animal suffering and the number of animals used.

In the present study, we used rabbit polyclonal antibodies to VGLUT1, VGLUT2 (Hioki et al., 2003), and Fluorogold (FG; AB153; Millipore, Billerica, MA); guinea pig polyclonal antibodies to VGLUT1 and VGLUT2 (Fujiyama et al., 2001); a mouse monoclonal antibody to neuronspecific nuclear protein (NeuN; MAB377; Millipore); and a goat polyclonal antibody to cholera toxin B subunit (CTb; 703: List Biological Laboratories, Campbell, CA) as primary antibodies. All antibodies have been characterized previously.

The VGLUT1 and VGLUT2 antibodies used in the present study were produced and characterized in previous...
studies (Fujiyama et al., 2001; Hioki et al., 2003). Briefly, peptides corresponding to the C-terminal 19 amino acids (residues 552–560) of rat VGLUT1 and the C-terminal 29 amino acids (554–582) of rat VGLUT2 were synthesized with addition of N-terminal cysteine for coupling of the peptides with a carrier protein. The peptides were conjugated with an equal weight of maleimide-activated bovine albumin (Pierce, Rockford, IL). The guinea pig and white rabbit were immunized by intracutaneous injections. The guinea pig and rabbit antisera were then affinity purified by column chromatography with antigen-conjugated columns. Results of immunoblotting tests using rat brain extracts indicated that VGLUT1 and VGLUT2 antibodies specifically recognized single bands that were in register with molecular weights of VGLUT1 and VGLUT2, respectively (for guinea pig antibodies see Fujiyama et al., 2001; for rabbit antibodies see Hioki et al., 2003). When the primary antibody was preincubated with an excess amount of the antigen peptide, no immunoreactivity was observed on the rat tissue sections.

A mouse monoclonal antibody against NeuN was originally made against cell nuclei purified from mouse brain (clone A60; Mullen et al., 1992). This antibody recognizes the nuclei and cell bodies of most neuronal cell types, but not glial fibrillary acidic protein (GFAP)-positive cells, throughout the CNS of rodents (Mullen et al., 1992). The antibody also detected several bands at 46–48 kDa on Western blots with isolated mouse brain nuclei, which were thought to reflect multiple phosphorylated isoforms of NeuN (Mullen et al., 1992; Lind et al., 2005). A rabbit polyclonal antibody to FG and a goat polyclonal antibody to CTb did not bind to any endogenous epitopes in the rat brain sections (Pang et al., 2006; Ito et al., 2007).

**Retrograde tract tracing with FG or anterograde tracing with CTb**

Three rats were deeply anesthetized with sodium pentobarbital (35 mg/kg body weight) and fixed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) in the prone position. About 0.3 μl of 4% (w/v) FG (80014; Biotum, Hayward, CA) dissolved in distilled water was injected into the Po and VPM in the thalamus (3.48 mm posterior to the bregma, 2.40 mm right to the midline, and 6.30 mm deep from the brain surface). Each injection was made by pressure through a glass micropipette (internal tip diameter 15–25 μm) attached to a 1-μl Hamilton microsyringe over a period of 10 minutes. After the injection, the micropipette was left in the place for additional 20 minutes. We also injected 0.25–0.3 μl of 2% (w/v) CTb (C9903; Sigma, St. Louis, MO) in 0.1 M sodium phosphate (PB; pH 7.3) into the Vp (9.70 mm posterior to the bregma, 2.95 mm left to the midline, and 9.00 and 8.30 mm deep from the brain surface) of three rats by pressure as described above. All the rats were allowed to survive for 6 days.

**Tissue preparation**

The rats injected with CTb and three untreated rats were deeply anesthetized with sodium pentobarbital (40 mg/kg body weight), and perfused transcardially with 200 ml of 5 mM sodium phosphate-buffered 0.9% (w/v) saline (PBS; pH 7.3). The rats were further perfused with 500 ml of 4% (w/v) paraformaldehyde and 75%-saturated picric acid in 0.1 M PB. The brains were removed, cut into several blocks, and postfixed at 4°C overnight with the same fixative. After cryoprotection with 30% (w/v) sucrose in 0.1 M PB, the brain blocks were cut into 20-μm-thick transverse sections on a cryostat.

To perform ISH histochemistry, we perfused FG-injected rats and three untreated rats with 500 ml of 4% (w/v) formaldehyde in 0.1 M PB as a fixative and postfixed the brain blocks with the same fixative for 3 days at 4°C. After cryoprotection with 30% (w/v) sucrose in PBS, the brain blocks of untreated rats were cut into 18-μm-thick transverse sections on a freezing microtome. In the case of the rats injected with FG, the brain blocks containing the injection sites or the brainstem were cut into 25- or 18-μm-thick transverse sections, respectively, on a cryostat. The sections containing the injection sites were counterstained with cresyl violet.

**ISH histochemistry**

**RNA probe preparation**

Complementary DNA fragment of VGLUT1 (nucleotides 855–1788; GenBank accession No. XM_133432.2; Watakabe et al., 2006) or VGLUT2 (848–2044; NM_080853.2; Nakamura et al., 2007) was cloned into a vector pBluescript II KS(+) (Stratagene, La Jolla, CA). By using the linearized plasmids as template, we synthesized sense and antisense single-strand RNA probes with a digoxigenin or fluorescein RNA labeling kit (Roche Diagnostics, Basel, Switzerland).

**Dual FISH**

The following hybridization procedure was based on that in the previous report (Hioki et al., 2010). Briefly, free-floating sections were hybridized for 20–24 hours at 60°C with a mixture of 1 μg/ml fluorescein-labeled RNA probe for VGLUT1 and 1 μg/ml digoxigenin-labeled RNA probe for VGLUT2 in a hybridization buffer, which consisted of 5× saline sodium citrate (SSC; 1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 2% (w/v) blocking reagent (Roche Diagnostics), 50% (v/v) formamide, 0.1% (w/v) N-lauroylsarcosine (NLS), and 0.1% (w/v) SDS. After two washes in 2× SSC, 50% (v/v) formamide,
and 0.1% (w/v) NLS for 20 minutes at 60°C, the sections were incubated with 20 μg/ml ribonuclease A (RNase A) for 30 minutes at 37°C in 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediamine tetraacetic acid, and 0.5 M NaCl, followed by two washes with 0.2 x SSC containing 0.1% (w/v) NLS for 20 minutes at 37°C. Subsequently, the sections were incubated overnight at room temperature with a mixture of 1:2,000-diluted peroxidase-conjugated antidigoxigenin sheep antibody (11-207-733-910; Roche Diagnostics) and 1:1,000-diluted alkaline phosphatase-conjugated antifluorescein sheep antibody (11-426-338-910; Roche Diagnostics) in 0.1 M Tris-HCl (pH 7.5)-buffered 0.9% (w/v) saline (TSB7.5) containing 1% blocking reagent (TSB). To visualize the signals for VGLUT2 mRNA efficiently, we performed the biotinylated tyramine (BT)-glucose oxidase (GO) amplification method (Kuramoto et al., 2009) with the reaction mixture containing 1.25 μM BT, 3 μg/ml GO, 2 mg/ml beta-D-glucose, and 1% bovine serum albumin (BSA) in 0.1 M PB for 30 minutes. The sections were then incubated with 5 μg/ml Alexa488-conjugated streptavidin (S-11223; Invitrogen, Eugene, OR) in TSB for 3 hours. The sections were finally reacted with a 2-hydroxy-3-naphthoic acid-2'-phenylalanine phosphate (HNPP) fluorescence detection kit (HNPP/FastRed; Roche Diagnostics) for several hours, to detect the signals for VGLUT1 mRNA.

Double labeling with FISH and immunofluorescence

The sections of FG-injected rats were hybridized with digoxigenin-labeled VGLUT1 or VGLUT2 riboprobe. After washes and RNase treatment, the hybridized sections were incubated overnight at room temperature with a mixture of 1:2,000-diluted peroxidase-conjugated antidigoxigenin sheep antibody and 1:1,000-diluted rabbit anti-FG antibody. To amplify the signals for VGLUT1 or VGLUT2 mRNA, we performed the BT-GO amplification method as described above. Subsequently, the sections were incubated with 10 μg/ml Alexa488-conjugated streptavidin in TSB for 2 hours and then with 10 μg/ml Alexa594-conjugated donkey antibody to rabbit IgG (A-21207; Invitrogen) in TSB for 4 hours. 10 μg/ml Alexa488-conjugated donkey antibody to rabbit IgG (A-21206; Invitrogen), and 10 μg/ml Cy5-conjugated donkey antibody to mouse IgG (AP192S; Millipore) in the presence of 10% (v/v) normal guinea pig serum. The incubation was carried out at room temperature in PBS containing 0.3% (v/v) Triton X-100, 0.25% (w/v) λ-carrageenan, and 1% (v/v) donkey serum (PBS-XCD).

The sections of CTb-injected rats were incubated at room temperature sequentially with 1a) a mixture of 0.5 μg/ml goat anti-CTb antibody, 1:1,000-diluted mouse anti-NeuN antibody, and 1 μg/ml rabbit anti-VGLUT1 or anti-VGLUT2 antibody or 1b) 0.5 μg/ml goat anti-CTb antibody, 1 μg/ml rabbit anti-VGLUT1, and 1 μg/ml guinea pig anti-VGLUT2 antibody overnight; 2) 10 μg/ml biotinylated donkey antibody to goat IgG (AP180B; Millipore) for 6 hours; or 3a) a mixture of 1 μg/ml Cy3-conjugated streptavidin, 10 μg/ml Alexa488-labeled donkey antibody to rabbit IgG, and 10 μg/ml Cy5-labeled donkey antibody to mouse IgG or 3b) a mixture of 1 μg/ml Alexa647-conjugated streptavidin (S-21374; Invitrogen), 10 μg/ml Alexa594-labeled donkey antibody to rabbit IgG, and 10 μg/ml DyLight488-labeled donkey antibody to guinea pig IgG (706-485-148; Jackson Immunoresearch) overnight in the presence of 10% (v/v) normal goat serum.

Micrographs

All the sections were mounted onto glass slides and coverslipped with an aqueous mounting medium (Permount; Beckman Coulter, Fullerton, CA) or 50% (v/v) glycerol and 2.5% (w/v) triethylenediamine in PBS. The sections were then observed under a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan) with appropriate laser beams and filter sets for Alexa488 and DyLight488 (excitation 488 nm, emission 510–530 nm), Alexa594, Cy3, and FastRed (excitation 543 nm, emission 590–615 nm), or Alexa647 and Cy5 (excitation 633 nm, emission ≥650 nm). The digital images were captured in software (FV10-ASW 1.6; Olympus), modified (15–20% contrast enhancement) in Photoshop CS2 (Adobe Systems, San Jose, CA), and then saved as TIFF files.

Counting procedures

We randomly selected five sections of 18-μm thickness from each rat (n = 3 rats; total 15 sections) and then counted the number of cells with a clear nucleus. The longest diameter of cell body through the nucleus was regarded as the major diameter of the cell. The Vp neurons counted in the present study were divided into large and small cells, the large ones with the major soma diameter of ≥20 μm and small ones with the major soma diameter of <20 μm.
The numbers of the counted cells were corrected according to the method of Abercrombie (Abercrombie, 1946; Guillery, 2002): We randomly selected 50 large or small cells, measured the major diameter of the nuclei, and calculated the mean nuclear diameter; the nuclear diameter of the large or small cells was 10.21 ± 1.20 μm or 6.36 ± 0.89 μm (mean ± SD), respectively. Then, the numbers of counted cells were corrected by use of Abercrombie’s equation: number of cell = number of cells counted × T/(T + h), where T = thickness of the sections and h = the mean diameter of the nuclei of the large or small cells.

Electron microscopy

Three adult rats were anesthetized and injected with CTb into the Vp as described above. After a survival period of 5–6 days, the rats were deeply anesthetized and perfused transcardially with 500 ml of 0.1 M PB that contained 4% (w/v) paraformaldehyde, 0.1% (w/v) glutaraldehyde, and 15% (v/v) saturated picric acid. The forebrains were cut serially into 50-μm-thick transverse sections on a Vibratome (Mircorslicer DTM-1000; Dosaka EM, Kyoto, Japan) and divided into three series. Subsequently, the sections were placed in 0.05 M PB containing 25% (w/v) sucrose and 10% (v/v) glycerol for 1 hour and then freeze-thawed with liquid nitrogen. The sections were then incubated at room temperature with 50 mM Tris-HCl-buffered saline (TBS; pH 7.4) containing 20% (v/v) normal goat serum for 1 hour and processed for double immunolabeling for CTb and VGLUT1 or VGLUT2. Briefly, the sections were incubated at room temperature for 24 hours with a mixture of 1:1,000-diluted goat anti-CTb antibody and 1 μg/ml guinea pig anti-VGLUT1 or anti-VGLUT2 antibody containing 2% (v/v) normal donkey serum (TBS-D). After washes with TBS, the sections were incubated with a mixture of 1:100-diluted 1.4-nm gold-particle-conjugated rabbit antibody to goat IgG (2005; Nanoprobes, Stony Brook, NY) and 1:100 diluted biotinylated donkey antibody to guinea pig IgG overnight in TBS-D. The sections were then postfixed with 1% (w/v) glutaraldehyde in 0.1 M PB (pH 7.4) for 10 minutes and washed in distilled water. After silver enhancement in the dark with an HQ Silver Kit (2012; Nanoprobes), the sections were incubated at room temperature with 0.05M Tris-HCl (pH 7.6) containing 0.02% (w/v) 3,3’-diaminobenzidine (DAB)-4HCl (Dojinodo, Tokyo, Japan) and 0.003% (v/v) H2O2 for 20–30 minutes. Subsequently, the sections were placed in 0.1 M PB (pH 7.4) containing 1% (w/v) OsO4 for 1 hour and then counterstained with 1% (w/v) uranyl acetate in 70% ethanol for 1 hour. After dehydration, the sections were mounted on silicon-coated glass slides and flat embedded in epoxy resin (Durcupan; Fluka, Buchs, Switzerland). Once the resin had polymerized, small pieces containing the VPM and Po were cut out from the flat-embedded sections, and selected tissue pieces were cut into 60-nm-thick sections on an ultramicrotome (Reichert-Nissei Ultracut S; Leica, Vienna, Austria). The ultrathin sections were mounted on single-slot grids coated with piloform membrane and examined with an electron microscope (CM100; Philips, Eindhoven, The Netherlands).

RESULTS

Expression of VGLUT1 and/or VGLUT2 mRNAs in the trigeminal sensory nuclear complex

Expression of VGLUT1 and VGLUT2 mRNAs in the Vp

The dual FISH histochemistry for VGLUT1 and VGLUT2 mRNAs labeled many neuronal cell bodies with hybridization signals throughout the Vp (Fig. 1a,b) and revealed three populations of Vp neurons: Vp neurons expressing VGLUT1 mRNA only, those expressing VGLUT2 mRNA only, and those showing coexpression of VGLUT1 and VGLUT2 mRNAs. The coexpression of VGLUT1 and VGLUT2 mRNAs was detected in the majority of single Vp neurons (Fig. 1c,c’). In total, 3,763 Vp neurons expressing VGLUT1 mRNA and/or VGLUT2 mRNA were examined in 15 sections, which were randomly selected from three rats (Table 1); VGLUT1 mRNA or VGLUT2 mRNA was expressed, respectively, in 78.9% or 85.4% of the Vp cells examined, and both VGLUT1 and VGLUT2 mRNA signals were detected in 63.3% of the Vp cells; 14.6% or 21.1% of the Vp cells expressed VGLUT1 or VGLUT2 mRNA alone, respectively. Thus the cells expressing both VGLUT1 and VGLUT2 mRNAs constituted 81.5% of cells positive for VGLUT1 mRNA and 75.3% of cells positive for VGLUT2 mRNA. These data indicated that glutamatergic Vp neurons might more frequently express VGLUT2 than VGLUT1 and that the majority of glutamatergic Vp neurons express both VGLUT1 and VGLUT2.

The glutamatergic Vp neurons counted in the present study were further classified into two groups on the basis of the major diameter of their cell bodies: large Vp cells with the major soma diameter of ≥20 μm and small Vp cells with the major diameter <20 μm; the large cells were seen mainly in the dorsomedial part of the Vp (Vpdm) and the dorsal aspects of the ventrolateral part of the Vp (Vpvl), whereas the small ones were distributed evenly throughout the Vp (Fig. 1a–c, a’–c’). The large and small cells constituted 8.6% and 91.4% of glutamatergic Vp cells and 77.8% of the small glutamatergic...
Vp cells, whereas those for VGLUT2 mRNA were found in 15.7% of the large glutamatergic Vp cells and 92.0% of the small glutamatergic Vp cells. Thus it appeared that the large glutamatergic Vp cells might express VGLUT1 much more frequently than VGLUT2, whereas the small glutamatergic Vp cells might express VGLUT2 a little more frequently than VGLUT1. The cells dually labeled with both VGLUT1 and VGLUT2 mRNA signals were also

Figure 1. Dual-fluorescence in situ hybridization histochemistry for VGLUT1 and VGLUT2 mRNAs in the Vp, Vo, Vi, and Vc. a–c,a′–c′: The signals for VGLUT1 and VGLUT2 mRNAs were visualized with FastRed (red fluorescence images converted to magenta ones) and Alexa488 (green), respectively. The fields in a′,b′ are the same as those in a,b; the identical fields were taken under different excitation. Merged fluorescence images are shown in c,c′. The framed area in c is magnified in c′. Arrows or single arrowheads in c′ point to cells positive for VGLUT1 mRNA only (magenta) or those positive for VGLUT2 mRNA only (green); cells positive for both VGLUT1 and VGLUT2 mRNAs (white) are indicated with double arrowheads. d–f: In contrast, colocalization of VGLUT1 and VGLUT2 mRNA signals is hardly observed in the Vo, Vi, or Vc. IRT, intermediate reticular nucleus; MdD, dorsal part of medullary reticular nucleus; RCRt, parvicellular reticular nucleus. For other abbreviations see list. Scale bars = 300 μm in a (applies to a–c); 20 μm in a′ (applies to a′–c′); 300 μm in d–f.
observed in 5.9% or 69.8% of the large or small glutamatergic Vp cells, respectively.

Expression of VGLUT1 and VGLUT2 mRNAs in the Vmes, Vo, Vi and Vc

The Vmes contained neuronal cells expressing VGLUT1 mRNA, but no Vmes cells were observed to express VGLUT2 mRNA (data not shown; cf. Fig. 4 of Pang et al., 2009). On the other hand, the signals for VGLUT1 and VGLUT2 mRNAs were distributed in the Vo, Vi, and Vc (Fig. 1d–f). The majority of neurons with VGLUT1 mRNA signals in the Vo, Vi, and Vc appeared to be of the large size. Moderate numbers of neurons with VGLUT1 mRNA signals were also distributed throughout the Vi (Fig. 1e), whereas rather small numbers of them were scattered within the Vo and Vc (Fig. 1d,f). In contrast, many neuronal cells with VGLUT2 mRNA signals were distributed in the Vo and Vc (Fig. 1d,f), and moderate numbers of them were observed in the Vi (Fig. 1e). The vast majority of Vo and Vc neurons with VGLUT2 mRNA signals were of the small size and were distributed throughout the Vo and Vc. In the Vi, VGLUT2 mRNA signals were found in both small and large neurons; these large Vi neurons with VGLUT2 mRNA signals were distributed mainly in the rostral part of the Vi. The colocalization of the signals for VGLUT1 and VGLUT2 mRNAs was hardly detected in the Vo, Vi, or Vc (Fig. 1d–f).

Immunoreactivities for VGLUT1 and VGLUT2 in the VPM and Po

The present data obtained by the dual FISH histochernistry for VGLUT1 and VGLUT2 mRNAs indicated the existence of many Vp neurons expressing VGLUT1 and VGLUT2. Thus, in turn, immunoreactivities for VGLUT1 and VGLUT2 were examined in the VPM and Po, i.e., the two major projection targets of trigeminothalamic fibers arising from the Vp (Smith, 1973; Fukushima and Kerr, 1979; Silverman and Kruger, 1985; Bruce et al., 1987; Kemplay and Webster, 1989; Chiaia et al., 1991a; Bennett-Clarke et al., 1992).

Immunoreactivities for VGLUT1 and VGLUT2 in the VPM and Po were observed as clusters of small granular structures, which were distributed throughout the neuropil but not in somatic or dendritic profiles of neuronal cells. Large numbers of small granular structures intensely positive for VGLUT1 were distributed densely throughout neuropil, whereas the density of VGLUT2-
immunopositive small granules was much lower than that of VGLUT1-immunopositive small ones. Triple immunofluorescence labeling for VGLUT1/VGLUT2/NeuN revealed that both VGLUT1-immunopositive small granules and VGLUT2-immunopositive ones were frequently in close apposition to somatic and dendritic profiles of neuronal cells positive for NeuN (Fig. 2). Colocalization of VGLUT1 and VGLUT2 immunoreactivities in single, small granules on neuronal profiles positive for NeuN was frequently detected in the VPM (Fig. 2a,a'), but rarely in the Po (Fig. 2b,b').

These results indicated that both VGLUT1-immunopositive axon terminals and VGLUT2-immunopositive ones might be in synaptic contact with neurons in the VPM and Po. Furthermore, the existence of single axon terminals positive for both VGLUT1 and VGLUT2 was indicated in the VPM but not in the Po.

Retrograde tract-tracing experiments combined with FISH histochemistry for VGLUT1 or VGLUT2 mRNA

To examine whether the Vp neurons expressing VGLUT1 or VGLUT2 mRNA might project contralaterally to the VPM and Po, retrograde tract-tracing combined with FISH histochemistry for VGLUT1 or VGLUT2 mRNA was performed in three rats after injecting FG unilaterally into the VPM and Po (Fig. 3). Many neuronal cell bodies were retrogradely labeled with FG throughout the Vp contralateral to the FG injection, whereas only small numbers
Figure 3. Retrograde labeling of neurons projecting to the VPM and Po. a,b: FG was injected into the VPM and Po on the right side (a). The cytoarchitectonic areas involved in the injection site was confirmed by observation of the sections counterstained with cresyl violet (b). Some layer VI neurons in the neocortex, presumably corticothalamic neurons, were labeled with FG (a). b,b′,c,c′: FG immunoreactivity and VGLUT1 or VGLUT2 mRNA signals were visualized with Alexa594 (red fluorescence images converted to magenta ones) and Alexa488 (green), respectively. Neuronal cells retrogradely labeled with FG were distributed throughout the Vp on the left side (b,c). Most of these FG-labeled cells were positive for VGLUT1 or VGLUT2 mRNA signals: In b′ and c′, FG-labeled neuronal cells positive for VGLUT1 or VGLUT2 mRNA are indicated by double arrowheads (white). Additionally, arrows point to cells positive for FG but negative for VGLUT1 or VGLUT2 mRNA (magenta), and single arrowheads indicate cells positive for VGLUT1 or VGLUT2 mRNA but not labeled with FG (green). For abbreviations see list. Scale bars = 1 mm in a (applies to a,a′); 350 μm in b (applies to b,c); 30 μm in b′ (applies to b′,c′).
of FG-labeled cells were observed in the Vp ipsilateral to the FG injection. The majority of these FG-labeled neurons were simultaneously labeled with VGLUT1 or VGLUT2 mRNA signals. The density of these labeled neurons appeared somewhat higher in the Vpvl than in the Vpdm (Fig. 3b,c). Most FG-labeled Vp neurons positive for VGLUT1 or VGLUT2 mRNA were small cells (Fig. 3b,c). On the other hand, FG-labeled Vp neurons of large size were hardly labeled with VGLUT1 or VGLUT2 mRNA signals.

In three rats, in which the site of FG injection covered largely the VPM and Po areas (cf. Fig. 3a,a'), the numbers of Vp neurons labeled with FG and/or VGLUT1 mRNA signals were counted on the side contralateral to the FG injection (Table 3): From among a total of 3,744 Vp neurons counted, 2,261 cells were dually labeled with VGLUT1 mRNA signals and FG, constituting 74.9% of Vp neurons labeled with VGLUT1 mRNA signals and 75.7% of FG-labeled Vp neurons. Counts were also made for Vp neurons labeled with FG and/or VGLUT2 mRNA signals (Table 4): From among a total of 3,706 Vp neurons labeled with VGLUT2 mRNA signals and/or FG, 2,599 cells were dually labeled with VGLUT2 mRNA signals and FG, constituting 79.9% of Vp neurons labeled with VGLUT2 mRNA signals and 85.2% of FG-labeled Vp neurons. These data indicated that about 75% of VGLUT1-expressing Vp neurons and about 80% of VGLUT2-expressing ones might send their axons to the contralateral VPM/Po and that VGLUT1 and VGLUT2 might be expressed, respectively, in about 76% and 85% of Vp neurons sending their axons to the contralateral VPM/Po.

Anterograde tract-tracing experiments combined with immunofluorescence staining for VGLUT1 or VGLUT2 Confocal laser scanning microscopy

Anterograde tract-tracing experiments combined with fluorescence immunohistochemistry for VGLUT1 and/or VGLUT2 were performed in three rats after unilateral injection of CTb into the Vp; neuronal profiles in the Vp were also immunostained with NeuN. The sites of CTb injection in the rats covered nearly the whole extent of the Vpdm and more than two-thirds of the Vpvl (Fig. 4a). The anterograde labeling in the VPM and Po contralateral to the Vp injected with CTb was observed as small granular structures labeled with CTb. Large numbers of small granular structures labeled with CTb were also detected in the Po; CTb-

| TABLE 3. Numbers of Vp Neurons Labeled With VGLUT1 mRNA and/or FG¹ |
|------------------|------------------|------------------|------------------|
| Rat 7 | Rat 11 | Rat 12 | Total of the three rats |
| 1) Cells singly labeled with VGLUT1 mRNA only | 259 | 275 | 223 | 757 |
| 2) Cells singly labeled with FG only | 246 | 265 | 215 | 726 |
| 3) Cells dually labeled with VGLUT1 mRNA signals and FG | 757 | 778 | 726 | 2,261 |
| 4) Cells labeled with VGLUT1 mRNA signals (1 + 3) | 1,016 | 1,053 | 949 | 3,018 |
| 5) Cells labeled with FG (2 + 3) | 1,003 | 1,043 | 941 | 2,987 |
| 6) Cells labeled with VGLUT1 mRNA and/or FG (1 + 2 + 3) | 1,262 | 1,318 | 1,164 | 3,744 |
| 7) Ratio of dually labeled cells in VGLUT1 cells (3/4 × 100%) | 74.5% | 73.9% | 76.5% | 74.9% |
| 8) Ratio of dually labeled cells in FG-labeled cells (3/5 × 100%) | 75.5% | 74.6% | 77.2% | 75.7% |

1Retrograde tract tracing was performed in three rats, which were injected with FG unilaterally into the VPM and Po; the Vp contralateral to the site of FG injection was examined. Counts were made on 15 sections of 18 μm thickness in three rats (five sections in each rat).

| TABLE 4. Numbers of Vp Neurons Labeled With VGLUT2 mRNA and/or FG¹ |
|------------------|------------------|------------------|------------------|
| Rat 7 | Rat 11 | Rat 12 | Total of the three rats |
| 1) Cells singly labeled with VGLUT2 mRNA only | 219 | 241 | 194 | 654 |
| 2) Cells singly labeled with FG only | 153 | 179 | 121 | 453 |
| 3) Cells dually labeled with VGLUT2 mRNA signals and FG | 867 | 893 | 839 | 2,599 |
| 4) Cells labeled with VGLUT2 mRNA signals (1 + 3) | 1,086 | 1,134 | 1,033 | 3,253 |
| 5) Cells labeled with FG (2 + 3) | 1,020 | 1,072 | 960 | 3,052 |
| 6) Cells labeled with VGLUT2 mRNA and/or FG (1 + 2 + 3) | 1,239 | 1,313 | 1,154 | 3,706 |
| 7) Ratio of dually labeled cells in VGLUT2 cells (3/4 × 100%) | 79.8% | 78.7% | 81.2% | 79.9% |
| 8) Ratio of dually labeled cells in FG-labeled cells (3/5 × 100%) | 85.0% | 83.3% | 87.4% | 85.2% |

1Retrograde tract tracing was performed in three rats, which were injected with FG unilaterally into the VPM and Po; the Vp contralateral to the site of FG injection was examined. Counts were made on 15 sections of 18 μm thickness in three rats (five sections in each rat).
Figure 4. Anterograde tracing of projection fibers from the Vp to the VPM/Po with CTb. a,b: After the injection of CTb into the Vp on the left side (a), immunoreactivities for CTb and NeuN were visualized with Cy3 (red fluorescence images converted to magenta ones) and Cy5 (converted to blue), respectively. The axon terminals were observed frequently in the VPM and less in the Po on the right side (b); the framed area in the Po in b is magnified in b’. c–d’: The immunoreactivities for VGLUT1 or VGLUT2, CTb, and NeuN were visualized with Alexa488 (green), Cy3 (magenta), and Cy5 (blue). Double arrowheads indicate the colocalization of immunoreactivities for CTb and VGLUT1 or VGLUT2 (white). Single arrows in c,d point to axon terminals labeled with CTb but not with VGLUT1 or VGLUT2 immunoreactivity. Arrowheads indicate axon terminals showing VGLUT2 immunoreactivity but not labeled with CTb. 3V, third ventricle; f, fornix; ic, internal capsule; mt, mammillothalamic tract. For other abbreviations see list. Scale bars = 500 μm in a; 1 mm in b; 100 μm in b’; 5 μm in c (applies to c–d’).
labeled granular structures, however, were apparently much less numerous in the Po than in the VPM (Fig. 4b, b').

Subsequently, the sections stained by triple-immunofluorescence labeling for VGLUT1/NeuN/CTb, VGLUT2/NeuN/CTb, or VGLUT1/VGLUT2/CTb were examined under a confocal laser scanning microscope. In the VPM, most of CTb-labeled small granules showed VGLUT2 immunoreactivity, and CTb-labeled small granules with VGLUT2 immunoreactivity were often in close apposition with neuronal profiles that are positive for VGLUT2 but not labeled with VGLUT1 is indicated by a double arrowhead, and one of the axon terminals that are positive for VGLUT2 but not labeled with CTb or VGLUT1 is indicated by a single arrowhead. Scale bar = 5 μm.

Figure 5. Triple-immunofluorescence labeling for VGLUT1, VGLUT2, and CTb in the VPM after CTb injection into the contralateral Vp. a–c: After the injection of CTb into the Vp on the left side (Fig. 4a), VGLUT1, VGLUT2, and CTb immunoreactivities in the VPM and Po on the right side were visualized with Alexa594 (red fluorescence images converted to magenta ones), DyLight488 (green), and Cy5 (converted to blue), respectively. d: In the VPM on the right side, most of the CTb-labeled axon terminals were positive for both VGLUT1 and VGLUT2 (arrows, calamine blue). Additionally, one of the CTb-labeled axon terminals that are positive for VGLUT2 but not labeled with VGLUT1 is indicated by a double arrowhead, and one of the axon terminals that are positive for VGLUT2 but not labeled with CTb or VGLUT1 is indicated by a single arrowhead. Scale bar = 5 μm.

labeled granular structures, however, were apparently much less numerous in the Po than in the VPM (Fig. 4b, b').

Subsequently, the sections stained by triple-immunofluorescence labeling for VGLUT1/NeuN/CTb, VGLUT2/NeuN/CTb, or VGLUT1/VGLUT2/CTb were examined under a confocal laser scanning microscope. In the VPM, most of CTb-labeled small granules showed VGLUT2 immunoreactivity, and CTb-labeled small granules with VGLUT2 immunoreactivity were often in close apposition with neuronal profiles labeled with NeuN (Fig. 4c'). CTb-labeled granular structures with VGLUT1 immunoreactivity in the VPM were apparently much less numerous than those with VGLUT2 immunoreactivity; some of these CTb-labeled small granules with VGLUT1 immunoreactivity were in close contact with neuronal profiles (Fig. 4c). Rather small numbers of CTb-labeled small granular structures with VGLUT2 immunoreactivity were also found in the Po; some of them were in close apposition to NeuN-labeled neuronal profiles (Fig. 4d'). On the other hand, no CTb-labeled granular structures with VGLUT1 immunoreactivity were detected in the Po (Fig. 4d). Con-

focal laser scanning microscopy of sections stained by triple-immunofluorescence labeling for VGLUT1/VGLUT2/CTb further revealed that the majority of single CTb-labeled small granules showed both VGLUT1 and VGLUT2 immunoreactivities in the VPM contralateral to the Vp injected with CTb (Fig. 5), indicating that Vp neurons expressing both VGLUT1 and VGLUT2 might send their axon terminals to the contralateral VPM.

We also counted the number of CTb-labeled axon terminals, including those labeled with CTb alone, those dually labeled with CTb and VGLUT1 or VGLUT2 immunoreactivity, and those triply labeled with CTb, VGLUT1, and VGLUT2 immunoreactivities in the VPM or Po areas where CTb-labeled axon terminals were distributed most densely: Among 800 CTb-labeled axon terminals counted in VPM areas, 72.3% (578/800) or 83.6% (669/800) also showed VGLUT1 or VGLUT2 immunoreactivity, respectively, and 79.9% (567/800) showed both VGLUT1 and VGLUT2 immunoreactivities. On the other hand, when 200 CTb-labeled axon terminals were counted in Po areas, 89.5% of them (179/200) also showed VGLUT2 immunoreactivity, but none of them showed VGLUT1 immunoreactivity.

Electron microscopy

Precise subcellular localization of VGLUT1 or VGLUT2 immunoreactivity in synaptic terminals of Vp neurons was examined by electron microscopy in the VPM and Po in additional rats. After injections of CTb unilaterally into the Vp, immunoreactivities for CTb and VGLUT1 or VGLUT2 were visualized by the immunogold-silver method and immunoperoxidase method, respectively, in the VPM and Po (Fig. 6). Axon terminals filled with clear, round synaptic vesicles were often labeled with dark products of the peroxidase reaction for VGLUT1 or VGLUT2 immunoreactivity in the VPM and Po, and some of these axon terminals were also labeled simultaneously with silver grains. These axon terminals labeled with both silver grains and peroxidase reaction products for VGLUT1 immunoreactivity were occasionally encountered in the VPM (Fig. 6a) but not in the Po. On the other hand, the axon terminals dually labeled with silver grains and peroxidase reaction products for VGLUT2 immunoreactivity were often observed in the VPM (Fig. 6a') and were occasionally found in the Po (Fig. 6b).

The electron microscopic analysis indicated that axon terminals of VGLUT2-expressing Vp neurons projecting to the contralateral thalamus might make synaptic contact on VPM and Po neurons and that VGLUT1-expressing Vp
neurons projecting to the contralateral thalamus might make synapses on VPM neurons. However, no data obtained in the present study indicated direct projections of VGLUT1-expressing Vp neurons to the Po.

DISCUSSION
Ascending projections from the Vp
Trigeminalthamic fibers arising from the Vp of the rat project primarily to the VPM (for review see Veinante and Deschênes, 1999; Waite, 2004). In addition to the VPM, however, the Vp of the rat has been reported to project to the Po (Smith, 1973; Chiaia et al., 1991a; Williams et al., 1994), medial geniculate nucleus (Peschanski, 1984), superior colliculus (Killackey and Erzurumlu 1981; Huerta et al., 1983; Bruce et al., 1987), ventral part of the zona incerta (Smith, 1973; Peschanski, 1984; Roger and Cadusseau, 1985; Shamah-Lagnado et al., 1985; Nicolelis et al., 1992; Lavallée et al., 2005), and anterior pretectal nucleus (Yoshida et al., 1992). These previous data indicate that the Vp sends its ascending projection fibers massively to the VPM and more sparsely to the Po; this was confirmed in the present study by the anterograde tract-tracing experiment with CTb.

Figure 6. Electron micrographs of axon terminals labeled for CTb and VGLUT1 or VGLUT2 in the VPM and Po after CTb injection into the contralateral Vp. CTb is labeled with silver grains by the immunogold-silver method, whereas VGLUT1 or VGLUT2 immunoreactivity was labeled with DAB reaction products by the immunoperoxidase method. a,a': Axon terminals (T) positive for CTb and VGLUT1 (a) or VGLUT2 (a') form asymmetrical synapses with dendritic profiles (Den). b: An axon terminal positive for CTb and VGLUT2 makes an asymmetrical synapse with a dendritic profile. Arrows indicate postsynaptic densities. Scale bars = 0.1 μm in a (applies to a,a'); 0.2 μm in b.
Cytoarchitectonic divisions of the Vp

The cat Vp is composed mainly of medium-sized nerve cells, and these Vp cells have been known to accumulate most densely in the dorsomedial part of the nucleus (Torvik, 1957). It has also been demonstrated that the dorsomedial part of the cat Vp provides the uncrossed trigeminothalamic fibers, whereas the ventral part provides crossed trigeminothalamic projections (Torvik, 1957; Burton and Craig, 1979; Kawamura et al., 1980; Matsushita et al., 1982; Shigenaga et al., 1983; Yasui et al., 1983). Thus, the cat Vp has usually been divided into the dorsal Vp (Vpd) and the ventral Vp (Vpv). A similar organization has also been observed in the monkey Vp (Walker, 1939; Carpenter, 1957; Mizuno, 1970; Smith, 1975). Electrophysiological studies have further indicated that the Vp of the cat projects heavily to the ventroposterior thalamus on both sides and that ipsilateral projections arise only from intraoral structures, including the oral mucosa, periodontal ligament, and dental pulp (Woda et al., 1977, 1983; Azerad et al., 1982). Transganglionic tract-tracing experiments in the dog and cat have also revealed that primary afferent fibers from the dental pulps terminate to the Vp as well as to all of the three divisions of the spinal trigeminal nuclear complex (Vo, Vi, Vc). In the monkey, primary afferent fibers from the dental pulps have been reported to end not only in the Vpd but also in the dorsal aspect of the Vpv (Takemura et al., 1993). In the cat, however, such fibers have been observed to terminate in the Vpd but not in the Vpv (Ishidori et al., 1986; Shigenaga et al., 1986, 1989; see also Marfurt, 1981). Thus, for the cat Vp, it has been presumed that the Vpd receives primary afferent fibers from the intraoral structures and sends ascending projection fibers mainly to the ipsilateral thalamus, whereas the Vpv receives primary afferent fibers mainly from the facial structures as well as from the intraoral structures and projects to the contralateral thalamus.

On the other hand, the rat Vp has been observed to send many projection fibers to the contralateral thalamus, in particular to the VPM and Po, but only a small amount of projections to the ipsilateral thalamus (Fukushima and Kerr, 1979; Silverman and Kruger, 1985; Bruce et al., 1987; Kemplay and Webster, 1989; Chiáia et al., 1991a; Bennett-Clarke et al., 1992). Fukushima and Kerr (1979) have reported that trigeminothalamic fibers projecting to the ipsilateral thalamus arise from the dorsal one-third of the most rostral part of the Vp. The rat Vp, however, exhibits a rather uniform cytoarchitectonic feature through its extent, and it seems impossible to divide the nucleus unequivocally into any cytoarchitectonic subdivisions (Torvik, 1956). It has also been reported that the primary afferent fibers from intraoral structures to the rat Vp terminate within a restricted area along the border of the most dorsomedial part of the Vp (Takemura et al., 1991; see also Marfurt and Turner, 1984). Thus, in the present study, the Vp was divided rather arbitrarily into the Vpd and the Vpv, according to a stereotaxic atlas (Paxinos and Watson, 1998).

Glutamatergic neurons in the Vp

The existence of glutamatergic neurons in the rat Vp was indicated immunohistochemically by use of monoclonal antibodies specific for fixative-modified glutamate (Magnusson et al., 1987) or phosphate-activated glutaminase (Kaneko et al., 1989) and more recently confirmed by ISH histochemistry for VGLUT1 or VGLUT2 mRNA (Furuta et al., 2008; Pang et al., 2009). The present study revealed three populations of Vp neurons by dual FISH histochemistry for VGLUT1 and VGLUT2 mRNAs: Vp neurons expressing VGLUT1 mRNA only, those expressing VGLUT2 mRNA only, and those expressing both VGLUT1 and VGLUT2 mRNAs (Fig. 1, Tables 1, 2). The data indicate that the majority of glutamatergic Vp neurons (64.3%) express both VGLUT1 and VGLUT2 (Table 2). The glutamatergic Vp cells positive for VGLUT1 and/or VGLUT2 mRNA signals were divided into two groups rather arbitrarily on the basis of their major diameter of the cell body: small cells with a major diameter of <20 μm and large cells with a major diameter of ≥20 μm. The large or small cells constituted about 9% and 91%, respectively, of cells positive for VGLUT1 and/or VGLUT2 mRNA signals (Table 2). It was further indicated that VGLUT1 and VGLUT2 were coexpressed in about 6% of large glutamatergic Vp neurons and in about 70% of small ones. The large glutamatergic Vp cells were observed mainly in the Vpd and the dorsal aspect of the Vpv, whereas the small ones were distributed throughout the Vp (Fig. 1a,b).

Glutamatergic projections from the Vp to the thalamus

**Glutamatergic Vp neurons projecting to the thalamus**

The existence of glutamatergic trigeminothalamic neurons in the sensory trigeminal complex was first suggested morphologically in the rat by means of retrograde tract-tracing combined with immunohistochemistry for glutamate after injecting wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) into the VPM (Magnusson et al., 1987); the greatest number of neuronal profiles dualy labeled with WGA-HRP and immunoreactivity for glutamate was found in the Vp. On the other hand, in the present study, glutamatergic neurons were identified on the basis of the expression of mRNA or immunoreactivity for VGLUT1 or VGLUT2. We performed
Axon terminals of glutamatergic Vp neurons projecting to the thalamus

This presents data suggest that almost all Vp neurons sending their axons to the VPM and/or Po are small neurons, although the Vp contained not only small glutamatergic neurons but also large glutamatergic neurons. Glutamatergic neurons projecting to the thalamus seem generally to be made up of axon collaterals of the large ones, were retrogradely labeled with FG throughout the Vp. The vast majority of the FG-labeled Vp neurons were distributed in the Vp contralateral to the VPM/Po injected with FG; 74.9% or 79.9% of these FG-labeled neurons positive for VGLUT1 or VGLUT2, respectively (Tables 3 and 4). A few of the FG-labeled small neurons positive for VGLUT1 or VGLUT2 were also scattered in the dorsal part of the Vp ipsilateral to the FG injection.

Thus the present data suggest that almost all Vp neurons sending their axons to the VPM and/or Po were small neurons, although the Vp contained not only small glutamatergic neurons but also large glutamatergic neurons. According to Fukushima and Kerr (1979), profuse crossed connections from the rat Vp to the ventrobasal thalamic Vp neurons arise exclusively from medium-sized and small cells but not from the large cells; these findings appear to be in good accordance with the results of retrograde tract-tracing in the present study. Bennett-Clarke et al. (1992) also reported that many small Vp cells, including parvalbumin-immunopositive cells, were retrogradely labeled by tracer injection into the ventrobasal and/or superior colliculus, whereas calbindin-immunopositive, large Vp cells in the dorsal half of the Vp were not labeled by such tracer injections. Insofar as the rat Vp has been reported to project to many brain regions other than the thalamus, including the zona incerta, superior colliculus, cerebellum, trigeminal motor nucleus, facial nucleus, hypoglossal nucleus, vestibular nuclei, and parabrachial nuclei (for review see Waite, 2004), large Vp cells, including glutamatergic ones, might send their axons to these brain regions other than the thalamus, although such an assumption has not been verified so far.

Axon terminals of glutamatergic Vp neurons projecting to the thalamus

Anterograde tract-tracing combined with immunofluorescence histochemistry for VGLUT1 or VGLUT2 was also performed in the present study after injection of an anterograde tracer CTb into the Vp. Confocal laser scanning microscopy of the sections stained for triple-immunofluorescence labeling for VGLUT1/VGLUT2/NeuN indicated that most of synaptic terminals of projection fibers from the Vp to the VPM might express VGLUT2 much more frequently than VGLUT1. In the Po, some synaptic terminals of the projection fibers from the Vp were also indicated to express VGLUT2, but none of these synaptic terminals was indicated to express VGLUT1 (Fig. 4). These results were further confirmed electron microscopically (Fig. 6), and, more importantly, coexpression of VGLUT1 and VGLUT2 in single Vp neurons projecting to the contralateral VPM was indicated by confocal laser scanning microscopy of the sections labeled for VGLUT1/VGLUT2/CTb: The majority of CTb-labeled single axon terminals showed both VGLUT1 and VGLUT2 immunoreactivities in the VPM contralateral to the Vp injected with CTb (Fig. 5). Thus the present study provides several lines of morphological evidence indicating that Vp neurons expressing both VGLUT1 and VGLUT2 might project directly to the VPM.

Possible functions of glutamatergic Vp neurons projecting to the thalamus

It has been known that trigeminothalamic Vp neurons largely respond to stimulation of a single vibrissa: According to Jacquin et al. (1988), 69% of the Vp cells that responded within 1.2 ± 0.2 msec after stimulation of the trigeminal ganglion were vibrissa-sensitive, and 80% of these vibrissa-sensitive cells responded to only a single vibrissa; the remaining responded only to guard hair, skin, or nociceptors. Veinante and Deschênes (1999) also observed that only 4% of axons of trigeminothalamic Vp neurons send their axons to both the VPM and Po. Thus the Po innervations of the Vp do not seem generally to be made up of axon collaterals of the Vp cells sending their axons to the VPM.

The Journal of Comparative Neurology | Research in Systems Neuroscience 3163
receptive fields projected to the VPM but not to the Po. On the other hand, the present data indicated that trigeminothalamic Vp cells expressing VGLUT1 alone projected to the VPM but not Po, although the Vp cells expressing VGLUT1 mRNA alone constituted only 14.6% of the total population of Vp cells expressing VGLUT1 and/or VGLUT2 mRNA (Table 1).

The present study, together with the reported data, suggests that the rat Vp contains many glutamatergic neurons that convey information from the vibrissae to the VPM and/or Po. It has been proposed that vibrissal information is conveyed to the somatosensory cortex via two parallel pathways; the lemniscal and the paralemniscal pathways, which ascend via the VPM and Po, respectively (for review see Diamond et al., 1992; Ahissar and Zacksenhouse, 2001; Waite, 2004). These two thalamic relay nuclei (VPM and Po) receive their main afferent input from the Vp and Vi (Diamond et al., 1992), and project to complementary regions of the somatosensory cortex (Lu and Lin, 1993): The VPM projects to the barrels in layer IV and layers Vb and Vla, whereas the Po projects to layers I and Va and to the interbarrel septa in layer IV (Koralek et al., 1988; Chmielowska et al., 1989; Lu and Lin, 1993; Land et al., 1995). The primary input to the VPM arrives from the Vp (Bruce et al., 1987; Peschanski, 1984; Veinante et al., 2000), whereas the primary input to the Po arrives from the Vi (Chiaia et al., 1991a; Williams et al., 1994; Pierret et al., 2000). The VPM of the rat is for the most part organized in so-called barreloids, which convey the information from single vibrissa to the barrels in layer IV of the somatosensory cortex (Land and Simons, 1985; Haidarliu and Ahissar, 2001). Such somatotopical organization is also observed in the sensory trigeminal nuclei, including the Vp (Arvidsson, 1982), but not in the Po. Although the Po receives the majority of its ascending input from the axonal branches of large Vc cells with receptive fields composed of multiple vibrissae (Jacquin et al., 1989; Williams et al., 1994; Veinante and Deschênes, 1999; Veinante et al., 2000), relay cells of the Po respond only poorly to vibrissa deflection (Chiaia et al., 1991a; Diamond et al., 1992); many Po cells respond to stimuli of the skin, mucosa, or muscle afferents, and a few are nociceptive (Chiaia et al., 1991b).

Thus it has been suggested that spatial resolution is higher in the lemniscal pathway than in the paralemniscal pathway and that the lemniscal or the paralemniscal system processes spatially encoded or temporally encoded information, respectively (Ahissar et al., 2000; Sosnik et al., 2001). It has also been proposed that signals conveyed by the lemniscal pathway via the VPM involve processing of object identity (Ahissar and Zackenhouse, 2001; Yu et al., 2006), whereas the type of information conveyed to the cortex by the paralemniscal pathway via Po relates to the processing of vibrissal information during active whisking (Pierret et al., 2000; Yu et al., 2006). Among the three classes of glutamatergic Vp neurons identified in the present study, Vp neurons expressing VGLUT1 alone projected to the VPM but not to the Po and therefore might constitute the lemniscal path. On the other hand, Vp neurons expressing VGLUT2, including those expressing both VGLUT1 and VGLUT2, projected to both the VPM and the Po and might participate in the formation of both the lemniscal and the paralemniscal pathways.

Functional significance of the existence of two classes of vesicular glutamate transporters, VGLUT1 and VGLUT2

It has been reported that the regional expressions of VGLUT1 and VGLUT2 are highly complementary, and the use of the proteins appears to be largely segregated to different neuronal populations in the CNS. VGLUT1 is expressed mainly in the telencephalic regions, whereas VGLUT2 is produced principally in the diencephalic and lower brainstem regions (Fremeau et al., 2001; Fujisawa et al., 2001; Kaneko and Fujisawa, 2002; Kaneko et al., 2002; Varoqui et al., 2002). Each population of neurons expressing VGLUT1 or VGLUT2 has often been assumed to define a distinct element in a functional system (Hioki et al., 2003; Fujisawa et al., 2006; Graziano et al., 2008). On the other hand, however, coexpression of VGLUT1 and VGLUT2 was also reported in the neocortex, hippocampus, cerebellum, and several thalamic nuclei in the adult and postnatal rodents (Sakata-Haga et al., 2001; Hisano et al., 2002; Hioki et al., 2003; Danik et al., 2005; Nakamura et al., 2005; Herzog et al., 2006; Barroso-Chinea et al., 2007); the present study has further provided new findings indicating coexpression of VGLUT1 and VGLUT2 in Vp neurons projecting to the VPM/Po.

Although VGLUT1 and VGLUT2 have been known to show 82% amino acid identity and no differences in biochemical and pharmacological properties (Ni et al., 1994; Aihara et al., 2000), functional differences between VGLUT1 and VGLUT2 have been suggested. Fremeau et al. (2001, 2004a,b) postulated that VGLUT1 or VGLUT2 might be expressed at synapses with low- or high-release probability, respectively. According to Varoqui et al. (2002), VGLUT1 is associated with many synapses exhibiting activity-dependent synaptic plasticity such as long-term potentiation, whereas VGLUT2 is expressed primarily in sensory and autonomic pathways that display high-fidelity neurotransmission. These presumptions, however, do not seem to be sufficiently verified so far. Thus, the functional significance of the existence of the three distinct populations of glutamatergic neurons, i.e., those
expressing VGLUT1, VGLUT2, or VGLUT1/VGLUT2, remains to be settled.

LITERATURE CITED


