

Title

Preprodynorphin-Expressing Neurons Constitute a Large Subgroup of Somatostatin-Expressing GABAergic Interneurons in the Mouse Neocortex

Running title:

Preprodynorphin interneurons in neocortex

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ABSTRACT

Dynorphins, leumorphin and neoendorphins are preprodynorphin (PPD)-derived peptides and ligands for kappa-opioid receptors. Using an antibody to PPD C-terminal, we investigated the chemical and molecular characteristics of PPD-expressing neurons in mouse neocortex. PPD-immunopositive neuronal somata were distributed most frequently in layer 5 and less frequently in layers 2–4 and 6 throughout neocortical regions. Combined labeling of immunofluorescence and fluorescent mRNA signals revealed that almost all PPD-immunopositive neurons expressed glutamic acid decarboxylase but not vesicular glutamate transporter, indicating their GABAergic characteristics, and that PPD-immunopositive neurons accounted for 15% of GABAergic interneurons in the primary somatosensory area. As GABAergic interneurons were divided into several groups by specific markers, we further examined the chemical characteristics of PPD-expressing neurons by the double immunofluorescence labeling method. More than 95% of PPD-immunopositive neurons were also somatostatin (SOM)-immunopositive in the primary somatosensory, primary motor, orbitofrontal and primary visual areas, but only 24% were SOM-immunopositive in the medial prefrontal cortex. In the primary somatosensory area, PPD-immunopositive neurons constituted 50%, 79%, 55% and 17% of SOM-immunopositive neurons in layers 2-3, 4, 5 and 6, respectively. Although SOM-expressing neurons contained calretinin-, neuropeptide Y- and nitric oxide synthase- and reelin-expressing neurons as subgroups, only reelin immunoreactivity was detected in many PPD-immunopositive neurons. These results indicate that PPD-expressing neurons constitute a large subgroup of SOM-expressing cortical interneurons, and the PPD/SOM-expressing GABAergic neurons might serve not only as inhibitory elements in the local cortical circuit, but also as modulators for cortical neurons expressing kappa-opioid and/or SOM receptors.

Abbreviations in the text

ABC	avidin-biotinylated peroxidase complex
BT	biotinylated tyramine
CR	calretinin
DAPI	4',6-diamidino-2-phenylindole
GAD67	glutamic acid decarboxylase of 67 kDa
GFP	green fluorescent protein
GO	glucose oxidase
M1	primary motor area
mPF	medial prefrontal area
NOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
OF	orbitofrontal area
PBS	5 mM sodium phosphate (pH 7.4)-buffered 0.9% (w/v) saline
PBS-X	PBS containing 0.3% (v/v) Triton X-100
PBS-XCD	PBS-X containing 0.1% (w/v) λ -carrageenan, 1% (v/v) donkey serum and 0.02% (w/v) sodium azide
PPD	preprodynorphin
PV	parvalbumin
S1	primary somatosensory area
SOM	somatostatin
TSA	tyramide signal amplification
V1	primary visual area
VGluT1	vesicular glutamate transporter 1
VIP	vasoactive intestinal polypeptide

INTRODUCTION

Four genes that encode endogenous opioid peptides are expressed in the mammalian brain (for review, see Höllt, 1983; Meis, 2003; Chavkin, 2013). Their transcripts are first translated into prepropeptides, and the prepropeptides are then processed to a variety of mature active peptides such as corticotropin, dynorphins, endorphins, enkephalins, leumorphin, lipotropins, melanotropins, neoendorphins and nociceptin (orphanin FQ). Of the four opioid prepropeptides, preprodynorphin (PPD; preproenkephalin B) is processed to generate several active peptides such as big dynorphin (dynorphin-32), dynorphin 1–8, dynorphin A (dynorphin 1–17), dynorphin B (rimorphin), leucine-enkephalin, leumorphin, α -neoendorphin and β -neoendorphin. In the rodent neocortex, immunoreactivity for PPD-derived peptides (McGinty JF et al., 1984; Fallon and Leslie, 1986) or mRNA signals for PPD (Alvarez-Bolado et al., 1990; Sato et al., 1991; Mansour et al., 1994b; Merchenthaler et al., 1997) have been detected in some cortical neurons. The sparse distribution of PPD-expressing neurons in the cerebral cortex suggests that most of them do not belong to the major population of principal cortical neurons, *i.e.* pyramidal neurons, but to a smaller group of interneurons. This is partially supported by the immunocytochemical report which shows that dynorphin B-immunoreactive cell bodies appear to be non-pyramidal (Fallon and Leslie, 1986).

It is well established that cortical GABAergic interneurons in the rat neocortex have been divided, mainly by their chemical and molecular characteristics, into three distinct subgroups: 1) parvalbumin (PV)-containing cells; 2) somatostatin (SOM)-expressing cells; and 3) calretinin (CR)-containing and/or vasoactive intestinal polypeptide (VIP)-expressing cells, which are partially immunopositive for cholecystokinin and choline acetyltransferase (Eckenstein and Baughman, 1984; Kosaka et al., 1987; Rogers, 1992; Chédotal et al., 1994; Kubota et al., 1994; Kawaguchi and Kubota, 1996; Bayraktar et al., 1997; Gonchar and Burkhalter, 1997; Kubota and Kawaguchi, 1997; Taki et al., 2000; Kawaguchi and Kondo, 2002; Uematsu et al., 2008). In addition, the fourth small subgroup has recently been recognized to be neurogliaform cells containing α -actinin in the perikaryal portion, and they are distributed mainly in layers 1–3 of the rat neocortex (Uematsu et al., 2008; Ma et al., 2011). Although the chemical classification of mouse neocortical interneurons mostly follows that of rat interneurons (Xu et al., 2006, 2010; Gonchar et al., 2007; Hioki et al., 2013), a clear difference between rat and mouse interneurons has been found in the co-occurrence of CR and SOM immunoreactivities: No SOM-expressing neurons in the rat neocortex show CR immunoreactivity in their cell bodies (Rogers, 1992; Gonchar and Burkhalter, 1997), whereas 15–44% of SOM-expressing neurons in the mouse neocortex display CR immunoreactivity (Xu et al., 2006, 2010; Gonchar et al., 2007), the co-expression rate being different between neocortical areas. In addition, this finding suggests that the SOM-expressing neuron group in the rodent neocortex is further divided into subgroups. Actually, immunoreactivities for neuropeptide Y (NPY) and neuronal nitric oxide synthase

(NOS) have been detected in a fraction of SOM-expressing neurons in the rat (Hendry et al., 1984; Papadopoulos et al., 1987; Kubota et al., 1994; Gonchar and Burkhalter, 1997; Kubota et al., 2011) and mouse neocortex (Gonchar et al., 2007; Xu et al., 2010; Perrenoud et al., 2012), indicating the heterogeneity of SOM-expressing neurons.

Prepropeptides in neurons are incorporated into the endoplasmic reticulum by the N-terminal signal peptide mechanism right after the translation, processed to propeptides by the deletion of the signal peptide, and then transported through the Golgi apparatus to axon terminals (Steiner et al., 1984). During the axonal transport, the propeptides in transport vesicles are further processed to mature active peptides with acid proteases. Thus, immunoreactivity for mature PPD-derived peptides is undetectable in the cell bodies without colchicine treatment, which blocks the axonal transport and enhances immunoreactivity for the mature peptides in the cell bodies (Hököfelt et al., 1971; McGinty JF et al., 1984; Fallon and Leslie, 1986). In order to detect the expression of PPD immunocytochemically in the cell bodies of striatal neurons, we produced antibodies to the C-terminal portion of PPD (Lee et al., 1997). Because the C-terminal portion was immunocytochemically detectable in the cell bodies after the completion of translation, these antibodies well visualized the cell bodies of PPD-expressing striatal projection neurons without colchicine treatment. In the subsequent study of mu-opioid receptor-expressing interneurons in the rat neocortex, we observed that some cell bodies of presumed interneurons were immunoreactive for the C-terminal portion of PPD, and that PPD-expressing neurons constituted a distinct population from preproenkephalin-expressing interneurons (Taki et al., 2000). Recently, we reported preliminarily that PPD was produced by some cortical interneurons that belonged to the group of SOM-expressing interneurons in the mouse neocortex (Sohn et al., 2012). In the present study, we conducted a comprehensive examination on the distribution and chemical/molecular characteristics of PPD-expressing neurons in the mouse primary somatosensory area (S1), partially comparing the results in the S1 with those in the primary motor (M1), primary visual (V1), medial prefrontal (mPF) and orbitofrontal areas (OF).

MATERIALS AND METHODS

Animals

This experiment was conducted on six male 8–12-week-old C57BL/6 mice (Japan SLC, Hamamatsu, Japan), weighing 20–30 g, with the approval of the Committee for Animal Care and Use (Med Kyo 12013) and that for Recombinant DNA Study (120093) at Kyoto University. All efforts were made to minimize animal suffering and the number of animals used.

Tissue preparation

Mice were deeply anesthetized with chloral hydrate (70 mg/100 g body weight),

perfused transcardially with 20 ml of 5 mM phosphate-buffered 0.9% saline (PBS; pH 7.4). For immunocytochemistry, the animals were further perfused for 5–10 minutes at room temperature with 20 ml of 4% formaldehyde, 0.9% picric acid (75%-saturated solution), and 0.1 M Na₂HPO₄ (adjusted to pH 7.0 with NaOH), and the brains were removed and post-fixed at 4°C for 3 hours in the same fixative. For in situ hybridization histochemistry, the animals were perfused with 20 ml of 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), followed by 3-day-long post-fixation at 4°C in the same fixative. After cryoprotection with 30% sucrose in PBS, the brain blocks containing the S1, M1, V1, mPF and OF were cut into 40- or 20- μ m-thick frontal sections on a freezing microtome.

Antibody characterization

The primary antibodies used in the present study are listed in Table 1 with the information for the immunogen, host animal, antibody condition and reference/manufacture. We raised a guinea pig antiserum against the C-terminal icosapeptide of rat PPD (FKVVTRSQENPNTYSEDLDV), which was identical with as the C-terminal icosapeptide of mouse PPD, and purified the crude gamma globulin fraction using the immunogen-conjugated column (Lee et al., 1997). The affinity-purified antibody specifically recognized immunogen peptide (PPD C-terminal icosapeptide), and did not cross-react with preproenkephalin C-terminal, preprotachykinin A C-terminal, dynorphin A1-17, leucine-enkephalin, methionine-enkephalin, substance P or neurokinin A in the dot blotting tests, and the immunoreactivity in the striatum was abolished by pre-incubation of the antibody with the immunogen peptide (Lee et al., 1997).

The specificities of the other commercially available antibodies to α -actinin, CR, NOS, NPY, PV, reelin, SOM and VIP have been examined by manufacturers or researchers (listed in Table 1). Most lines of evidence are described in the data sheets which were provided by the manufacturers unless otherwise indicated: 1) Monoclonal anti- α -actinin mouse IgG1 recognized skeletal and cardiac α -actinins of many kinds of vertebrates, including those of rats, and stained Z lines and dots in stress fibers of myotubes. In immunoblotting tests with rat brain membrane, the antibody labeled a protein band of ~100 kDa, which was consistent with the known and predicted size of α -actinin (Wyszynski et al., 1998): 2) Affinity-purified anti-CR rabbit antibody specifically recognized calretinin in immunoblotting tests, and the positive band was suppressed by pre-incubation of the immunogen peptide. It did not cross-react with calbindin-D28k: 3) Anti-CR goat serum showed no immunoreactivity in the cerebral cortex of CR knock-out mice. The pre-incubation of the antiserum with recombinant human CR completely blocked the immunoreactivity (Xu et al., 2010): 4) Anti-NOS sheep serum recognized a protein band of 150 kDa in Western blot tests with rat brain extract, and the positive band in the blot as well as the immunoreactivity in the central and peripheral nervous tissue was abolished by the pre-

incubation of the antiserum with the immunogen (Anderson et al., 1995): 5) In enzyme-linked immunosolvent assay, anti-NPY rabbit serum recognized NPY, NPY13-36 and NPY18-36, cross-reacted with peptide YY, but not with neurokinin B, neurokinin A, substance P, calcitonin, SOM or bovine serum albumin. The immunolabeling in the mouse cortex was blocked by pre-incubation of the antibody with NPY (Zhao et al., 2008): 6) The specificity of anti-NPY sheep serum was determined by radioimmunoassay. Angiotensin II, enkephalins, oxytocin, pancreatic polypeptide, SOM, substance P, vasopressin and VIP showed less than 0.05% cross-reactivity. Only Peptide YY displayed 36% cross-reactivity. In the forebrain of NPY knock-out mice, no immunoreactivity was seen with the antibody (Glavas et al., 2008). The distribution of immunoreactivity with the anti-NPY sheep serum in the mouse cortex was almost the same as that with the anti-NPY rabbit serum (the present observation): 7) In immunoblotting tests, anti-PV mouse monoclonal IgG recognized PV (12 kDa) originating from many vertebrates including rat, but did not cross-react with other members of the EF-hand family such as calmodulin, intestinal calcium-binding protein, S-100, myosin light chain and troponin. The pre-adsorption of the antibody with an excess amount of recombinant rat muscle PV eliminated the immunolabeling in the rat cochlea and cerebellar tissue (Hackney et al., 2005). Furthermore, no immunoreactivity was observed with the antibody in the brain tissue of PV knock-out mice (Burette et al., 2009): 8) Anti-reelin mouse IgG1 recognized a protein band of ~388 kDa in the immunoblotting tests of mouse brain membrane lysates: 9) Anti-SOM rabbit serum recognized SOM-14, SOM-28, SOM-25 and [Des-Ala¹]-SOM-14 in radioimmunoassay tests. It did not cross-react with D-Trp⁸-SOM-14, prosomatostatin 1-32, SOM analogs (RC-160, CROP-NH₂), substance P, NPY, VIP, insulin or glucagon 1–29. The distribution of immunoreactivity with the anti-SOM rabbit serum in the mouse cortex was almost the same as that with the anti-SOM rat IgG (the present observation): 10) Anti-SOM rat monoclonal IgG showed no cross-reactivity to calcitonin-gene related peptide, endorphins, enkephalins or substance P. It partially cross-reacted with SOM fragments. When the antibody was pre-adsorbed with synthetic SOM, the immunoreactivity in the mouse neocortex was completely blocked (Xu et al., 2006): 11) The immunoreactivity with anti-VIP rabbit serum in the rat amygdala, cortex and suprachiasmatic nucleus was completely abolished by pre-adsorption with VIP, but not with corticotropin, calcitonin gene-related peptide, FMRF-amide, gastric inhibitory peptide, gastrin 34, glucagon, growth hormone-releasing hormone, insulin, motilin, NPY, pancreatic polypeptide, peptide histidine isoleucine 27, peptide YY, secretin, SOM or substance P. The immunoreactivity in the mouse cerebral cortex was also blocked in immunoadsorption tests with synthetic VIP (Xu et al., 2006).

Immunoperoxidase staining

To enhance immunoreactive sensitivity for PPD, we applied the biotinylated

tyramine (BT)-glucose oxidase (GO) amplification method (Furuta et al., 2009; Kuramoto et al., 2009; Ge et al., 2010; Ma et al., 2011; Ohno et al., 2011). The following incubations were performed at room temperature. Brain sections with 40 μm thickness were incubated overnight with 1.0 $\mu\text{g}/\text{ml}$ affinity-purified anti-PPD guinea pig antibody in PBS containing 0.3% Triton X-100, 0.12% λ -carrageenan, 0.02% sodium azide and 1% normal donkey serum (PBS-XCD). After being washed with PBS containing 0.3% Triton X-100 (PBS-X), they were incubated for 1 hour in PBS-XCD with 10 $\mu\text{g}/\text{ml}$ biotinylated donkey antibody to guinea pig IgG (706-065-148; Jackson ImmunoResearch, West Grove, PA), and for another 1 hour in PBS-X with 1/100-diluted avidin-biotinylated peroxidase complex (ABC) (Elite variety; Vector, Burlingame, CA). Subsequently, the sections were reacted for 30 minutes with a mixture containing 31 μM BT (Kuramoto et al., 2009), 3 $\mu\text{g}/\text{ml}$ of GO (257 U/mg; 16831-14; nacalai tesque, Kyoto, Japan), 2 mg/ml of β -D-glucose and 2% bovine serum albumin in 0.1 M phosphate buffer (pH 7.4). The sections were again incubated for 1 hour with ABC in PBS-X, and the bound peroxidase was finally developed brown by reaction for 30–60 minutes with 0.02% diaminobenzidine-4HCl (347-00904, Dojindo, Kumamoto, Japan) and 0.0001% H_2O_2 in 50 mM Tris-HCl (pH 7.6). The stained sections were mounted onto the gelatinized glass slides, dehydrated in an ascending ethanol series, cleared in xylene and coverslipped. The specificity of the BT-GO method was examined by pre-incubation with an excess amount (100–1000-fold in mol) of the immunogen peptide. Monochrome images were taken with digital camera QICAM (QImaging, Surrey, BC, Canada) attached to microscope FX (Nikon, Tokyo, Japan). After the plotting of PPD-immunopositive cell bodies, the sections were counter-stained for Nissl with 0.2% cresyl violet to identify cytoarchitecture of the neocortex. Cortical areas and their layers were determined according to Caviness (1975), Lorente de N  (1992), Van De Werd et al. (2010) and Franklin and Paxinos (2008).

Combined labeling of immunofluorescence staining and fluorescent in situ hybridization

The complementary DNA fragment corresponding to a region of glutamic acid decarboxylase 67 kDa isoform (GAD67; nucleotides 855–1788 in GenBank accession no. XM_133432.2; Tamamaki et al., 2003) or vesicular glutamate transporter 1 (VGluT1; nucleotides 43–661 in GenBank Y12257; Nakamura et al., 2007) was cloned into pBluescript II SK (+) vector (Stratagene, La Jolla, CA). Sense and antisense single-strand riboprobes were synthesized with a digoxigenin RNA labeling kit (Roche Diagnostics, Basel, Switzerland). The following hybridization procedure was carried out as reported in previous studies (Hioki et al., 2010; Ma et al., 2011). Briefly, 20- μm -thick free-floating sections were hybridized for 16–20 hours at 60°C with 1 $\mu\text{g}/\text{ml}$ digoxigenin-labeled sense or antisense riboprobes in a hybridization buffer. After washes and ribonuclease A treatment, the sections

were incubated overnight at room temperature with a mixture of 1/1000-diluted alkaline phosphatase-conjugated anti-digoxigenin sheep antibody (11-093-274-910; Roche Diagnostics) and one of the following antibodies: 1.0 $\mu\text{g/ml}$ affinity-purified anti-PPD guinea pig antibody; 1/1000-diluted affinity-purified anti-CR rabbit antibody; 1/1000-diluted anti-NOS sheep serum; 1/1000-diluted anti-NPY sheep serum; and 1/2000-diluted anti-SOM rabbit serum. The sections treated with the primary antibody against CR, NOS, NPY or SOM were then incubated for 2 hours with 5 $\mu\text{g/ml}$ AlexaFluor488-conjugated donkey antibody to rabbit (A-11034; Invitrogen, Carlsbad, CA) or sheep IgG (A-11015; Invitrogen). In the case of PPD immunolabeling, we applied BT-GO method as described in the immunoperoxidase staining section; after incubations with the biotinylated secondary antibody, ABC and BT-GO reaction mixture, the sections were incubated for 2 hours at room temperature with 5 $\mu\text{g/ml}$ AlexaFluor488-conjugated streptavidin (S11223; Invitrogen). All the sections were finally reacted overnight at 4°C with a 2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate Fluorescence Detection kit (HNPP/FastRed; Roche Diagnostics). The sections were mounted onto 3-aminopropyltriethoxysilan-coated glass slides and coverslipped with CC/Mount (K002; Diagnostic Biosystems, Pleasanton, CA). We detected no higher signals with the sense probe than the background labeling.

Double immunofluorescence labeling

For double immunofluorescence labeling for PPD and either α -actinin, NOS, NPY, PV, SOM, VIP or reelin, 40- μm -thick brain sections were incubated overnight in PBS-XCD with 1.0 $\mu\text{g/ml}$ anti-PPD guinea pig antibody and one of the following antibodies: 1/4000-diluted anti- α -actinin mouse ascites, 1/1000-diluted anti-NOS sheep serum, 1/1000-diluted anti-NPY sheep serum, 1/4000-diluted anti-PV mouse ascites, 1/2000-diluted anti-SOM rabbit serum, 1/1000-diluted anti-VIP rabbit serum and 10 $\mu\text{g/ml}$ anti-reelin mouse IgG1. The following incubations were conducted at room temperature in PBS-XCD unless otherwise stated. After washes with PBS-X, the sections were incubated for 1 hour with a mixture of 10 $\mu\text{g/ml}$ biotinylated donkey anti-[guinea pig IgG] antibody and 10 $\mu\text{g/ml}$ AlexaFluor594-conjugated donkey or goat antibody to mouse (A-21203; Invitrogen), sheep (A-11016; Invitrogen) or rabbit IgG (A-11037; Invitrogen). The sections were subsequently incubated with ABC in PBS-X, reacted with the BT-GO mixture, as described above, and finally incubated for 1 hour with 5 $\mu\text{g/ml}$ AlexaFluor488-conjugated streptavidin. In the case of double labeling for PPD and CR, because CR immunoreactivity was weak without heat treatment, we used the tyramide signal amplification (TSA) method for CR immunoreactivity. The sections were incubated overnight with a cocktail of 1.0 $\mu\text{g/ml}$ anti-PPD guinea pig antibody and 1/1000-diluted affinity-purified anti-CR rabbit antibody, and processed for the biotinylated secondary antibody to guinea pig IgG, ABC, and BT-GO reaction mixture as described above. After inactivation of bound peroxidase by incubation for 1 hour with 2%

sodium azide in PBS, the sections were further incubated for 1 hour in PBS-X with 5 $\mu\text{g/ml}$ AlexaFluor594-conjugated streptavidin (S11227; Invitrogen) and 5 $\mu\text{g/ml}$ peroxidase-conjugated donkey antibody to rabbit IgG (AP182P; Millipore), and finally reacted for 20–30 minutes with the TSA Fluorescein kit (NEL701; Perkin Elmer, Boston, MA).

For double labeling between NOS, NPY and SOM, the following combinations of 1/1000-diluted primary antibodies were used: anti-SOM rabbit serum and anti-NOS sheep serum; anti-SOM rabbit serum and anti-NPY sheep serum; or anti-NPY rabbit serum and anti-NOS sheep serum. After incubation with the primary antibodies overnight, the sections were incubated for 1 hour with a mixture of 5 $\mu\text{g/ml}$ DyLight594-conjugated donkey antibody to rabbit IgG (711-515-152; Jackson ImmunoResearch) and 5 $\mu\text{g/ml}$ AlexaFluor488-conjugated donkey antibody to sheep IgG. In the case of double labeling for CR and either NOS, NPY or SOM, the normal (heat-untreated) sections were incubated overnight with 1/1000-diluted anti-CR rabbit antibody and either 1/1000-diluted anti-NPY sheep serum, 1/1000-diluted anti-NPY sheep serum or 1/50-diluted anti-SOM rat monoclonal antibody. The sections were then incubated for 1 hour in PBS-X with a mixture of 5 $\mu\text{g/ml}$ peroxidase-conjugated donkey antibody to rabbit IgG and 5 $\mu\text{g/ml}$ AlexaFluor594-conjugated donkey antibody to sheep or rat IgG, and finally reacted with the TSA Fluorescein kit for CR immunoreactivity. After the immunolabeling, the sections were mounted onto gelatin-coated glass slides, and coverslipped with 50%(v/v) glycerol and 2.5% (w/v) triethylenediamine (anti-fading reagent) in PBS.

To check the specificity of CR immunoreactivity with the anti-CR rabbit antibody purchased from Sigma-Aldrich (Table 1), CR immunoreactivity with the Sigma-Aldrich rabbit antibody was compared to that with the anti-CR goat serum purchased from SWANT (Table 1), which was used in the previous report (Xu et al., 2010). The mouse cortical sections were doubly immunolabeled with 1/1000-diluted Sigma-Aldrich rabbit antibody and 1/2000-diluted SWANT goat serum, applying the same method except the use of 5 $\mu\text{g/ml}$ AlexaFluor594-conjugated donkey antibody to goat IgG (A-11058; Invitrogen). The two immunofluorescences for CR were almost completely colocalized in single neocortical neurons; 200 cortical neurons immunoreactive with the SWANT antibody were immunoreactive with the Sigma-Aldrich antibody, and vice versa (data not shown). This suggests the high reliability of the present TSA-enhanced method with the Sigma-Aldrich anti-CR rabbit antibody for detecting CR immunoreactivity.

In control experiments of double immunofluorescence labeling, one of the primary antibodies was omitted or replaced with the normal antibody or serum in the first incubation, and the sections were processed in the same manner as above. The sections were always negative for immunoreactivity associated with the omitted or replaced antibody.

Image acquisition and cell counting

The fluorescence-labeled sections were observed under epifluorescence microscope Axiophot (Zeiss, Oberkochen, Germany) with appropriate filter sets for AlexaFluor488 and fluorescein (excitation, 450–490 nm; emission, 515–565 nm), and for AlexaFluor594, DyLight594 and Fast Red (excitation, 530–585 nm; emission, $\geq$ 615 nm). Pseudocolor images were taken by QICAM FAST digital monochrome camera (QImaging), modified ($\pm 20\%$ contrast and brightness enhancement) in software Canvas X (ACD Systems, Saanichton, Canada), and saved as TIFF files. Cortical lamination in fluorescence-labeled sections was determined in reference to counter-staining with 1 $\mu\text{g/ml}$ 4',6-diamidino-2-phenylindole (DAPI; excitation, 359–371 nm; emission, $\geq$ 397 nm) in PBS. Cortical lamination in DAPI staining was pre-determined in the control sections with the cytoarchitecture revealed by co-staining with 1/200-diluted NeuroTrace 500/525 green fluorescent Nissl stain solution (Invitrogen) in PBS.

To acquire numerical data for each combination of double labeling in each cortical area of interest, we labeled the frontal sections of 3 mice, and acquired the data from a mouse using at least three sections that were located more than 100 μm apart from each other. In the 3 frontal sections of a mouse, 6 vertical cortical strips (0.8–1.2 mm wide x cortical depth), which covered all the layers, were selected bilaterally in the cortical area of interest, and used for the data acquisition for each combination of double labeling in order to reduce the effect of region-to-region variability within the area. After counting the number of fluorescence-labeled neuronal cell bodies with a clear nucleolus under the epifluorescence microscope with reference to Guillery (2002), the sum of the counted numbers in each cortical layer of the 6 strips was recorded as a numerical result of the layer from the mouse, and the same counting was repeated in the 3 mice.

RESULTS

The distribution and GABAergic characteristics of PPD-immunopositive neurons in the neocortex

Neurons immunopositive for the C-terminal portion of PPD were distributed in all the areas of the mouse neocortex, showing some differences in density among cortical areas (Fig. 1a–d). The density of PPD-immunopositive neurons was higher in somatosensory, auditory and visual areas than in motor and frontal areas. Almost no immunoreactivity for PPD in the neocortex was observed in neuropil, but its immunoreactivity was located mainly in the cell bodies and proximal dendrites of neurons (Fig. 1d, e, g–k), which often emitted multipolar (Fig. 1g–i) and occasionally bipolar/bifurcated dendrites (Fig. 1j, k). Of six neocortical layers, PPD-immunopositive neurons were distributed most densely in layer 5, moderately in layers 2–4 and sparsely in layer 6 (Fig. 1e; Table 2). Almost no PPD-immunopositive neurons were found within layer 1 in the present study (Fig. 1e; Table 2).

Almost no or very few PPD-immunopositive neurons were observed in the piriform or entorhinal areas (Fig. 1a–c). The immunoreactivity in the cortical regions was completely blocked by pre-incubation of the antibody with a 100–1000-fold excess amount (in mol) of the C-terminal icosapeptide of PPD (Fig. 1f).

More than 97.6% of these PPD-immunopositive neurons showed mRNA signals for GAD67, and less than 0.1% displayed signals for VGluT1 (Fig. 2; Table 2) in the S1, suggesting that almost all PPD-expressing neurons were GABAergic in the neocortex. In addition, a considerable portion (about 15%) of neocortical GABAergic neurons were immunopositive for PPD in all the cortical layers of the S1, particularly amounting to 26% in layer 5 (Table 2). These results indicate that PPD-expressing neurons constitute a substantial population of cortical GABAergic neurons particularly in layer 5. Because the counting was performed along the vertical cortical strips covering all the neocortical layers, the relative laminar compositions of marker-immunopositive neurons in all the layers were estimated by dividing the number of neurons in each cortical layer by the total number in all the layers; 62% (998/1619) of PPD-immunopositive neurons in the S1 were located at layer 5, while only 36% (3820/10705) of GAD67-expressing neurons were found at layer 5 (Table 2). It should be noted that the counting in Table 2 and in the following tables was performed in the cortical strips that were selected non-randomly, although we tried to make an unbiased collection of information by using 6 strips in a given cortical area. A possible method for estimation of the numbers or densities of neurons might be random sampling with an adequate stereologic design, but we did not find out a sufficient method for random sampling in the cortical area of interest. Thus, the data in the tables did not exactly represent the information on actual numbers and densities in the areas of interest, but were only local numbers and densities in the sampled regions.

PPD-expressing neurons in GABAergic subpopulations

As almost all PPD-expressing neurons were revealed to be GABAergic, we next examined which group of the four major GABAergic populations (PV-, SOM-, VIP- and α -actinin-immunoreactive neuron groups) included PPD-expressing neurons. In Figure 3a–c, of the four markers, SOM immunoreactivity was almost always co-detected in PPD-immunopositive neurons ($\geq 97.4\%$; Table 3). Conversely, about half of SOM-immunopositive neurons were immunoreactive for PPD in the S1 with variable colocalization rates depending on cortical layers. PPD immunoreactivity was observed in SOM-immunopositive neurons most frequently at layer 4 (79%), less frequently at layers 2-3 and 5 (50–55%), and infrequently at layers 1 and 6 (10–17%) (Table 3). In contrast, only few or no PPD-immunopositive neurons showed immunoreactivity for PV or VIP, respectively (Fig. 3d–i; Table 3). It was unexpected that almost no small neuronal profiles showed α -actinin-like immunoreactivity in the mouse neocortex, although α -actinin-like immunoreactivity was

detected in many small neurons of the rat neocortex (Ma et al., 2011). Since neuropil α -actinin-like immunoreactivity in the mouse neocortex (data not shown) was very similar to that in the rat neocortex (Wyszynski et al., 1998), neurogliaform cells of the mouse cortex were likely to lack α -actinin-like immunoreactivity in their cell bodies as reported in the mouse piriform cortex (Suzuki and Bekkers, 2010).

We then examined whether or not PPD-immunopositive neurons were included in SOM-immunopositive neurons in other cortical areas, such as the M1, V1, OF and mPF (prelimbic and infralimbic areas). As shown in Figure 4 and Table 4, most PPD-immunopositive neurons (95.9–98.8%) were immunoreactive for SOM in the M1, V1 and OF. It was surprising, however, that the mPF contained many PPD-immunopositive neurons without SOM immunoreactivity. Only 24.1% of PPD-immunopositive neurons showed SOM immunoreactivity, and only 13.3% of SOM-immunopositive neurons displayed PPD immunoreactivity, suggesting that the mPF was different from the other neocortical areas. In fact, the colocalization rate of PPD and SOM immunoreactivities in single neurons of the mesocortical areas, such as the cingulate area, was between the mPF and the other neocortical areas (data not shown).

Because SOM-expressing neurons were heterogenous and some of them showed immunoreactivity for NPY, CR or NOS in the mouse neocortex (Xu et al., 2006, 2010; Gonchar et al., 2007), we further examined the colocalization of PPD immunoreactivity with these markers in the S1 (Fig. 5). Only 7% of PPD-immunopositive neurons showed CR immunoreactivity, and almost no PPD-immunopositive neurons displayed immunoreactivity for NPY or NOS (Table 4). These results suggest that PPD immunoreactivity is a marker for a newly recognized subgroup of SOM-expressing neurons in the S1, and that the subgroup constitutes about half (34.9–54.9%) of SOM-expressing population in many neocortical areas except the mPF (Tables 3 and 4).

The population analysis of known subgroups in SOM-expressing neurons and the comparison of the population size of PPD-expressing neurons with those of the subgroups

To reveal the relative population sizes of SOM-, NPY-, CR- and NOS-expressing neuron groups in GABAergic cortical neurons, we performed combined fluorescence labeling for immunoreactivity of those markers and in situ hybridization signals of GAD67 in the S1 (Fig. 6). As shown previously in the mouse neocortex (Gonchar et al., 2007; Perrenoud et al., 2012), almost all SOM-, NPY-, CR- and NOS-immunopositive neurons were positive for mRNA signals of GAD67 in the S1. Conversely, SOM-, NPY-, CR- and NOS-immunopositive neurons were 32%, 13%, 8% and 2% of GABAergic neurons, respectively (Table 6). There were differences in the percentages of SOM-immunopositive neurons between the superficial and deep cortical layers; 15–20% of GAD67-expressing neurons were immunopositive for SOM in layers 2–4, and 41–49% in layers 5–6. Similarly, NOS

immunoreactivity was found less frequently in the superficial layers than in the deep layers; 5% and less than 1% of GAD67-expressing neurons were immunopositive for NOS in layer 6 and in layers 2–5, respectively. In contrast, CR immunoreactivity was observed in GAD67-expressing neurons more frequently in superficial layers 2–4 (11–15%) than in deep layers 5–6 (3–6%). The occurrence of NPY immunoreactivity in GAD67-expressing neurons was higher in layers 2–3 and 6 (17–21%) than in layers 4–5 (8–10%).

In addition, the relative population sizes of NPY-, CR- and NOS-immunopositive neurons in SOM-immunopositive neurons were examined in the S1 by the double immunofluorescence labeling method (Fig. 7). NPY-, CR- and NOS-immunopositive neurons occupied only 6%, 7% and 6%, respectively, of SOM-expressing neurons in the S1 (Table 7). Conversely, 19% of NPY-immunopositive neurons and 25% of CR-immunopositive neurons were immunoreactive for SOM. Only 5–6% of CR- or NPY-immunopositive neurons displayed NPY or CR immunoreactivity, respectively. Almost all NOS-immunoreactive neurons were immunopositive for SOM and NPY. The co-occurrence of NOS and NPY immunoreactivities was highest (47% of NPY-immunopositive neurons) in layer 6. NOS immunoreactivity was observed in 4% of CR-immunopositive neurons, and CR immunoreactivity was found in 31% of NOS-immunopositive neurons.

Finally, using the present data, we summarize the relative size of each GABAergic subpopulation in the S1 (Fig. 8). This figure clearly shows that PPD-expressing neurons occupy a unique position in cortical GABAergic interneurons; they belong to the SOM-expressing neuron group in the neocortex, and constitute a large subgroup of the group in layers 2–5. PPD-expressing neurons, except those in layers 2–3, are mostly distinguished from small known subgroups of SOM-expressing neurons, *i.e.* CR-containing and NPY/NOS-expressing subgroups. PPD-expressing neurons are most abundant in layer 5, although the percentage of PPD-expressing neurons in SOM-expressing ones is larger in layer 4 than in layer 5. On the other hand, from the viewpoint on somatostatin-expressing neurons, this second largest population of cortical GABAergic neurons, accounting for 32% of GABAergic neurons, is divided into three or four subgroups: 1) a large subgroup of PPD-expressing neurons, amounting to 46% of SOM-expressing neurons; 2) a small subgroup of NPY/NOS-expressing neurons (~6%); 3) neurons positive for CR alone (~2%); and 4) other SOM-expressing neurons without PPD, NPY, NOS or CR immunoreactivity (~46%). CR-containing neurons are most heterogeneous in SOM-expressing neurons because small fractions of PPD-expressing and NPY/NOS-expressing subgroups express CR, and hence do not seem to constitute an entity as a subgroup.

Reelin immunoreactivity in PPD-expressing neurons

It was recently reported that reelin immunoreactivity was frequently detected in more than 2/3 of SOM-expressing neurons in the S1 of 21-day-old mice (Miyoshi et al.,

2010), although only a small portion (less than 20%) of SOM-expressing neurons showed mRNA signals for reelin in the S1 of adult mice (Alcántara et al., 1998). Thus, we examined the presence of reelin immunoreactivity in PPD- and SOM-immunopositive neurons in the S1 of adult mice by the double labeling method (Fig. 9, Table 8). Reelin immunoreactivity was detected in 67% of SOM-immunopositive neurons in our samples, supporting the recent results of Miyoshi et al. (2010). In addition, about 60% of reelin-immunopositive neurons displayed SOM immunoreactivity, which was also compatible with the results shown by Miyoshi et al. (2010). On the other hand, about 90% of PPD-immunopositive neurons were immunoreactive for reelin (Table 8), indicating that the population of reelin- and SOM-double expressing neurons largely overlapped with that of PPD-expressing neurons in the S1.

DISCUSSION

The type of interneurons expressing PPD

The present results revealed that PPD-expressing neurons constituted a substantial population of GABAergic cortical neurons, especially of SOM-expressing neurons, in the mouse neocortex except the mPF. It is well known that SOM is commonly expressed by Martinotti cells (Kawaguchi and Kubota, 1996, 1997; Goldberg et al., 2004; Wang et al., 2004; Toledo-Rodriguez et al., 2005; Xu et al., 2006; Marinelli et al., 2008), which are multipolar or bitufted neurons with sparsely spiny dendrites, and specifically send local-ascending axon fibers that arrive and ramify in layer 1 (for review, see Fairén et al., 1984). Martinotti cells are ubiquitous components of mammalian neocortex, and distributed more frequently in layer 5 than in superficial layers. Thus, PPD/SOM-expressing neurons, especially those in layer 5, are likely candidates for Martinotti cells.

However, SOM-expressing neurons are known to include non-Martinotti cells as well as Martinotti cells. Recently, in the three distinct lines of transgenic mice expressing green fluorescent protein (GFP) under a short promoter for GAD67 gene, almost all GFP-immunopositive cortical neurons showed SOM immunoreactivity. Interestingly, the distribution of GFP-immunopositive neurons was different among the mouse lines; GFP-immunopositive neurons of lines GIN, X94 and X98 were distributed mainly in layers 2-3 and 5, in layers 4-5 and in layers 5-6, respectively (Ma et al., 2006). Furthermore, GFP-immunopositive neurons of lines GIN and X98 sent dense axonal arborization to layer 1, whereas those of line X94 projected few axon fibers to layer 1, clearly indicating that SOM-expressing neurons in the mouse neocortex included both Martinotti and non-Martinotti cells. In addition, a recent study revealed that all of 16 layer 4 SOM-expressing neurons examined morphologically after whole cell recording sent axonal arborization restrictedly to layer 4 in X94 and SOM/Cre mice (Xu et al., 2013). This is supported in the rat neocortex by the presence of SOM-immunopositive wide arbor cells in layers 2-3 (Kawaguchi and Kubota, 1998), and by the frequent expression of SOM mRNA in non-Martinotti cells such as bitufted,

large basket and nest basket cells (Markram et al., 2004; Toledo-Rodriquez et al., 2005). These findings indicate that SOM-expressing neurons are heterogenous in the rodent neocortex, being compatible with the present results.

Since some SOM-expressing neurons are known to send projecting axons to remote areas in the neocortex (Tomioka et al., 2005), PPD/SOM-immunopositive neurons might contain these long-projecting GABAergic neurons. However, most of the long-projecting GABAergic neurons produce not only SOM but also NPY and NOS, and they are frequently located in layer 6 and less so in layers 2-3 in the rat neocortex (Tomioka et al., 2005). The present study revealed that NOS/NPY/SOM-immunopositive neurons were distributed mainly in layer 6 and partially in layers 2-3 and 5 of the mouse neocortex, suggesting that these neurons might be the same long-projecting GABAergic neurons as those in the rat neocortex. In contrast, because PPD/SOM-immunopositive neurons constituted a distinct population from NOS/NPY/SOM-immunopositive neurons and were distributed mainly in layers 4–5 (Fig. 5), the majority of PPD/SOM-immunopositive neurons might not be long-projecting GABAergic neurons, but are likely to serve as cortical local circuit neurons. Thus, although PPD-expressing neurons are probable candidates for Martinotti cells as discussed above, the exact morphological nature of PPD-expressing neurons remains to be clarified.

CR immunoreactivity in SOM- and PPD-expressing neurons

SOM/CR-immunopositive neurons were more frequently distributed in layers 2–4 than in layer 5 of the S1 (Table 7 in the present study; Xu et al., 2006, 2010), suggesting that SOM/CR-immunopositive neurons included many non-Martinotti cells. Although 15–23% of SOM neurons in the S1 were reported to express CR (Table 1 of Xu et al., 2006; Table 3 of Xu et al., 2010), only 6.7% of SOM-immunopositive neurons in the S1 displayed CR immunoreactivity in the present study. This difference in the colocalization rate might be caused by the use of different antibodies. However, as described in the MATERIALS AND METHODS, neurons showing immunofluorescence with the rabbit anti-CR antibody, which is mainly used in the present study, were identical to those exhibiting immunofluorescence with the goat anti-CR antibody that was used by Xu et al. (2010). Thus, the use of different antibodies does not account for the difference in the CR colocalization rate. In addition, by comparing the method of Xu et al. (2006, 2010) with the method used in this study, the tissue fixation, sectioning thickness and counting method do not appear to explain the difference in the CR colocalization rate. Instead, the age of mice could be one of the factors affecting CR expression in SOM-immunopositive neurons, because the CR colocalization rate was higher in 21-day-old mice (23.4%; Xu et al., 2006) than in adult (15.1%; Xu et al. 2010) and 8–12-week-old mice (6.7%; the present result). However, the difference between the present result and that of Xu et al. (2010) in the S1 still remains to be accounted for.

PPD-immunopositive neurons showed almost the same co-localization rate for CR

(6.7%; Table 5) as SOM-immunopositive neurons in the present study. The vast majority of PPD-immunopositive neurons were included in SOM-expressing neurons in the S1, which suggests that CR was expressed in SOM-immunopositive neurons with a small probability (~7%) irrespective of PPD expression.

PPD-expressing neurons in the mPF

It is surprising that PPD-expressing neurons in the mPF showed different molecular characteristics, although PPD-expressing neurons in another prefrontal area, the OF, exhibited similar characteristics to those in the S1, M1 and V1. Although we do not have a specific idea for the cause of the difference, SOM-expressing neurons in the mPF might constitute a different population from those in the other neocortical areas. SOM/PPD-expressing neurons might simply be a minor population, compared with the majority of neurons expressing SOM alone in the mPF. Furthermore, about 3/4 of PPD-expressing neurons were not included in SOM-expressing neurons, suggesting that PPD-expressing neurons in the mPF chiefly consist of a different type, such as non-Martinotti type, of interneurons from those in the other neocortical areas, or that they might contain non-GABAergic neurons. Consistent with the possibility that some PPD-immunopositive neurons in mPF are glutamatergic, a preliminary study revealed the presence of VGluT1 mRNA in some PPD-immunopositive neurons. Although it is interesting to examine PPD immunoreactivity in the mPF, the classification of mPF PPD-expressing neurons needs further examinations including the comparative data on PPD-expressing neurons in the mesocortical areas, such as the cingulate, retrosplenial and insular regions.

Functional targets of PPD-derived peptides

The rodent brain has been reported to express four kinds of opioid receptors in the cerebral cortex: they are delta-, kappa- and mu-opioid receptors and nociceptin/orphanin FQ receptors (for review, see Mansour et al., 1995a; Meis, 2003). For example, mu-opioid receptors are expressed by some neocortical GABAergic interneurons, which belong to a different group from PV-containing and SOM-expressing interneurons; the mu-opioid receptor-expressing interneurons were immunopositive for VIP, corticotropin releasing factor, choline acetyltransferase, calretinin and cholecystokinin in the rat (Taki et al., 2000). Furthermore, the mu-opioid receptor-expressing interneurons are selectively excited by the nicotinic agonists, and the excitatory responses are suppressed through the activation of mu-opioid receptors (Férezou et al., 2007). Thus, opioid signaling is important for the control of a specific subgroup of cortical interneurons. However, the natural ligands for mu-opioid receptor are unlikely to be PPD-derived peptides, but instead they are preproenkephalin-derived peptides in the neocortical region.

Of the four opioid receptors, the major functional target of PPD-derived peptides is

kappa-opioid receptors, although the peptides can bind weakly to mu- and delta-opioid receptors (Mansour et al., 1995b; for review, see Janecka et al., 2004). Dense mRNA signals for kappa-opioid receptors have been observed in layers 5–6 of the rat (Meng et al., 1993; Minami et al., 1993; Mansour et al., 1994a, b) and mouse neocortex (Yasuda et al., 1993; DePaoli et al., 1994). Judging from the high density of mRNA-expressing neurons, many pyramidal neurons in layers 5–6 are supposed to express kappa-opioid receptors. In accordance with the localization of mRNA signals, binding autoradiography with radiolabeled kappa-opiate ligands shows high binding activity in the deep layers of the rodent cerebral cortex (Goodman and Snyder, 1982; for review, see Mansour et al., 1988), although immunoreactivity for kappa-opioid receptors is scanty in the rodent cerebral cortex (Arvidsson et al., 1995; Drake et al., 1996). The localization of kappa-binding activity in the deep cortical layers further suggests that kappa receptors are located mainly on the basal dendrites rather than on the apical dendrites of those pyramidal neurons. Martinotti cells in layer 5 are known to emit axon collaterals not only in layer 1 but also in the vicinity of their cell bodies (Kawaguchi and Kubota, 1996, 1997). Thus, PPD-derived peptides may be released mainly from the perisomatic axon fibers of Martinotti cells, and work on the basal dendrites of layer 5 pyramidal neurons. However, as discussed above, it is probable that most layer 4 PPD/SOM-expressing neurons emit axon fibers within layer 4. In this case, the targets of PPD-derived peptides released from layer 4 PPD-expressing neurons may be the apical dendrites of deep layer pyramidal neurons. In this case, the weak kappa-binding activity in the layers 1–4 may be caused by the difference in density of kappa-opioid receptor-expressing dendrites. This is because the density of the apical dendrites of deep layer pyramidal neurons is decreased in layers 1–4 by the dendrites of spiny neurons in layers 2–4. Alternatively, PPD-derived peptides may be used as a volume transmitter (for review, cf. Agnati et al., 2010) from layer 4 interneurons to deep layer pyramidal neurons, moving by diffusion to and working on the basal dendrites of these pyramidal neurons. In any case, it is likely that the functional targets of PPD-derived peptides are pyramidal neurons in layers 5–6.

The effects of kappa-opioid receptor activation are similar to those of the mu- or delta-opioid receptor activation; the effects are mediated through G protein-coupled signaling, and basically suppressive and long-lasting in receptor-expressing neurons, including the inhibition of cAMP accumulation, inhibition of phosphatidyl inositol hydrolysis, Ca channel inhibition and K channel stimulation (for review, see Standifer and Pasternak, 1997). Interestingly, the effects of SOM receptor 2, which is the main receptor for SOM in the murine brain (for review see, Videau et al., 2003), are also suppressive in receptor-expressing neurons not only presynaptically by inhibiting presynaptic transmitter release, activating leak K currents, and inhibiting voltage-gated Ca currents, but also postsynaptically by activating K currents and inhibiting voltage-gated Ca currents (for review see, Viollet et al., 2008). Furthermore, the expression of SOM receptors in the neocortex has been observed mainly in

pyramidal neurons of the deep cortical layers (Breder et al., 1992; Dournaud et al., 1996; Helboe et al., 1999; Schindler et al., 1997, 1999; Allen et al., 2003) and the binding activity for SOM was dense in the deep layers (Reubi and Maurer, 1985; Dournaud et al., 1996) as kappa-opioid receptors. Thus, both the targets and effects of SOM are very similar to those of PPD-derived peptides. This may account for the co-production of PPD and SOM in some neocortical interneurons, which have been identified in the present study. If PPD/SOM-expressing GABAergic interneurons are Martinotti cells, the interneurons not only inhibit the apical dendrites of layer 5 pyramidal neurons by their ascending axons, but also may suppress kappa-opioid and/or SOM receptor-expressing pyramidal neurons in layers 5–6 in a long-lasting manner through the local co-release of PPD-derived peptides and SOM.

CONFLICT OF INTEREST

There are no conflicts of interest.

CONTRIBUTION OF AUTHORS

All the authors have full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study design and concept: JS, HH and TK. Acquisition of data: JS, HH and SO. Analysis, interpretation of the data and drafting of article: JS and TK. Critical revision of article for intellectual content: JS, HH, SO and TK.

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Table 1. Primary antibodies used

Antigen	Immunogen	Host, condition	Reference or Manufacturer with catalog No.
PPD	C-terminal portion of rat PPD (residues 229–248; FKVVTRSQENPNTYSEDLDV) conjugated with carrier protein keyhole limpet hemocyanin	Guinea pig, affinity-purified polyclonal antibody	Lee et al., 1997
α -actinin	Purified rabbit skeletal (sarcomeric) α -actinin	Mouse, monoclonal IgG1 in ascites (clone EA-53)	Sigma-Aldrich (St. Louis, MO), catalog No. A7811
CR	Synthetic peptide corresponding to the C-terminal region of rat CR (amino acids 194-209) conjugated to keyhole limpet hemocyanin	Rabbit, affinity-purified polyclonal antibody	Sigma-Aldrich, catalog No. C7479
CR	Human recombinant calretinin	Goat, polyclonal serum	SWANT, catalog No. CG1
NOS	21 amino acid peptide (1409-1429) of the neuronal form of NOS from rat cerebellum, coupled to keyhole limpet hemocyanin	Sheep, polyclonal serum	Millipore (Billerica, MA), catalog No. AB1529
NPY	Synthetic NPY (porcine) conjugated to keyhole limpet hemocyanin	Rabbit, polyclonal serum	Sigma-Aldrich, catalog No. N9528
NPY	Synthetic NPY conjugated to bovine thyroglobulin	Sheep, polyclonal serum	Millipore, catalog No. AB1583
PV	Purified frog muscle PV	Mouse, monoclonal IgG1 in ascites (clone PARV-19)	Sigma-Aldrich, catalog No. P 3088
Reelin	Recombinant reelin amino acids 164–496	Mouse, monoclonal IgG1	Millipore, catalog No. MAB5364
SOM	SOM-14	Rabbit, polyclonal serum	Peninsula Laboratories (Belmont, CA), catalog No. T-4103
SOM	Synthetic peptide corresponding to amino acids 1-14 of cyclic SOM conjugated to bovine thyroglobulin using carbodiimide.	Rat, monoclonal IgG2b in culture supernatant	Millipore, catalog No. MAB354
VIP	Synthetic VIP coupled to bovine thyroglobulin with carbodiimide linker	Rabbit, polyclonal serum	ImmunoStar (Hudson, WI), catalog No. 20077

CR, calretinin; NOS, nitric oxide synthase; NPY, neuropeptide Y; PPD, prodynorphin; PV, parvalbumin; SOM, somatostatin; VIP, vasoactive intestinal polypeptide.

Table 2. Colocalization of PPD immunoreactivity with mRNA signals for GAD67 or VGluT1 in the mouse S1.

Layer	PPD/GAD67	GAD67/PPD	PPD/VGluT1	VGluT1/PPD
1	— (0/435) ^a	— (0/0)	— (0/0)	— (0/1)
2-3	6.4 ± 1.7 ^b (167/2647)	99.3 ± 0.1 (167/168)	0.014 ± 0.020 (2/12075)	3.5 ± 5.0 (2/68)
4	18.6 ± 2.5 (282/1543)	97.6 ± 0.4 (282/289)	0.024 ± 0.021 (3/10848)	1.5 ± 1.3 (3/181)
5	25.6 ± 1.4 (978/3820)	98.2 ± 1.3 (978/998)	0.084 ± 0.062 (5/7310)	0.9 ± 0.6 (5/602)
6	7.1 ± 0.3 (161/2260)	98.2 ± 1.7 (161/164)	0.005 ± 0.007 (1/19343)	0.8 ± 1.2 (1/96)
total	14.9 ± 1.0 (1588/10705)	98.2 ± 1.0 (1588/1619)	0.022 ± 0.016 (11/49576)	1.2 ± 0.9 (11/948)

^aThe numerator and denominator in the parentheses of column “X/Y” are the total number of cell bodies positive for X and Y and Y-positive cell bodies, respectively, observed in the examined cortical strips of three mice.

^bPercentages are given as mean ± standard deviation (SD) in three mice. The percentages of Layer 1 are not evaluated mainly because the numbers of cell bodies were small.

GAD67, glutamic acid decarboxylase of 67kDa; S1, primary somatosensory area; VGluT1, vesicular glutamate transporter 1.

Table 3. Colocalization of PPD immunoreactivity in SOM-, PV- or VIP-immunopositive neurons in the S1.

Layer	PPD/SOM	SOM/PPD	PPD/PV	PV/PPD	PPD/VIP	VIP/PPD
1	— (1/9) ^a	— (1/1)	— (0/3)	— (0/4)	— (0/7)	— (0/1)
2-3	49.9 ± 8.8 ^b (375/761)	97.4 ± 0.2 (375/385)	0.13 ± 0.19 (3/1776)	0.27 ± 0.38 (3/917)	0 (0/1407)	0 (0/680)
4	78.5 ± 3.1 (582/742)	99.5 ± 0.7 (582/585)	0.31 ± 0.22 (5/1824)	0.62 ± 0.47 (5/924)	0 (0/262)	0 (0/609)
5	54.5 ± 4.4 (1629/3014)	97.4 ± 1.4 (1629/1672)	0.22 ± 0.09 (9/3992)	0.40 ± 0.18 (9/2183)	0 (0/252)	0 (0/1865)
6	17.4 ± 1.8 (314/1831)	98.5 ± 1.5 (314/319)	0.06 ± 0.08 (1/2111)	0.26 ± 0.36 (1/450)	0 (0/248)	0 (0/334)
total	46.0 ± 4.1 (2901/6357)	97.9 ± 0.9 (2901/2962)	0.19 ± 0.02 (18/9706)	0.40 ± 0.04 (18/4478)	0 (0/2176)	0 (0/3489)

^aThe numerator and denominator in the parentheses of column “X/Y” are the total number of X- and Y-immunopositive cell bodies and Y-positive cell bodies, respectively, in the examined cortical strips of three mice.

^bPercentages are given as mean ± standard deviation (SD) in three mice. The percentages of Layer 1 are not evaluated mainly because the numbers of cell bodies were small.

Table 4. Colocalization of PPD and SOM immunoreactivity in the mouse mPF, OF, M1 and V1.

	M1		V1		OF		mPF	
Layer	PPD/SOM	SOM/PPD	PPD/SOM	SOM/PPD	PPD/SOM	SOM/PPD	PPD/SOM	SOM/PPD
1	– (0/13) ^a	– (0/0)	– (1/6)	– (1/1)	– (6/11)	– (6/6)	– (1/4)	– (1/1)
2-3	39.8 ± 5.4 ^b (296/754)	96.7 ± 1.7 (296/306)	49.3 ± 1.0 (218/441)	91.3 ± 1.3 (218/238)	59.7 ± 10.1 (166/280)	96.9 ± 3.0 (166/172)	26.8 ± 3.1 (117/450)	43.3 ± 8.7 (117/273)
4	–	–	85.8 ± 3.1 (399/467)	99.1 ± 0.7 (399/403)	–	–	–	–
5	46.1 ± 4.6 (1001/2168)	99.4 ± 0.2 (1001/1007)	58.4 ± 3.7 (825/1437)	96.0 ± 0.9 (825/858)	63.0 ± 3.7 (547/870)	97.8 ± 1.5 (547/560)	13.6 ± 7.3 (136/985)	17.6 ± 11.8 (136/822)
6	9.6 ± 2.3 (107/1090)	99.0 ± 1.5 (107/108)	15.3 ± 1.1 (65/432)	92.6 ± 10.5 (65/73)	8.8 ± 1.3 (31/358)	96.7 ± 4.7 (31/32)	1.0 ± 0.0 (5/522)	27.8 ± 3.9 (5/18)
total	34.9 ± 3.6 (1404/4025)	98.8 ± 0.3 (1404/1421)	54.9 ± 2.8 (1508/2783)	95.9 ± 0.5 (1508/1573)	49.7 ± 4.4 (750/1519)	97.4 ± 1.4 (750/770)	13.3 ± 4.5 (259/1961)	24.1 ± 10.4 (259/1114)

^aThe numerator and denominator in the parentheses of column “X/Y” are the total number of cell bodies positive for X and Y and Y-positive cell bodies, respectively, in the examined cortical strips of three mice.

^bPercentages are given as mean ± standard deviation (SD) in three mice. The percentages of Layer 1 are not evaluated mainly because the numbers of cell bodies were small.

M1, primary motor area; mPF, medial prefrontal area; OF, orbitofrontal area; V1, primary visual area.

Table 5. Colocalization of PPD immunoreactivity in NPY-, CR- or NOS-immunopositive cell bodies in the S1.

Layer	PPD/NPY	NPY/PPD	PPD/CR	CR/PPD	PPD/NOS	NOS/PPD
1	— (0/7) ^a	— (0/4)	— (0/27)	— (0/2)	— (0/1)	— (0/4)
2-3	0.15 ± 0.22 ^b (1/676)	0.19 ± 0.26 (1/564)	16.7 ± 0.2 (153/934)	25.8 ± 3.4 (153/593)	0 (0/41)	0 (0/505)
4	0.98 ± 1.39 (2/194)	0.36 ± 0.51 (2/521)	6.4 ± 2.5 (18/277)	3.7 ± 1.5 (18/485)	0 (0/3)	0 (0/574)
5	0.53 ± 0.41 (2/413)	0.12 ± 0.09 (2/1811)	11.5 ± 4.4 (33/269)	1.9 ± 0.9 (33/1646)	0 (0/80)	0 (0/1802)
6	0.29 ± 0.41 (1/438)	0.34 ± 0.48 (1/277)	0 (0/79)	0 (0/300)	0 (0/198)	0 (0/225)
total	0.39 ± 0.33 (6/1728)	0.20 ± 0.15 (6/3177)	12.8 ± 0.5 (204/1586)	6.7 ± 1.3 (204/3026)	0 (0/323)	0 (0/3110)

^aThe numerator and denominator in the parentheses of column “X/Y” are the total number of X- and Y-immunopositive cell bodies and Y-positive cell bodies, respectively, in the examined cortical strips of three mice.

^bPercentages are given as mean ± standard deviation (SD) in three mice. The percentages in Layer 1 are not evaluated mainly because the numbers of cell bodies were small.

Table 6. Colocalization of SOM, NPY, CR or NOS immunoreactivity in cortical neurons expressing GAD67 mRNA signals in the S1.

Layer	SOM/GAD67	GAD67/SOM	NPY/GAD67	GAD67/NPY	CR/GAD67	GAD67/CR	NOS/GAD67	GAD67/NOS
1	– (6/444) ^a	– (6/6)	– (24/457)	– (24/24)	– (29/440)	– (29/31)	– (0/457)	– (0/0)
2-3	14.5 ± 2.0 ^b (420/2933)	99.1 ± 1.2 (420/424)	21.1 ± 0.5 (681/3223)	99.4 ± 0.2 (681/685)	14.8 ± 1.9 (492/3283)	99.5 ± 0.4 (492/494)	0.53 ± 0.8 (18/3410)	100.0 ± 0.0 (492/494)
4	20.0 ± 0.7 (380/1912)	98.8 ± 1.2 (380/385)	9.8 ± 0.2 (199/2016)	99.6 ± 0.5 (199/200)	10.6 ± 1.5 (181/1712)	99.6 ± 0.6 (181/182)	0.06 ± 0.08 (1/1714)	– ^c (1/1)
5	49.0 ± 4.1 (1921/3928)	99.6 ± 0.2 (1921/1929)	8.1 ± 0.8 (361/4417)	99.8 ± 0.4 (361/362)	5.7 ± 1.0 (219/3782)	99.4 ± 0.9 (219/221)	0.93 ± 0.10 (38/4104)	100.0 ± 0.0 (4104/4104)
6	40.6 ± 4.7 (971/2379)	97.4 ± 0.4 (971/997)	17.3 ± 2.7 (461/2730)	98.6 ± 1.4 (461/469)	3.0 ± 0.8 (78/2665)	93.7 ± 4.6 (78/84)	4.99 ± 0.38 (151/2982)	100.0 ± 0.0 (151/151)
total	31.9 ± 2.3 (3698/11596)	98.9 ± 0.3 (3698/3741)	13.4 ± 0.1 (1726/12843)	99.2 ± 0.4 (1726/1740)	8.3 ± 0.9 (999/11882)	98.7 ± 0.7 (999/1012)	1.63 ± 0.20 (208/12667)	100.0 ± 0.0 (208/208)

^aThe numerator and denominator in the parentheses of column “X/Y” are the total number of cell bodies positive for X and Y and Y-positive cell bodies, respectively, in the examined cortical strips of three mice.

^bPercentages are given as mean ± standard deviation (SD) in three mice. The percentages of Layer 1 are not evaluated mainly because the numbers of cell bodies were small.

^cThe percentage in layer 4 was not calculated, because the data contained the denominator = 0.

Table 7. Colocalization between SOM, NPY, CR and NOS immunoreactivities in single neurons of the S1.

Layer	NPY/SOM	SOM/NPY	CR/SOM	SOM/CR	NOS/SOM	SOM/NOS
1	– (1/7) ^a	– (1/3)	– (0/4)	– (0/42)	– (4/10)	– (4/4)
2-3	6.0 ± 1.0 ^b (49/819)	7.2 ± 0.8 (49/680)	25.3 ± 3.2 (245/948)	30.3 ± 2.8 (245/794)	3.3 ± 0.4 (25/756)	100.0 ± 0.0 (25/25)
4	1.0 ± 0.4 (6/574)	3.2 ± 1.3 (6/187)	4.9 ± 0.4 (32/648)	10.7 ± 0.9 (32/298)	0.8 ± 0.6 (5/637)	– ^c (5/5)
5	3.4 ± 0.4 (83/2475)	22.9 ± 2.5 (83/365)	3.5 ± 1.3 (91/2709)	26.5 ± 4.9 (91/340)	3.4 ± 0.9 (85/2431)	100.0 ± 0.0 (85/85)
6	13.9 ± 3.6 (174/1246)	38.0 ± 9.2 (174/454)	2.1 ± 1.1 (33/1681)	30.9 ± 2.6 (33/104)	13.4 ± 3.5 (176/1317)	100.0 ± 0.0 (176/176)
total	6.2 ± 1.4 (313/5121)	18.5 ± 3.0 (313/1689)	6.7 ± 1.2 (401/5990)	25.2 ± 1.5 (401/1578)	5.7 ± 1.2 (295/5151)	100.0 ± 0.0 (295/295)

Layer	NPY/CR	CR/NPY	NPY/NOS	NOS/NPY	NOS/CR	CR/NOS
1	– (0/35)	– (0/8)	– (1/2)	– (1/4)	– (0/40)	– (0/4)
2-3	3.8 ± 0.3 (28/724)	3.5 ± 0.5 (28/789)	97.4 ± 26.2 (39/40)	7.0 ± 0.1 (39/556)	1.8 ± 0.5 (14/749)	66.9 ± 17.0 (14/21)
4	0.3 ± 0.5 (1/295)	0.8 ± 1.1 (1/186)	– ^c (2/2)	1.6 ± 1.2 (2/146)	0 (0/268)	– ^c (0/2)
5	9.1 ± 3.9 (30/334)	8.6 ± 3.5 (30/350)	100.0 ± 0.0 (87/87)	21.8 ± 4.5 (87/389)	8.1 ± 0.5 (23/285)	36.8 ± 4.2 (23/63)
6	22.1 ± 1.2 (38/173)	8.1 ± 0.6 (38/467)	99.2 ± 1.1 (207/209)	47.2 ± 1.6 (207/437)	27.5 ± 6.1 (26/98)	23.0 ± 1.5 (26/114)
total	6.3 ± 0.8 (97/1561)	5.5 ± 0.6 (97/1800)	99.0 ± 0.9 (336/340)	21.8 ± 1.1 (336/1532)	4.4 ± 0.2 (63/1440)	30.8 ± 1.8 (63/204)

^aThe numerator and denominator in the parentheses of column “X/Y” are the total number of X- and Y-immunopositive cell bodies and Y-positive cell bodies, respectively, in the examined cortical strips of three mice.

^bPercentages are given as mean ± standard deviation (SD) in three mice. The percentages of Layer 1 are not evaluated mainly because the numbers of cell bodies were small.

^cThe percentage in layer 4 was not calculated, because the data contained the denominator = 0.

Table 8. Colocalization of reelin immunoreactivity with SOM or PPD immunoreactivity in the S1.

Layer	Reelin/PPD	PPD/Reelin	Reelin/SOM	SOM/Reelin
1	^a — (3/4) ^b	— (3/559)	56.9 ± 5.6 (12/20)	2.2 ± 1.3 (12/543)
2-3	89.0 ± 4.4 (350/394)	39.0 ± 6.7 (350/914)	61.0 ± 7.8 (426/696)	41.5 ± 2.6 (426/1019)
4	97.4 ± 1.4 (420/431)	65.0 ± 7.7 (420/654)	88.7 ± 3.3 (515/581)	63.5 ± 9.4 (515/812)
5	88.1 ± 3.9 (1208/1376)	52.8 ± 1.1 (1208/2289)	71.9 ± 1.3 (1799/2499)	82.0 ± 3.0 (1799/2201)
6	92.6 ± 4.9 (141/150)	18.2 ± 4.8 (141/761)	45.0 ± 7.9 (466/1024)	60.2 ± 15.4 (466/800)
total	90.3 ± 3.1 (2122/2355)	41.1 ± 1.7 (2122/5177)	66.7 ± 2.1 (3218/4820)	60.2 ± 4.2 (3218/5375)

^aPercentages are given as mean ± standard deviation (SD) in three mice. The percentages of Layer 1 are not evaluated mainly because the numbers of cell bodies were small.

^bThe numerator and denominator in the parentheses of column “X/Y” are the total number of cell bodies positive for X and Y and Y-positive cell bodies, respectively, observed in the examined cortical strips of three mice.

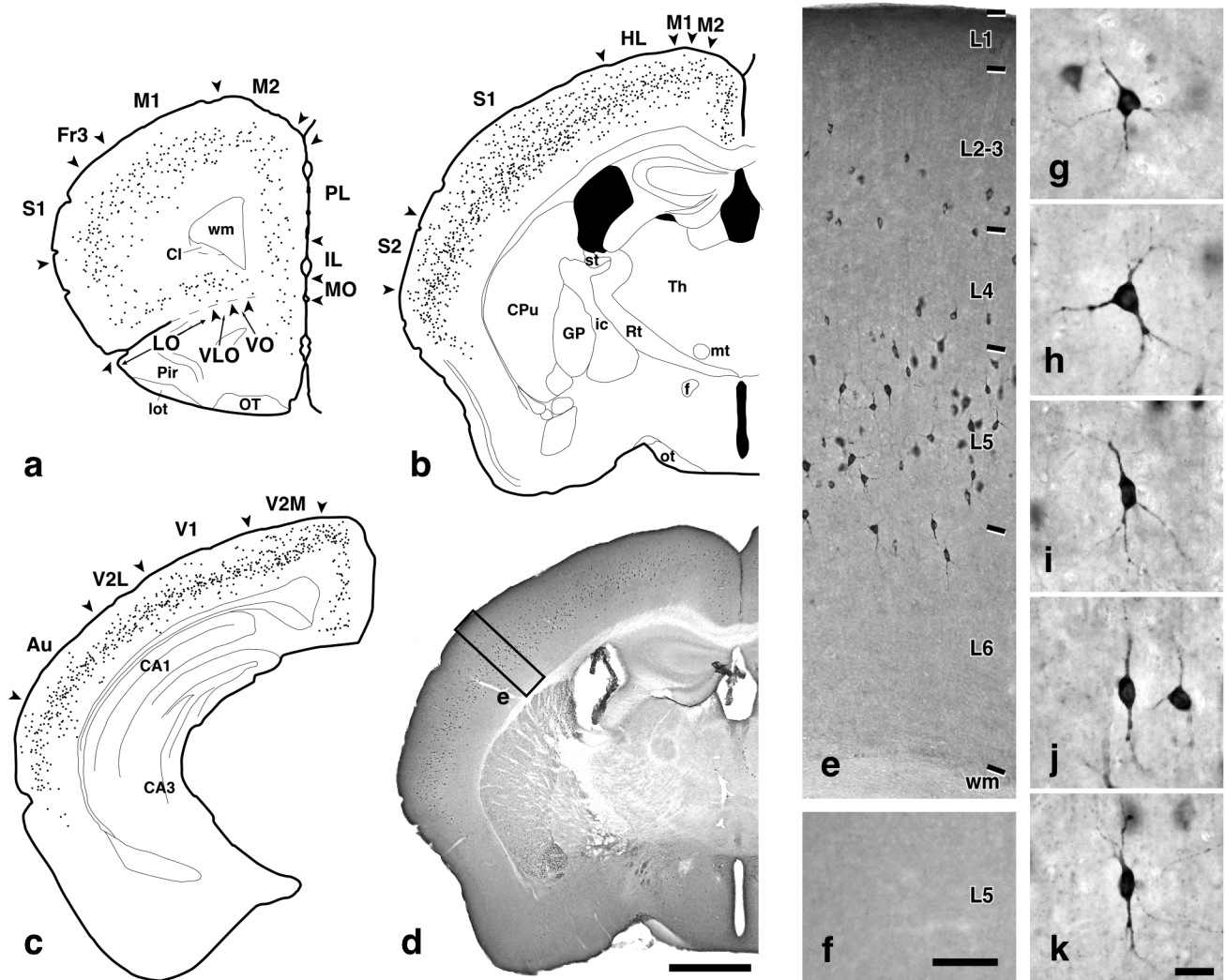


Figure 1. The distribution of preprodynorphin (PPD)-immunopositive neurons in the mouse neocortex. PPD-immunopositive neurons were distributed throughout the neocortical areas of rostral (a), middle (b) and caudal frontal sections (c). PPD-immunopositive neurons were plotted on all the cortical regions shown in a–c except the hippocampal region. d; The photograph corresponding to drawing b. e; The photograph of vertical strip e indicated with a rectangle in d. The immunoreactivity was completely blocked by pre-incubation of the antibody with an excess amount of the immunogen peptide (f). High power views of PPD-immunopositive cortical neurons showed multipolar (g–i) and bipolar dendrites (j, k). After taking images, the sections were counter-stained for Nissl with cresyl violet, and the cortical areas and layers (L1–L6) were determined by the cytoarchitecture. ac, anterior commissure; Au, auditory areas; CA, cornu ammonis; Cl, claustrum; CPu, caudate-putamen; f, fornix; Fr3, area 3 of the frontal cortex; GP, globus pallidus; HL, hindlimb area; ic, internal capsule; IL, infralimbic area; LO, lateral orbital area; lot, lateral olfactory tract; M2, secondary motor area; MO, medial orbital area; mt, mammillothalamic tract; OT, olfactory tubercle; ot, optic tract; Pir, piriform area; PL, prelimbic area; Rt, thalamic reticular nucleus; S2, secondary somatosensory area; st, stria terminalis; Th, thalamic nuclei; V2L, lateral part of the secondary visual area; V2M, medial part of the secondary visual area; VLO, ventrolateral orbital area; VO, ventral orbital area; wm, white matter. Scale bar = 1 mm in d applies to a–d, 100 μ m in f applies to e, f, and 20 μ m in k applies to g–k.

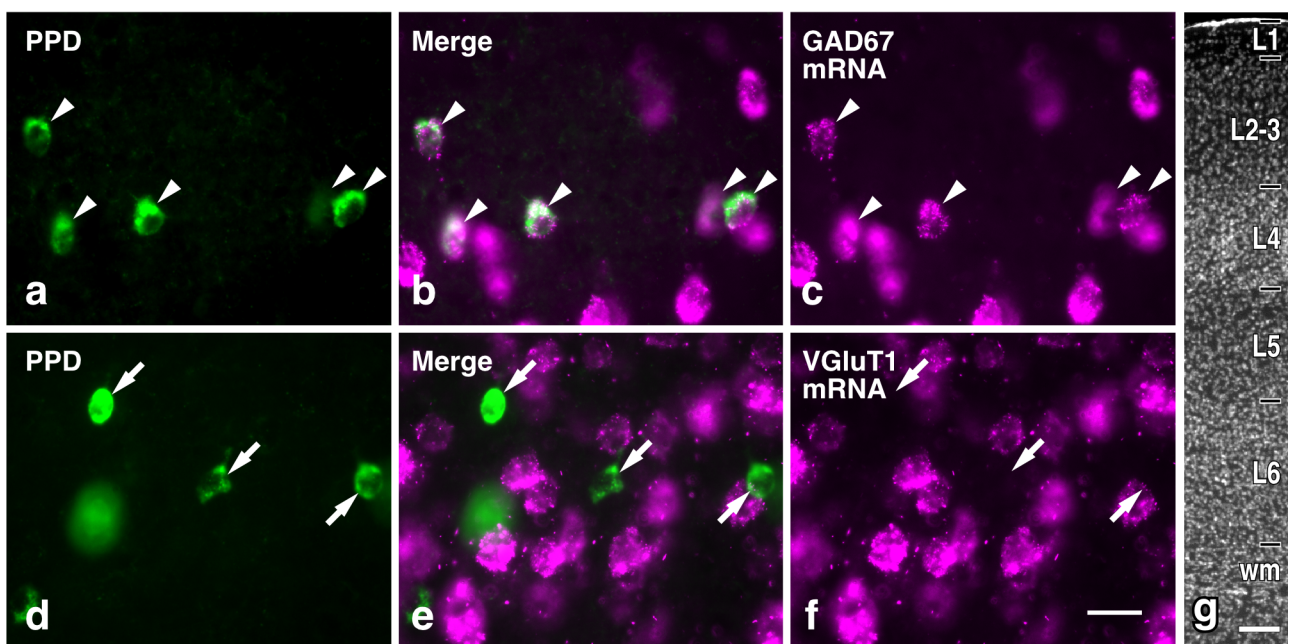


Figure 2. PPD immunoreactivity in inhibitory and excitatory neurons of the primary somatosensory area (S1). PPD immunoreactivity invariably showed mRNA signals for glutamic acid decarboxylase of 67 kDa (GAD67) (arrowheads in **a–c**), but not for vesicular glutamate transporter 1 (VGluT1) (arrows in **d–f**). Cortical layers were determined with the assistance of the counter-staining with 4',6-diamidino-2-phenylindole (DAPI) (**g**). L, layer; wm, white matter. Scale bar = 20 μ m in **f** applies to **a–f**; 100 μ m in **g**.

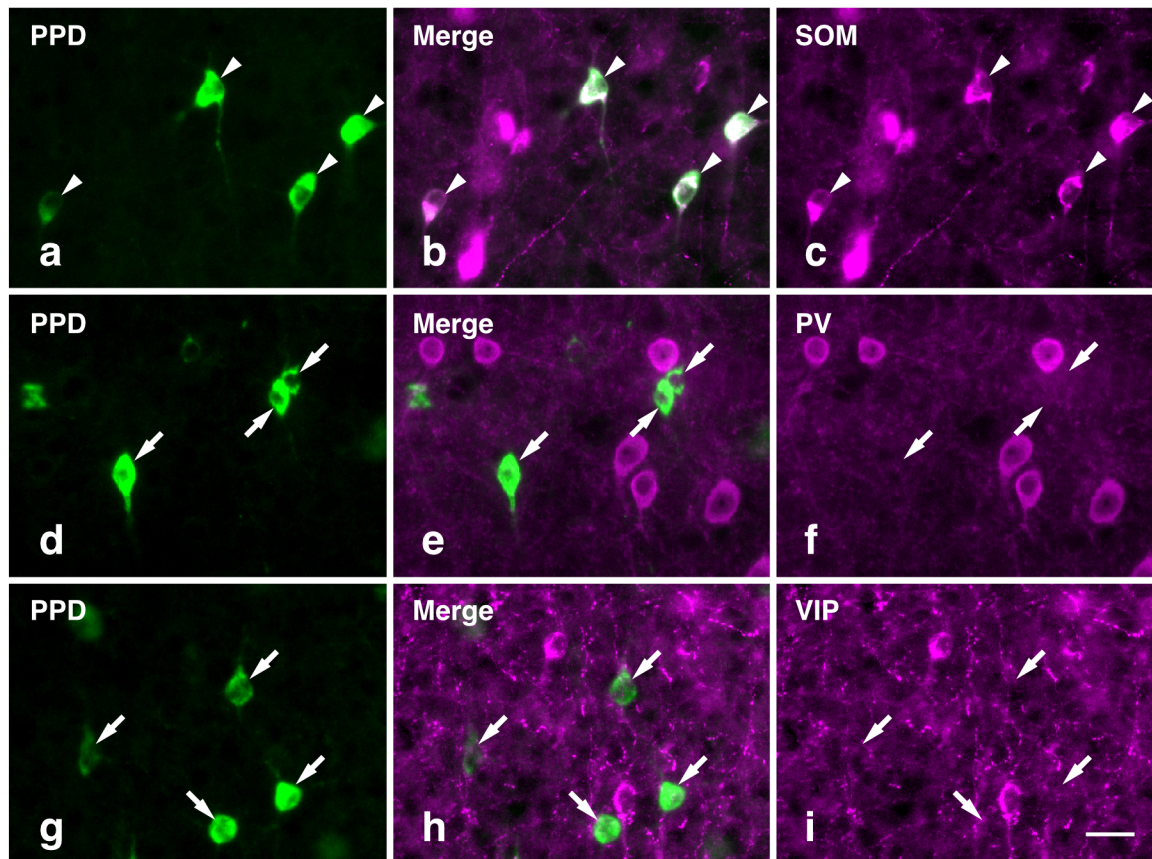


Figure 3. PPD immunoreactivity in GABAergic neuron subgroups of the S1. Of the major subgroup markers of cortical GABAergic neurons, only somatostatin (SOM) immunoreactivity was found in PPD-immunopositive cell bodies (arrowheads in **a–c**). No PPD-immunopositive neurons showed parvalbumin (PV) or vasoactive intestinal polypeptide (VIP) immunoreactivity (arrows in **d–i**). Scale bar = 20 μ m in **i** applies to all the images.

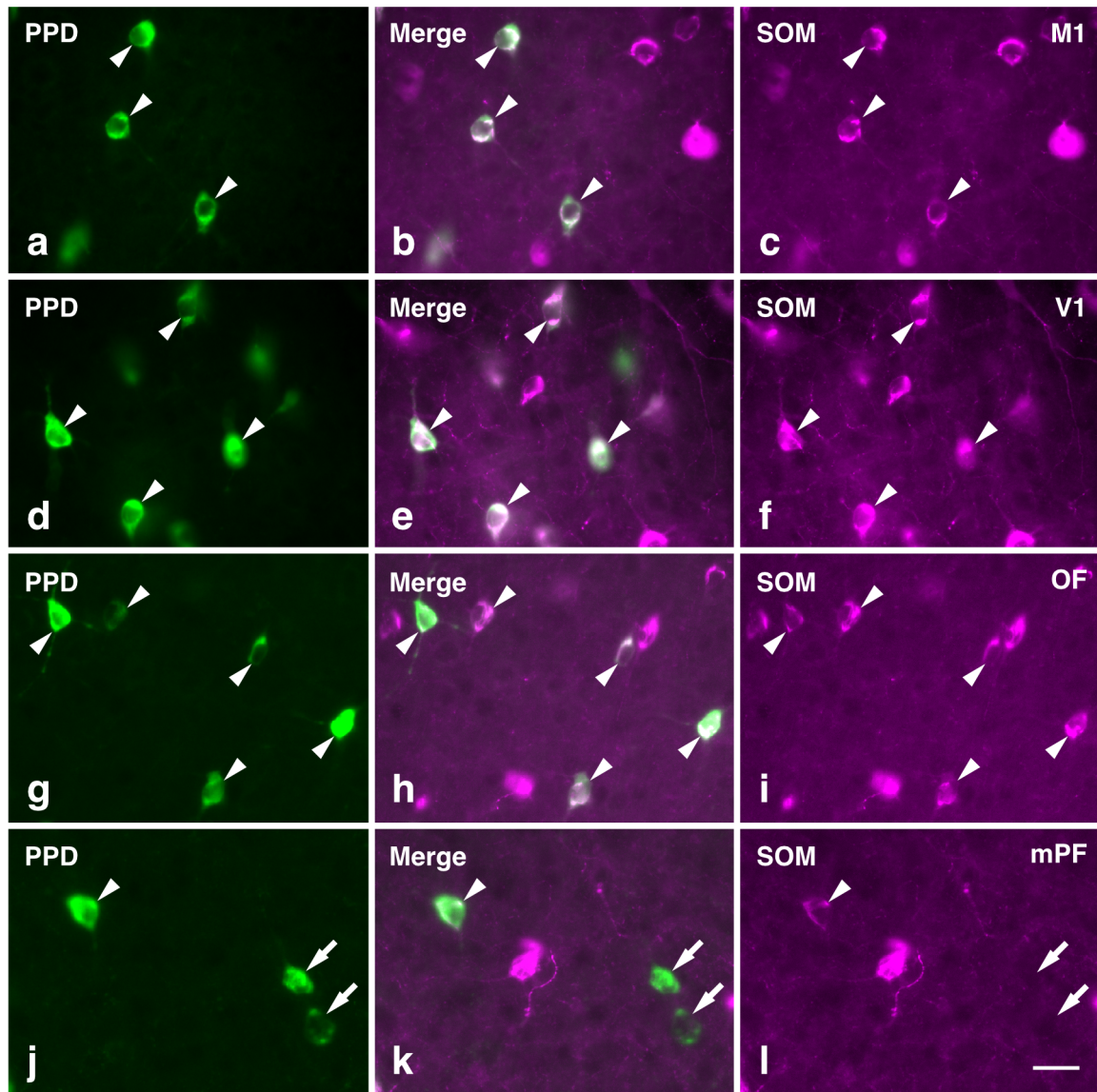


Figure 4. Colocalization of PPD and SOM immunoreactivities in the primary motor (M1), primary visual (V1), orbitofrontal (OF) and medial prefrontal areas (mPF). Almost all PPD-immunopositive neurons were immunoreactive for SOM in the OF, M1 and V1 (arrowheads in **a–i**) as in the S1 (Fig. 3). In contrast, some PPD-immunoreactive neurons were negative for SOM in the mPF (arrows in **j–l**). Scale bar = 20 μ m in **l** applies to all the images.

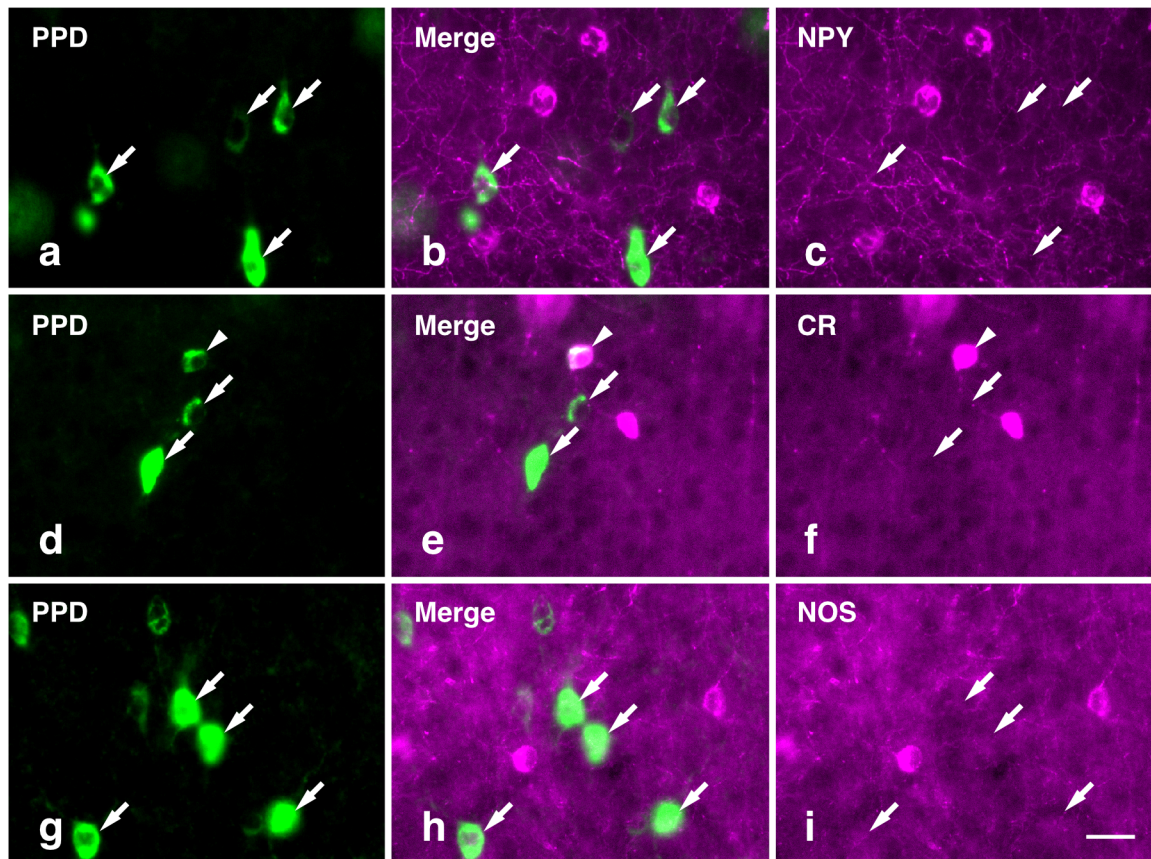


Figure 5. Infrequent colocalization of PPD immunoreactivity with subpopulation markers of SOM-immunopositive neurons in the S1. Almost no PPD-immunopositive neurons showed neuropeptide Y (NPY) (arrows in **a–c**) or nitric oxide synthase (NOS) immunoreactivity (**g–i**). A few PPD-immunopositive neurons were immunoreactive for calretinin (CR) (arrowheads in **d–f**), although the majority of PPD-immunopositive neurons were negative for CR immunoreactivity (arrows). Scale bar = 20 μ m in **i** applies to all the images.

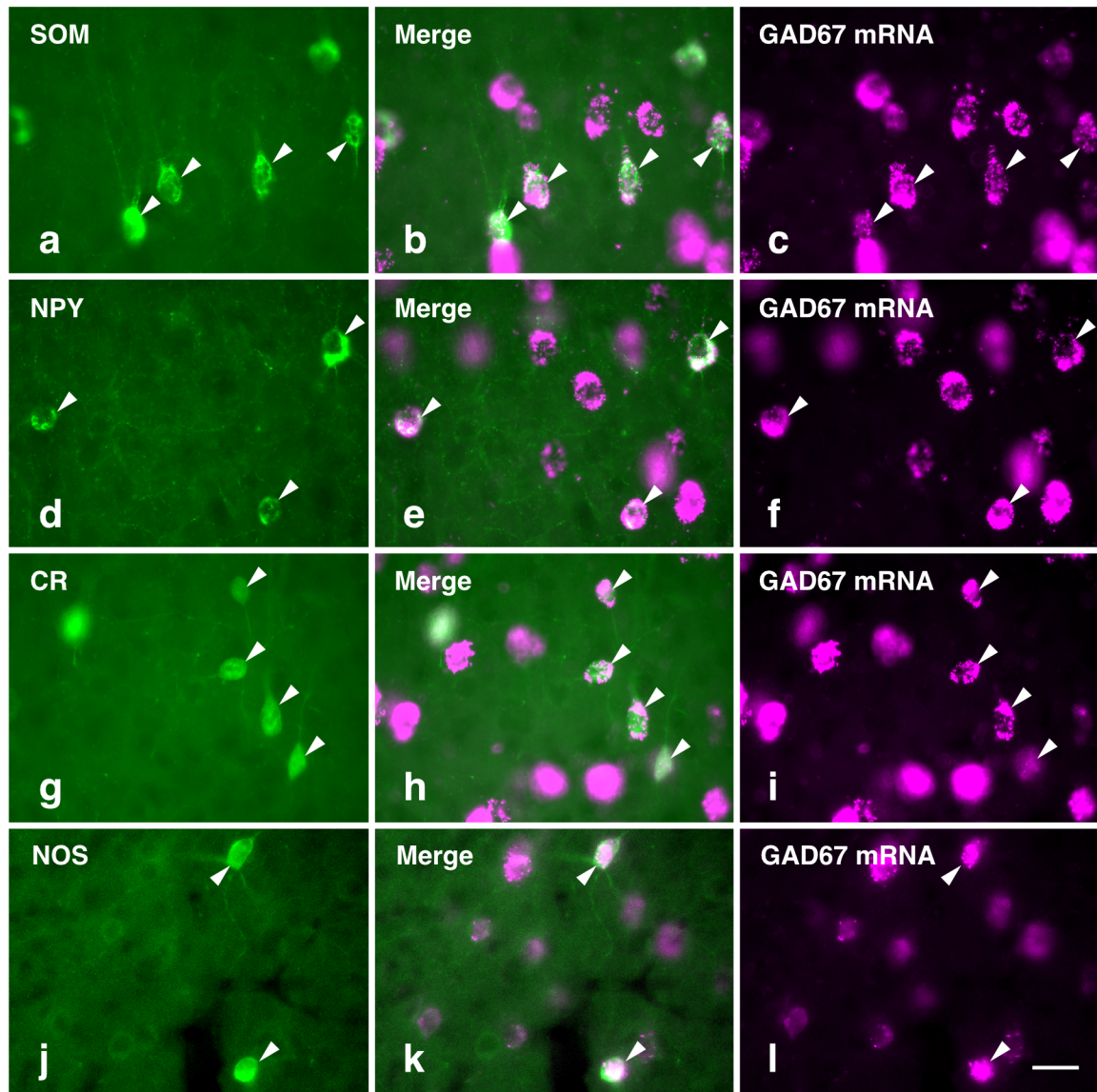


Figure 6. GABAergic characteristics of SOM-, NPY-, CR- and NOS-immunoreactive neurons in the S1. Almost all SOM- (arrowheads in a–c), NPY- (d–f), CR- (g–i) and NOS-immunoreactive neurons (j–l) showed mRNA signals for GAD67. Scale bar = 20 μ m in l applies to all the images.

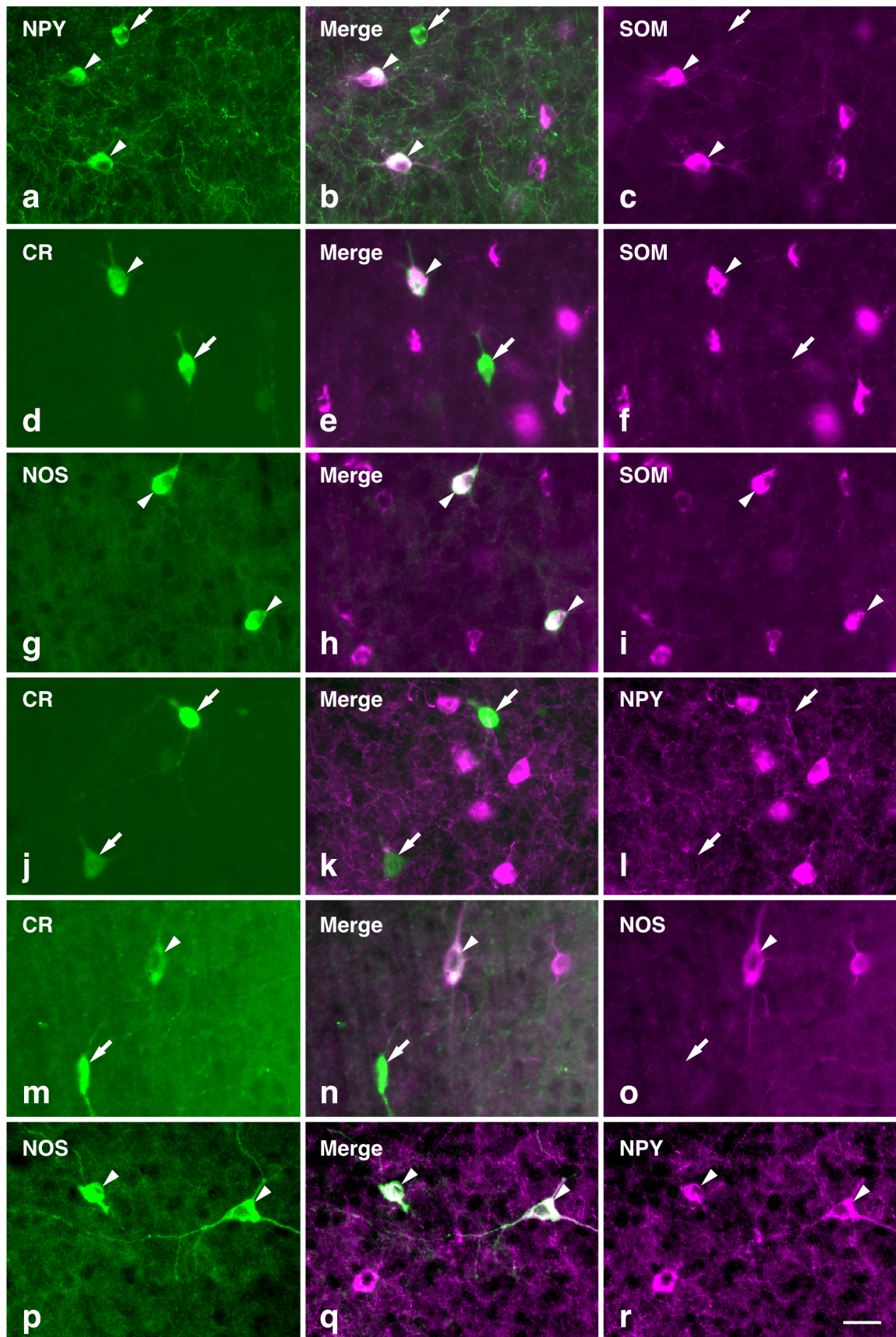


Figure 7. Mutual relationships of subpopulation markers of the SOM-immunoreactive neurons in the S1. A part of NPY- and CR-immunopositive neurons showed SOM immunoreactivity in their cell bodies, and all NOS-immunopositive neurons displayed SOM immunoreactivity (arrowheads in **a–i**). Many CR-immunopositive neurons were negative for NPY and NOS (arrows in **j–o**), whereas almost all NOS-immunopositive neurons were positive for NPY (arrowheads in **p–r**). Scale bar = 20 μ m in **r** applies to all the images.

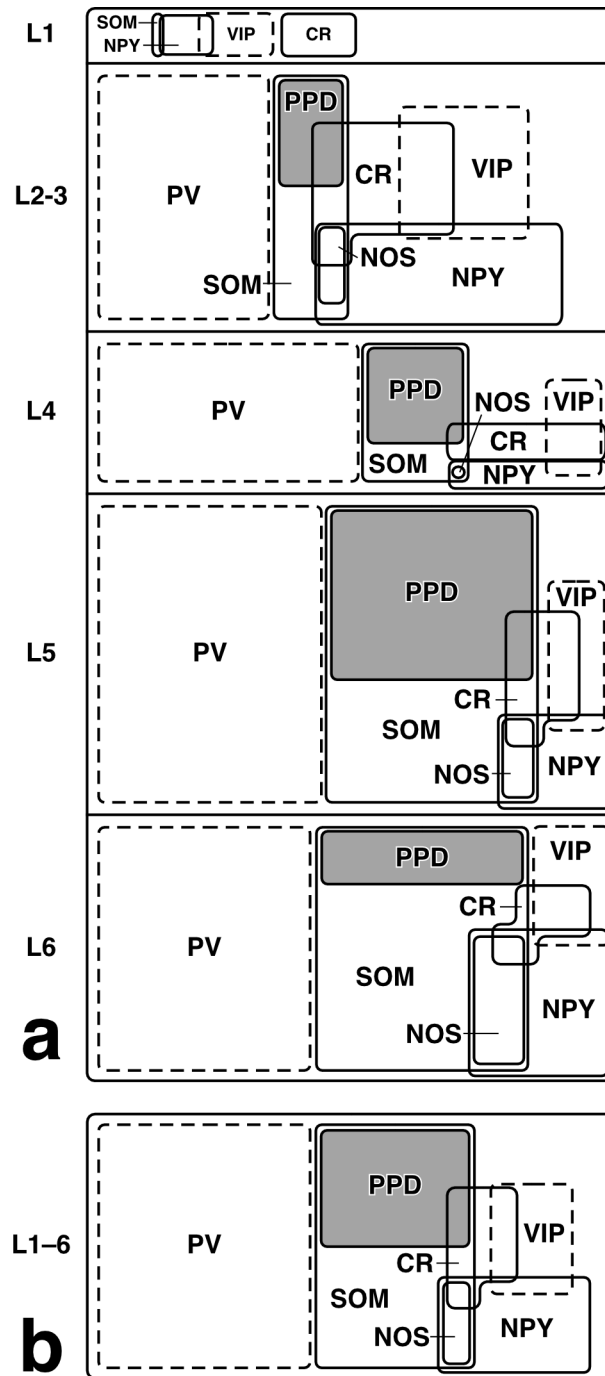


Figure 8. Relative distribution of GABAergic subpopulation sizes with the emphasis on the relationship of PPD- and SOM-expressing subgroups. The rectangle size associated with each cortical layer in **a** indicates the relative population size of GABAergic neurons among the layers. The relative sizes of GABAergic subpopulations in all the cortical layers are summarized in **b**. The relative population size of PV- or VIP-containing neurons was calculated from the data reported previously in the mouse neocortex (Hioki et al., 2013 or Xu et al. 2010, respectively). For more detail, see text.

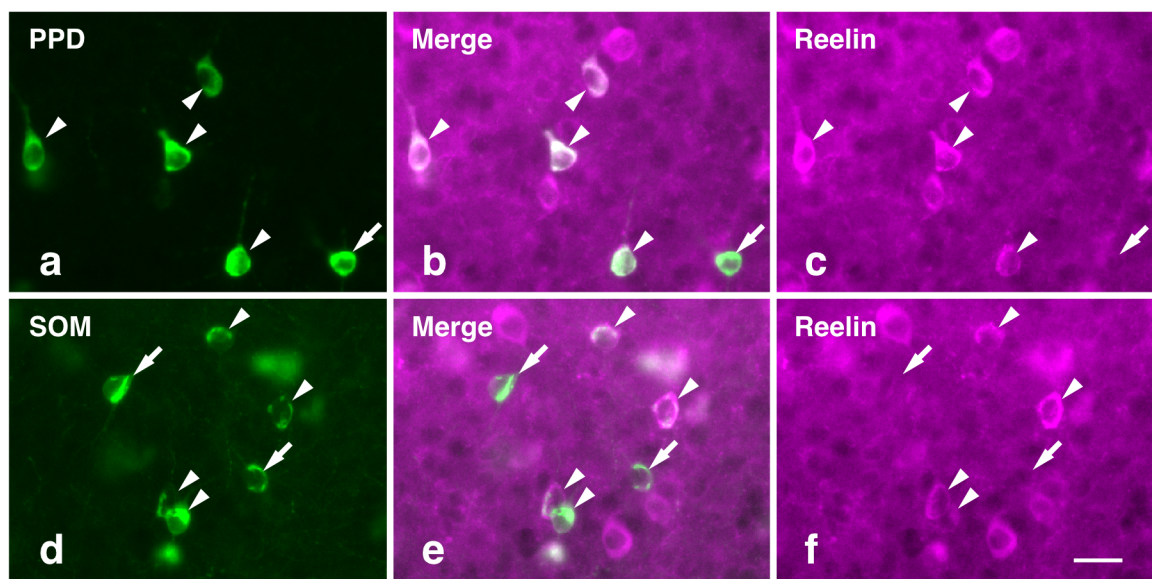


Figure 9. Reelin immunoreactivity in the PPD- and SOM-immunopositive neurons of the S1. Although most PPD-immunopositive neurons were positive for reelin (arrowheads in **a–c**), but a few neurons negative for reelin (arrow). About 2/3 of SOM-immunopositive neurons were immunoreactive for reelin and vice versa (arrows in **d–f**). Scale bar = 20 μm in **r** applies to all the images.