Three Distinct Families of GABAergic Neurons in Rat Visual Cortex

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In the cortex inhibition is mediated predominantly by GABAergic interneurons. Although all of these neurons use the same neurotransmitter, studies in the rat frontal cortex have shown that they are molecularly and physiologically diverse. It is not known whether similar subgroups of GABAergic neurons exist in primary visual cortex and how these different inhibitory neurons are inserted into specific cortical circuits. We have used immunostaining with antibodies against gamma aminobutyric acid (GABA), parvalbumin (PV), calretinin (CR), somatostatin (SOM), calbindin (CB) and nitric oxide synthase (NOS) to probe for colocalization of known markers of GABAergic interneurons. The results show that the majority of PV (100%), SOM (89.8%) and CR (93.9%) staining neurons are GABA positive. PV immunoreactive neurons constitute a distinct group that show no overlap with CR, SOM and NOS expressing cells and only a minor overlap (5.3%) with CB. PV immunoreactive cells account for 50.8% of GABAergic neurons. A second group of SOM expressing neurons accounts for 16.9% of GABAergic cells. None of these cells colocalize PV or CR, but 1.7% of SOM neurons stain for NOS and 86.3% show CB immunoreactivity. The third distinct group of CR expressing cells accounts for 17.0% of GABAergic neurons. All of these are PV, CB, SOM and NOS negative. CB expressing neurons represent a heterogeneous group that includes GABAergic and non-GABAergic cells. Our findings indicate that GABAergic neurons in rat area 17 are organized in at least three separate families that can be identified by the expression of PV, CR and SOM. These cells account for 84.9% of GABAergic neurons. These results extend previous observations in rat frontal agranular cortex and suggest that in visual cortex the inhibitory network is composed of similar cell types.

In mammalian visual cortex the main source of inhibition is provided by neurons that use GABA as neurotransmitter. These neurons are morphologically distinct from pyramidal cells and account for 15–25% of all cortical neurons (Jones, 1993). Although they share nonpyramidal morphology it is well established that they are anatomically, molecularly and physiologically heterogeneous (Fairen *et al.*, 1984; Jones and Hendry, 1986; Naegele and Barnstable, 1989; Kawaguchi, 1995). In an effort to eliminate ambiguities inherent in classification schemes based on axonal and dendritic morphology (Fairen *et al.*, 1984) an important advance was made by the discovery that many nonpyramidal and GABAergic neurons colocalize peptides such as somatostatin (SOM), cholecystokinin (CCK), neuropeptide Y (NPY), vasointestinal polypeptide (VIP) and the neurotransmitter markers choline acetyltransferase, nitric oxide synthase (NOS) and NADPH-diaphorase (Hendry *et al.*, 1984; Somogyi *et al.*, 1984; Jones *et al.*, 1987; Kosaka *et al.*, 1987; Rogers, 1992; Vincent and Kimura, 1992; Dennison-Cavanagh *et al.*, 1993; Valtschanoff *et al.*, 1993). A significant extension of these studies was provided by probing GABAergic neurons for the expression of the calcium binding protein parvalbumin (Celio, 1986). Subsequent studies have shown that parvalbumin (PV) and calbindin-D28 (CB) label two groups of cells that account for a substantial proportion of GABAergic neurons in rat, cat and monkey cerebral cortex (Demeulemeester *et al.*, 1989; Hendry *et al.*, 1989; Celio, 1990; Van Brederode *et al.*, 1990, 1991; Jones, 1993; DeFelipe, 1993; Condé *et al.*, 1994). Although the majority of cells expressed either PV or CB, enthusiasm over the identification of cell specific markers was attenuated by the finding that PV and CB were coexpressed in some GABAergic neurons and that CB was not exclusively confined to nonpyramidal cells (Hendry *et al.*, 1989; Van Brederode *et al.*, 1990; DeFelipe, 1993; Jones, 1993; Kubota *et al.*, 1994). The partial success of PV and CB immunostaining initiated studies with antibodies against calretinin (CR), which indicated the existence of an additional group of GABAergic neurons (Jacobowitz and Winsky, 1991; Rogers, 1992; Rogers and Résibois, 1992). However, GABA was detected only in a small proportion of CR cells (Rogers, 1992). Until recently it remained largely unknown what substances mark separate, nonoverlapping classes of GABAergic neurons. Thus, considerable attention was received by the observation that PV, CR and SOM immunoreactive cells in rat frontal cortex constitute distinct, nonoverlapping groups of GABAergic neurons (Kubota *et al.*, 1994).

Motivated by a long-term interest in understanding how inhibitory neurons are integrated into distinct visual cortical circuits (Johnson and Burkhalter, 1996), the goal of the present study was to test the hypothesis derived from the work in rat frontal cortex (Kubota *et al.*, 1994) that GABAergic neurons in sensory cortex contain molecularly distinct cell types. For this purpose we have probed GABAergic neurons in rat primary visual cortex for the colocalization of different markers using antibodies against GABA, PV, CR, SOM, CB and NOS in pairs of all possible combinations.

Materials and Methods

Experiments were performed on 21 adult Long–Evans rats. Animals were anesthetized with sodium pentobarbital (80 mg/kg i.p.) and perfused through the aorta first with heparinized 0.1 M phosphate buffer (PB), followed by a mixture of 4% paraformaldehyde and 0.2–0.3% glutaraldehyde in PB, pH 7.4. The brain was removed from the skull, postfixed for 2–4 h in the same fixative (4°C) and then washed thoroughly in PB. Coronal sections were cut on a vibratome at 20 μm and collected in PB.

Immunocytochemistry

To reduce free aldehydes, sections were treated for 30 min in 1% phosphate buffered sodium borohydride. For single substance immunolabeling, sections were transferred for 1 h into 10% normal serum derived from the species that supplied the secondary antibody. Incubations were performed (overnight, with agitation at 4°C) in primary antibodies against gamma aminobutyric acid (rabbit anti-GABA, 1:500; Sigma, St Louis, MO; mouse anti-GABA, 1:500, Chemicon,

Temecula, CA), PV (rabbit anti-PV, 1:2000, Sigma; mouse anti-PV, 1:1000, Swant, Bellinzona, Switzerland), CR (rabbit anti-CR, 1:2000; Swant), CB (rabbit anti-CB, 1:500; Swant), SOM (rat anti-SOM, 1:500; Chemicon) and NOS (rabbit anti-NOS, 1:250; Chemicon). In some cases 0.1% Triton X-100 was included in the preincubation and incubation solutions. This, however, did not significantly improve staining with any of the antibodies and was therefore omitted in the material used for quantitative analyses. The next day sections were washed in PB containing 2% normal serum of the species from which the secondary antibody was derived. To visualize primary antibodies we used both immunofluorescence and immunoperoxidase procedures. For immunofluorescence, sections were incubated for 2 h in optimal dilutions (1:300) of Cy-3 labeled secondary antibody [goat anti-rabbit (Chemicon), goat anti-mouse (Chemicon); donkey anti-rat (Cappel, Durham, NC)]. For immunoperoxidase, sections were first treated for 2 h with biotinylated secondary antibody [1:200 biotinylated goat anti-rabbit (Chemicon); 1:200 biotinylated horse anti-mouse (Chemicon)], washed in PB and then incubated for 1 h in avidin and biotinylated HRP (Vectastain Elite Kit, Vector, Burlingame, CA). Sections were then exposed for 15 min to a Tris buffered solution (pH 7.4) of 0.04% $Ni(NH_4)(SO_4)_2$ and 0.05% $CoCl_2$. After that HRP activity was visualized in the presence of 0.01% H_2O_2 and 0.05% 3,3′-diaminobenzidine in PB.

For double immunolabeling in all combinations of antibodies against GABA, PV, CR, CB, SOM and NOS sections were incubated for 30 min in a 5%/5% mixture of two normal sera derived from the host species that provided the secondary antibodies. This step was followed by an overnight incubation (4°C) in a mixture of two primary antibodies raised in different species containing 0.25% normal serum from each of the species that supplied the secondary antibodies. The next day, sections were washed in a mixture of two normal sera (1%/1% in PB). This was followed by a 1 h treatment with an optimal concentration of fluorescein isothiocyanate (FITC) labeled goat anti-rabbit IgG (1:200; Vector, Burlingame, CA), followed by 1 h in biotinylated secondary antibody and an additional 1 h in avidin-neutralite conjugated to Texas Red (1:1000; Molecular Probes, Eugene, OR). Finally sections were washed, mounted on gelatin coated slides, air dried and coverslipped with Vectashield (Vector) mountant. Stained neurons were viewed and photographed under a fluorescence microscope equipped with rhodamine (for visualization of Texas Red and Cy3) and fluorescein (for visualization of FITC) optics. For visualization of layers, sections were counterstained with an aqueous solution of 0.001% bisbenzimide and viewed under UV optics.

Immunoperoxidase labeled sections were mounted on gelatinized slides, air dried, dehydrated in a series of graded ethanol, cleared in xylene and coverslipped with DPX mountant. For visualization of laminar and areal boundaries parallel sections were stained for Nissl substance (Fig. 1A). Microscopic examination and photography was performed under bright field illumination.

Quantification of Labeled Neurons

Stained sections were viewed at ×250 under a fluorescence microscope equipped with fluorescein, rhodamine and UV optics. Laminar distribution, percent colocalization of antigens and numerical densities of immunoreactive cells were studied in at least nine different, randomly selected sections taken from at least three different animals. Stained cells were counted in 410 μm wide vertical strips across all layers of area 17. To determine the laminar position of labeled neurons we measured for each cell the distance from the pial surface. Parallel sections stained with bisbenzimide to reveal the cytoarchitecture of area 17 (Paxinos and Watson, 1986) were used to convert distances into laminar positions and to determine the overall thickness of layers.

The quantitative analysis of double labeled neurons was performed by switching between fluorescein and rhodamine illumination and checking each cell stained with the first antibody for staining with a second antibody. After that neurons immunoreactive for the second substance were counted. Only cells with visible nuclei were scored. As a result we obtained for each 410 μm wide column and each antibody the number of single (e.g. PV only, GABA only) and double labeled cells (e.g. PV + GABA). From these data we calculated the percentage of colocalization: for example, the proportion of PV cells that express GABA and the proportion of GABA cells that colocalize PV. In some cases, to efficiently assess in a large number of cells whether they are GABAergic, CR, CB, SOM and NOS stained neurons were checked only for the coexpression of GABA. Numerical densities of immunolabeled neurons in different layers of area 17 were performed using the optical disector method (West, 1993; Coggeshall and Lekan, 1996). For this purpose we used a 41 × 41 μm counting frame and scored all labeled cells whose nuclei were in sharp focus between 5 and 15 μm below the surface of the section. Counts were made according to the rules of West and Gundersen (1990). A total of ~0.1 mm^3 of tissue was analyzed for each substance. Numerical densities were corrected for an estimated 6% linear and 17% volume shrinkage of the coverslipped tissue.

Controls

To control for specific labeling, the primary antibodies were omitted from the staining procedure. Sections treated in this manner showed no detectable labeling.

Results

Markers of GABAergic Neurons

GABA

Consistent with previous reports (Meinecke and Peters, 1987), our results show that all GABA immunopositive cell bodies were shaped like nonpyramidal neurons. The staining intensity of individual neurons varied over a wide range. Because weakly labeled neurons were often found near the cut surface of the section this property appeared unrelated to antibody penetration. Rather, it seemed to reflect different levels of GABA expression. GABA immunoreactive boutons often decorated GABA-negative pyramidal cell bodies, giving rise to neuropil labeling patterns that resembled pericellular baskets. Numerical densities of GABA immunoreactive neurons were similar in

Table 1

Numerical densities (no. of neurons/mm^3) of GABA, parvalbumin, calretinin, somatostatin, calbindin and nitric oxide synthase immunoreactive neurons in rat area 17

Cortical layer	GABA	Parvalbumin	Calretinin/*	Somatostatin/**	Calbindin	NOS
Layer 1	6168 ± 1725	0	1145 ± 793/1234 ± 440	0	occasional cells	0
Layer 2/3	12226 ± 2508	4540 ± 1377	2912 ± 720/2567 ± 970	2254 ± 564/1972 ± 438	1127 ± 106	193 ± 68
Layer 4	15605 ± 2998	11378 ± 2377	2482 ± 1345/2482 ± 1345	2689 ± 1033/2170 ± 1240	2379 ± 724	104 ± 43
Layer 5	12844 ± 2165	8979 ± 1407	1478 ± 829/1659 ± 685	2812 ± 721/2416 ± 613	2812 ± 865	95 ± 29
Layer 6	11002 ± 3986	6779 ± 1266	1174 ± 401/958 ± 371	2658 ± 773/2317 ± 587	1483 ± 649	120 ± 42
All layers	11836 ± 3327	6018 ± 637	1822 ± 106/1725 ± 416	2326 ± 292/1999 ± 230	1681 ± 186	121 ± 53
Under 1 mm^2 of pial surface	16317 ± 3207	8293 ± 878	2512 ± 146/2378 ± 573	3207 ± 402/2756 ± 317	2317 ± 280	168 ± 59
	$n = 1067$	$n = 612$	$n = 177$	$n = 271$	$n = 152$	$n = 238$

In the calretinin and somatostatin columns, the top number indicates densities of all CR and SOM cells, the bottom number represents CR and SOM cells that coexpress GABA. Mean number and standard deviation are presented. *Number of calretinin immunoreactive neurons showing GABA immunoreactivity;**number of somatostatin immunoreactive neurons showing GABA immunoreactivity.

layers 2/3–6 and only layer 1 contained substantially fewer cells (Fig. 1*B*, Table 1). A similar picture was seen in plots of the relative distribution of labeled cells across different layers (Fig. 2).

Parvalbumin

Fewer neurons were labeled with antibodies to parvalbumin than to GABA (Table 1). Intense staining of nonpyramidal cell bodies (Figs 1*C* and 3*B*) in layers 2/3–6 suggested that this was

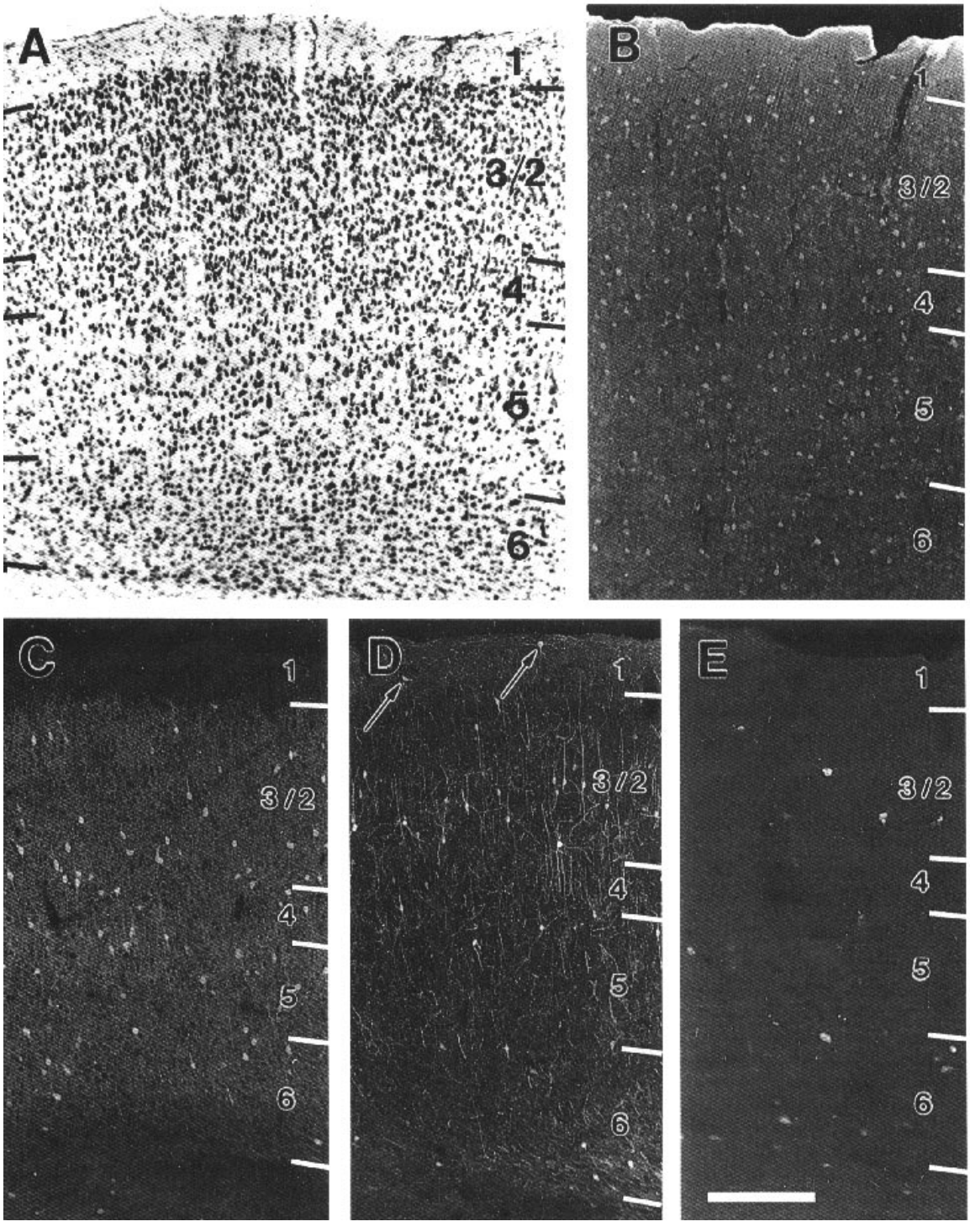

Figure 1. Photomicrographs of coronal sections through rat area 17, showing laminar distribution of different markers of GABAergic neurons. (*A*) Layers of area 17 revealed in Nissl stained section. (*B*) GABA immunoreactive neurons are distributed quite uniformly across all layers. (*C*) Parvalbumin (PV) immunoreactive neurons are absent from layer 1. PV stained neurons are slightly more numerous in deep than in superficial layers. Fibers of the neuropil are intensely stained in all layers, except for layer 1. Staining is most intense in layer 5. (*D*) Calretinin (CR) immunoreactive neurons and fibers are more numerous in superficial layers than in deep layers. CR stained neurons are found in layer 1 (arrows). (*E*) Somatostatin (SOM) immunoreactive neurons of different sizes are seen in layers 2/3–6. Layer 1 is devoid of labeling. Scale bar: 250 μm. *A–E* same magnification.

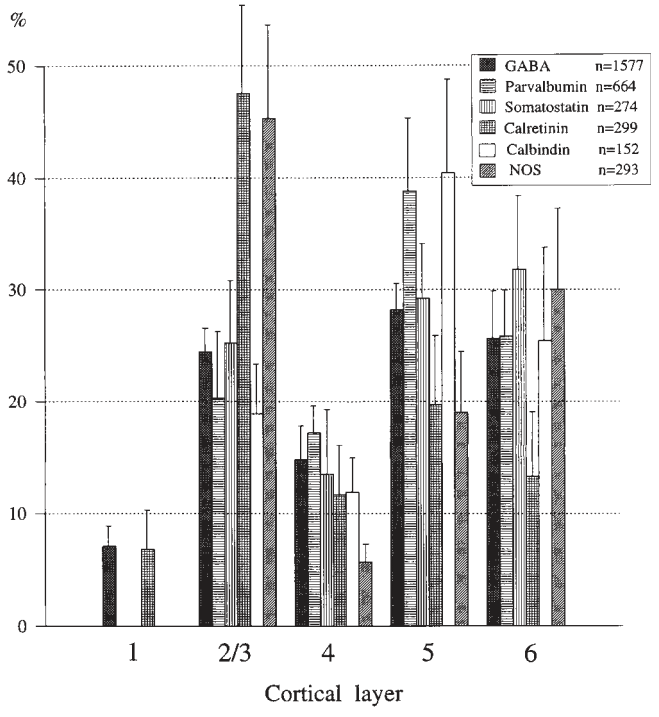

Figure 2. Laminar distribution of GABA, parvalbumin, somatostatin, calretinin, calbindin and nitric oxide synthase immunoreactive neurons in different layers of rat area 17. Ordinate indicates percent immunoreactive neurons. Error bars: SD.

not due to a lesser sensitivity of PV compared with GABA immunoreactivity (Fig. 1*B*). It is more likely that PV was expressed in a subpopulation of intensely GABA immunoreactive neurons (Fig. 3*A*, *B*) with a different laminar distribution (Fig. 2). This is best illustrated by the complete absence of PV staining neurons in layer 1 (Figs 1*C* and 4*A*). Indeed, double labeling experiments with GABA antibodies demonstrated that PV was expressed in only 50.8% (589/1160) of GABA immunoreactive neurons (Fig. 3*A*, *B*, Table 2). All of the PV positive neurons, however, colocalized GABA (100%, 664/664).

PV labeled fibers and terminals surrounded pyramidal cell profiles in layers 2/3–6. A particularly prominent plexus was seen in layer 5 (Fig. 1*C*).

Calretinin

In layers 2/3–6 the density of CR staining neurons was considerably lower than that for PV. Only layer 1 was different, in that it contained a small but reproducible number of CR labeled neurons (Figs 1*D* and 4*B*, Table 1). Similar to PV, CR antibodies exclusively stained non-pyramidal cells. Most of these cells expressed GABA (93.9%, 177/182; Table 2) to varying degrees of intensities (Fig. 3*C*, *D*). Although both CR and PV immunoreactive neurons were GABAergic, they had different morphologies and laminar distributions. Compared with PV positive neurons, cell bodies stained for CR tended to be slightly smaller. Unlike the uniformly multipolar-shaped PV cells (Figs 3*B* and 4*A*, *C*), CR immunoreactive neurons were evenly split between multipolar cells that were intensely stained for GABA and neurons with bipolar and bitufted dendrites in which GABA was expressed more weakly (Figs 3*C*, *D* and 4*D*). Weakly GABA immunoreactive cells were typically found in superficial layers. These layers also contained most CR expressing cells (Fig. 2). CR and PV expression patterns further differed in the neuropil: CR immunostaining was associated with vertical fibers (Figs 1*D* and 4*D*) that often seemed to contact other CR-positive profiles, and contrasted with the more irregular fiber labeling pattern and the stained pericellular baskets seen in PV stained sections (Figs 3*B* and 4*C*, *E*). The difference in PV and CR neuropil staining was particularly striking in layer 1. Here, CR immunoreactive axons and dendrites were abundant (Figs 1*D* and 4*B*) and could be followed spreading horizontally for up to 2 mm from the cell body. In contrast, PV stained processes in layer 1 were sparse and more narrowly confined (Fig. 4*A*). These diverse staining patterns suggest that CR staining neurons are distinct from PV immunoreactive cells and that they constitute a separate, smaller group of GABAergic neurons that account for 17% (102/538) of GABAergic neurons (Table 2).

Somatostatin

The numerical density of somatostatin expressing neurons in different layers was comparable to that seen with antibodies against CR (Fig. 1*E*, Table 1). As with PV and CR, SOM staining was confined to nonpyramidal cells. A large fraction of these coexpressed GABA (89.8%, 103/115; Fig. 3*E*, *F*). Interestingly, in all of these cells GABA expression was low. SOM immunoreactive neurons were among the largest GABAergic neurons. They typically had multipolar and bitufted morphologies (Figs 6*B* and 7*A*). Most SOM immunoreactive cells were located in layers 5 and 6 (Fig. 2). Fewer cells were found in layers 2/3 and 4, and staining was completely absent from layer 1 (Fig. 2, Table 1). SOM neurons accounted for 16.9% (180/1067) of all GABA-positive neurons in striate cortex (Table 2).

Calbindin

Calbindin antibodies labeled a large number of neurons, particularly in superficial layers. Labeled cells fell into two subgroups: intensely labeled and weakly labeled cells (Fig. 5*A*). Intensely labeled CB immunoreactive neurons were relatively rare and, typically, had large nonpyramidal cell bodies. Weakly labeled neurons were much more numerous and comprised both pyramidal and nonpyramidal cells. Most of the intensely CB immunoreactive cells coexpressed GABA (97%, 149/154; Table 2) and accounted for ~19% (108/569) of GABAergic interneurons. GABA staining, however, was not restricted to intensely CB labeled cells and many weakly CB stained neurons expressed variable levels of GABA immunoreactivity (Fig. 5*A*, *B*). However, many weakly CB positive cells in superficial layers were GABA negative. Because many weakly CB positive cells did not stain for GABA we focused our analyses on intensely CB immunoreactive neurons. These counts have shown that the numerical density of intensely stained CB cells was comparable to that of CR and SOM expressing neurons (Table 1). The laminar distribution of CB staining also showed similarities to SOM; however, the bias towards lower layers differed from the pattern seen in CR stained sections (Fig. 2). Occasional CB positive neurons were observed in layer 1.

Nitric Oxide Synthase

Numerically, NOS expressing neurons represented the smallest group of cells studied. Stained cell bodies tended to be large and resembled nonpyramidal neurons with bitufted or multipolar morphologies (Fig. 6*A*). Most of them were found in layer 2/3, with few cells being located in the middle and deep layers. NOS immunoreactive cells were absent from layer 1 (Fig. 2). A large fraction of NOS positive neurons coexpressed GABA (88%,

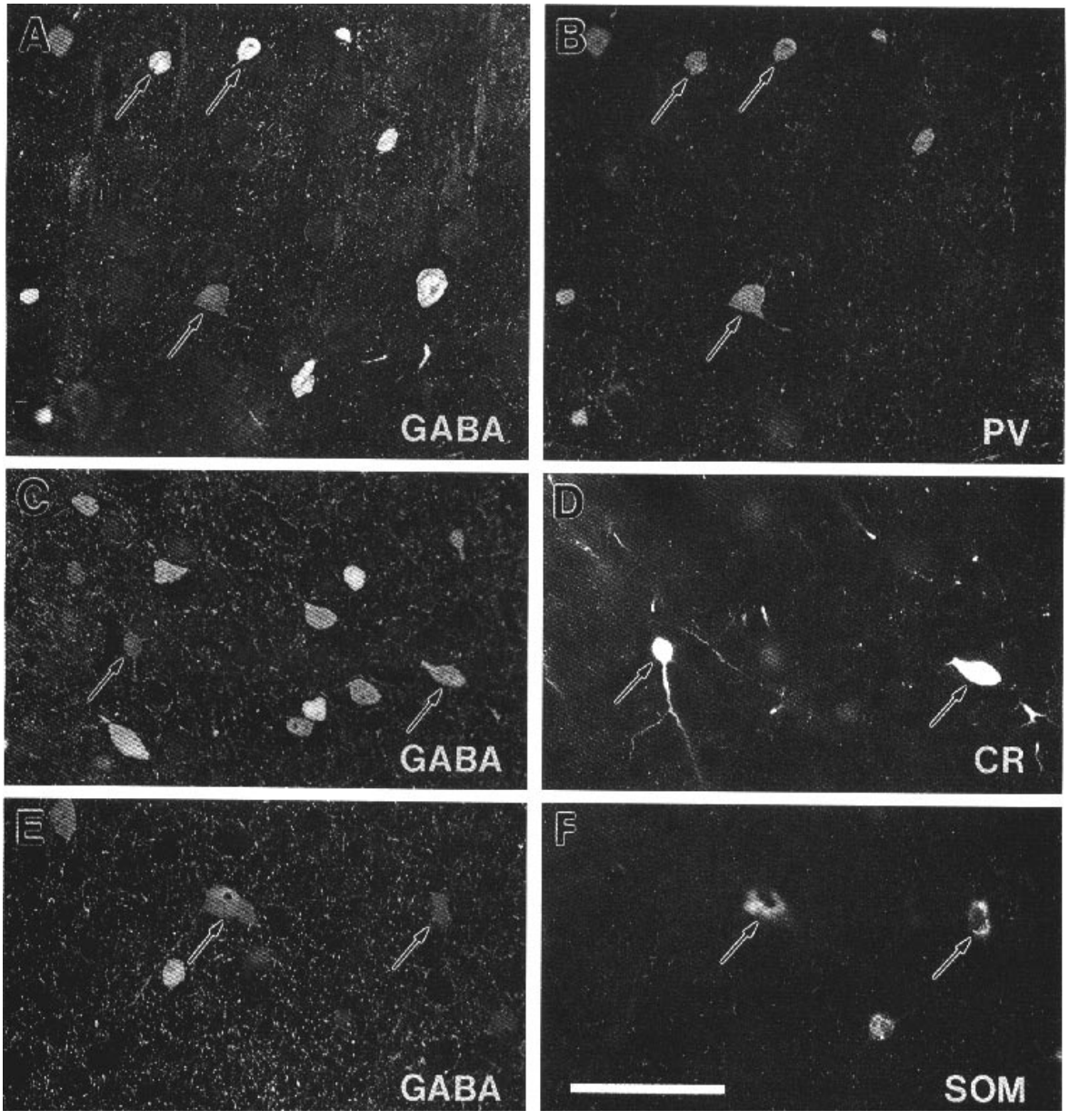

Figure 3. Colocalization of parvalbumin (PV), calretinin (CR) and somatostatin (SOM) immunofluorescence in GABAergic neurons of rat area 17. (*A*) GABA immunoreactivity in layer 5 revealed with a FITC labeled secondary antibody. (*B*) PV immunoreactivity in the same field shown in (*A*), seen under rhodamine illumination. All of the PV stained neurons coexpress GABA. Note that some of the most intensely GABA immunopositive neurons do not colocalize PV. (*C*) GABA immunofluorescence in layer 2/3 seen under fluorescein illumination. (*D*) CR immunoreactivity in same field shown in (*C*), seen under rhodamine illumination. All of the CR stained neurons coexpress GABA. (*E*) GABA immunolabeling in layer 5, seen under fluorescein illumination. (*F*) SOM immunoreactivity in the same section shown in (*E*), revealed under rhodamine illumination. Most SOM stained neurons coexpress low levels of GABA immunoreactivity. Arrows mark selected examples of double labeled cells. Scale bar: 50 μm. *A–F* same magnification.

53/60), but NOS staining neurons accounted for only 0.5% (51/10162) of GABAergic neurons in area 17. NOS immunoreactive fibers formed diffuse networks extending throughout all layers.

Colocalization of Markers

To assess the degree of overlap between different groups of GABAergic neurons that coexpress calcium binding proteins and/or peptides we examined the colocalization of PV, CR, SOM, CB and NOS in all possible pairs of combinations (Table 2).

Parvalbumin

As suggested by the distinct cell body morphology, the patterns of laminar distribution and the presence of stained pericellular baskets in the neuropil, colocalization experiments showed that PV was expressed by a separate group of GABAergic neurons that did not show immunoreactivity for CR (0%, 0/538; Fig. 4*C*, *D*), SOM (0%, 0/342; Fig. 4*E*, *F*) and NOS (0%, 0/1621; Table 2). However, a small number (5.3%, 19/357) of PV immunopositive neurons in layers 2/3, 5 and 6 expressed CB (Fig. 5*E*, *F*).

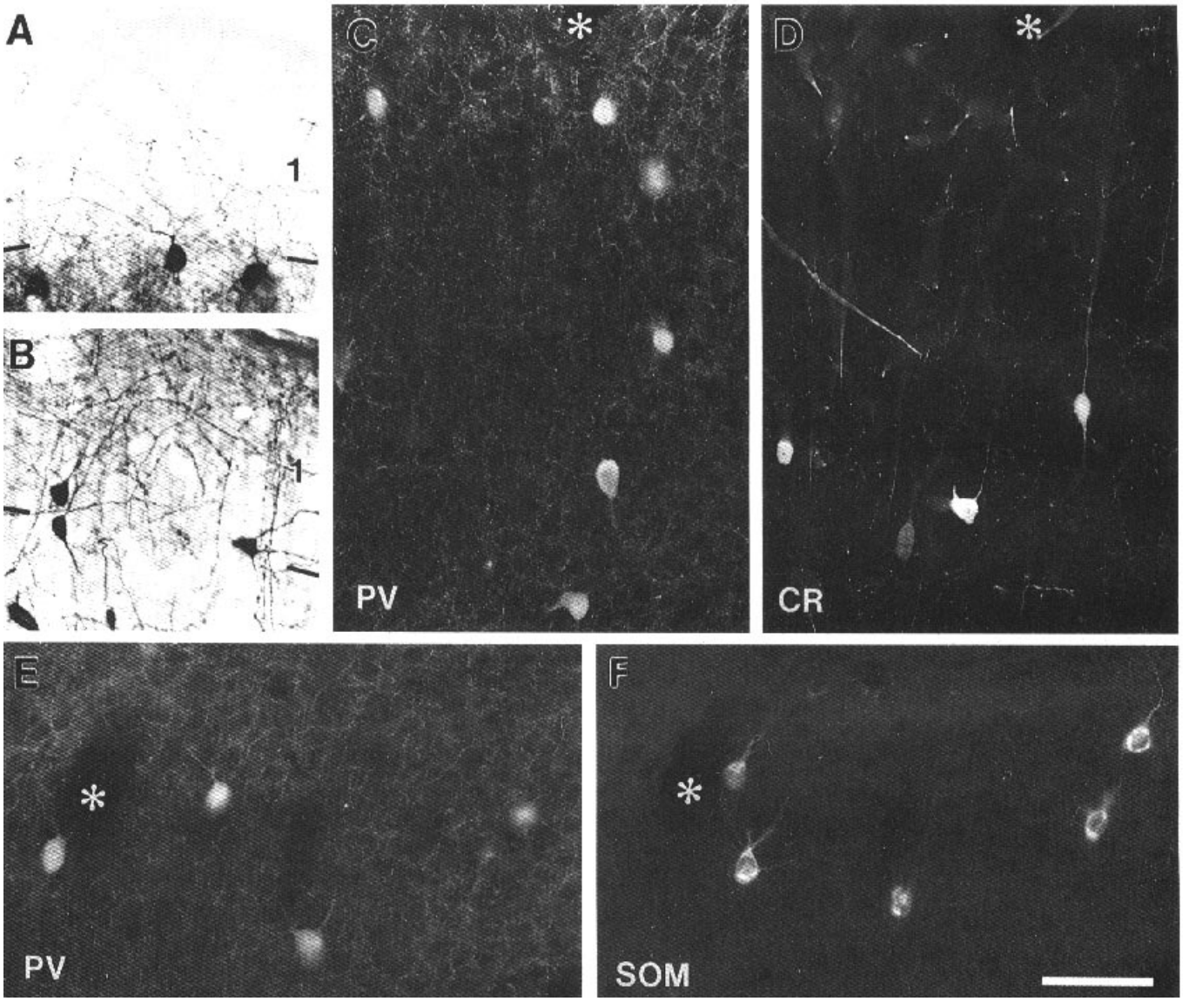

Figure 4. Parvalbumin (PV), calretinin (CR) and somatostatin (SOM) immunolabeled neurons in rat primary visual cortex. (*A*) Immunoperoxidase staining of PV expression in layer 1. Labeling is sparse and confined to axons and dendrites in the inner half of layer 1. (*B*) Immunoperoxidase staining of CR in layer 1. Intense labeling of cell bodies, axons and dendrites is seen throughout the thickness of layer 1. (*C*) FITC immunofluorescence of PV in layer 2/3. (*D*) CR stained neurons in the same field shown in (*C*), seen under rhodamine illumination. CR and PV stain two distinct populations of GABAergic neurons. (*E*) FITC immunofluorescence of PV in layer 5. (*F*) SOM stained neurons in the same field shown in (*E*), seen under rhodamine illumination. PV and SOM stain two distinct populations if GABAergic neurons. Asterisks mark matching blood vessels. Scale bar: 50 μm. *A–F* same magnification.

Table 2
Colocalization of GABA, parvalbumin, calretinin, somatostatin, calbindin and nitric oxide synthase in neurons of rat area 17

First marker	Second marker					
	GABA	Parvalbumin	Calretinin	Somatostatin	Calbindin	NOS
GABA	****	589/1160 (50.8 ± 5.4%)	102/538 (17.0 ± 4.9%)	180/1067 (16.9 ± 1.9%)	108/569 (19.0 ± 2.3%)	51/10162 (0.5 ± 0.3%)
Parvalbumin	664/664 (100%)	****	0/568 (0%)	0/342 (0%)	19! 357 (5.3 ± 2.1%)	0/1621 (0%)
Calretinin	177/182 (93.9 ± 5.3%)	0/190 (0%)	****	0/106 (0%)	0/183 (0%)	0/193 (0%)
Somatostatin	103/115 (89.8 ± 3.3%)	0/130 (0%)	0/144 (0%)	****	63/74 (86.3 ± 5.7%)	11/655 (1.7 ± 0.9%)
Calbindin (intensely)	149/154 (97.0 ± 2.8%)	19/157 (12.1 ± 4.6%)	0/162 (0%)	63/100 (69.2 ± 15.0%)	****	9/721 (1.2 ± 1.0%)
NOS	53/60 (88.3 ± 2.9%)	0/170 (0%)	0/52 (0%)	60/60 (100%)	37/58 (63.7%)	****

Absolute numbers of cells checked for the presence of immunomarkers are given in the format: second marker/first marker. Percent indicates proportion of neurons immunoreactive for the first marker expressing second marker; number of neurons expressing first marker equals 100%.

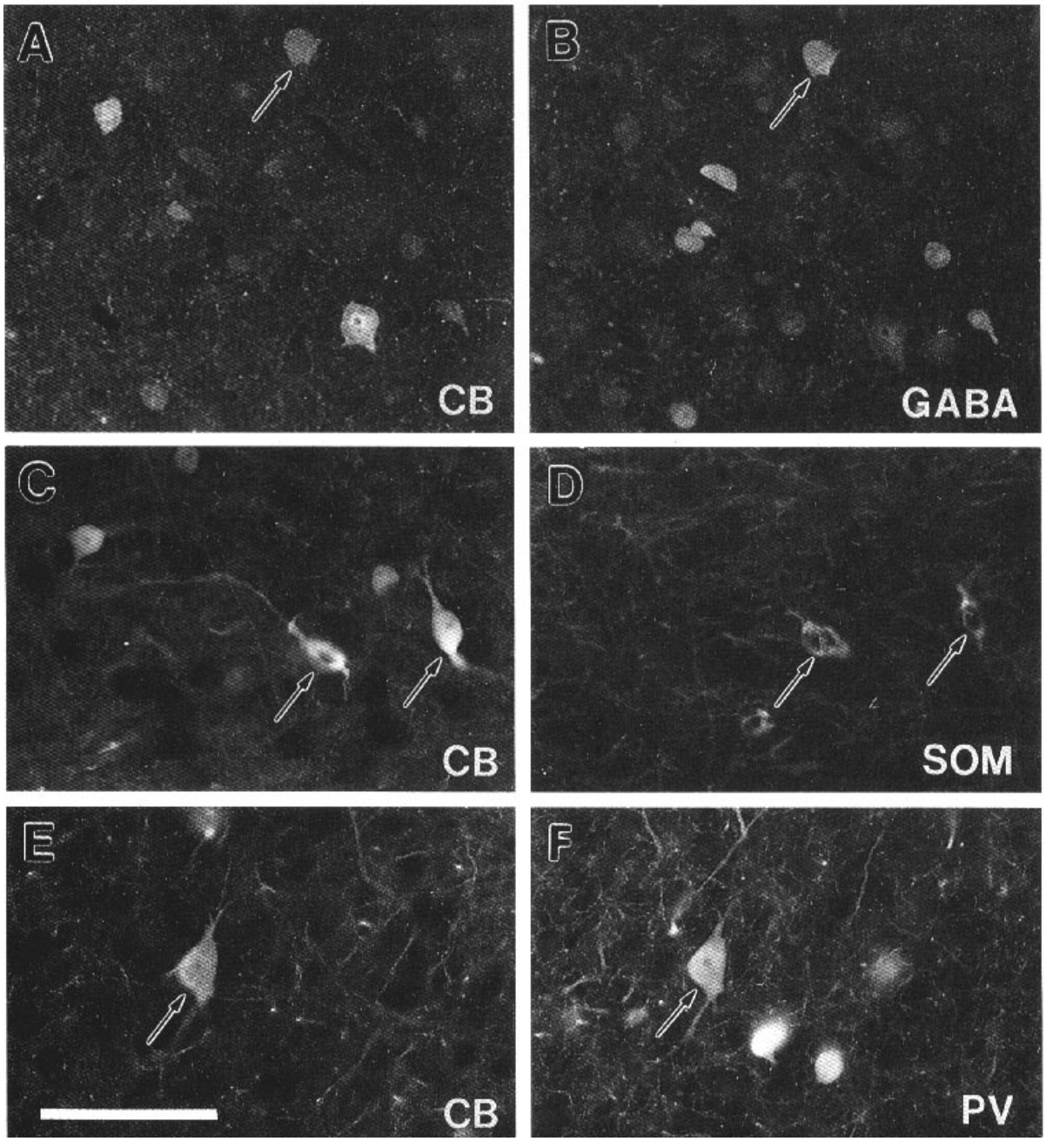

Figure 5. Immunolabeling of calbindin (CB) expressing neurons with antibodies against GABA, somatostatin (SOM) and parvalbumin (PV) in rat area 17. (*A*) CB staining neurons in layer 2/3 seen under fluorescein illumination. (*B*) GABA staining in the same field shown in (*A*), seen under rhodamine illumination. Some of the intensely labeled CB expressing cells colocalize GABA. (*C*) CB immunoreactive neurons in layer 6 seen under rhodamine illumination. (*D*) SOM staining in the same field shown in (*C*), revealed with FITC labeled secondary antibody. The CB and SOM staining populations are partially overlapping. (*E*) CB labeled neurons in layer 5 seen under fluorescein illumination. (*F*) PV staining in the same field shown in (*E*), seen under rhodamine illumination. The CB and PV staining populations are partially overlapping. Arrows mark selected examples of double labeled cells. Scale bar: 50 μm. *A–F* same magnification.

Calretinin

None of our tests revealed colocalization of CR with PV, SOM, CB or NOS (Figs 4*C*, *D* and 7*A*, *B*, Table 2). We therefore consider CR positive cells a separate group of GABAergic neurons.

Somatostatin

All of the SOM expressing neurons examined for colocalization of PV (Fig. 4*E*, *F*) and CR (Fig. 7*A*, *B*) tested negative (Table 2). This molecular distinction was often underscored by the segregation into topographically distinct clusters of SOM and CR stained neurons (Fig. 7). However, most SOM immunoreactive cells coexpressed CB (86.3%, 63/74; Table 2). A small but consistent proportion of SOM positive neurons expressed NOS (1.7%, 11/655; Fig. 6, Table 2).

Calbindin (Intensely Stained)

Unlike PV, CR and SOM, which stained distinct, non-overlapping populations of GABAergic interneurons, CB immunoreactivity

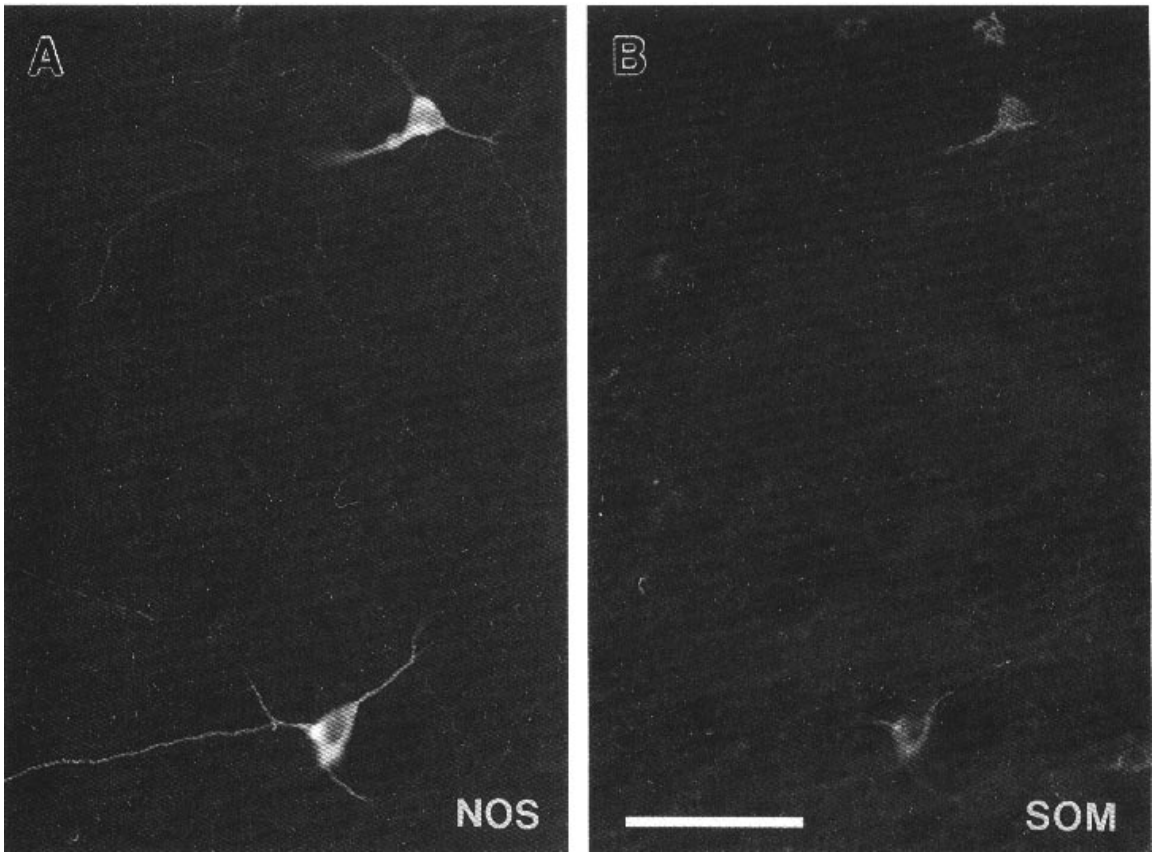

Figure 6. Double immunolabeling with antibodies against nitric oxide synthase (NOS) and somatostatin (SOM) in rat area 17. (*A*) NOS expression in layer 6 seen under rhodamine illumination. (*B*) SOM expression in the same field shown in (*A*), seen under fluorescein illumination. All NOS stained cells express SOM. Scale bar: 50 μm.

was more promiscuous and showed colocalization with PV, SOM and NOS. Specifically, 12.1% (19/157) of intensely staining CB neurons also expressed PV (Fig. 5*E*, *F*, Table 2). Even greater overlap was found with SOM, where 69.2% (63/100) of CB immunoreactive neurons colocalized the peptide (Fig. 5*C*, *D*, Table 2). The overlap with NOS was negligible (1.2%, 9/721; Table 2) and no colocalization was detected with CR (0%, 0/162; Table 2).

Nitric Oxide Synthase

All of the NOS positive cells examined (100%, 60/60) also stained for SOM (Fig. 6, Table 2). However, NOS immunoreactive neurons account for only a very small proportion (1.7%, 11/655) of SOM expressing cells (Table 2). CB was colocalized in 63.7% (37/58) of NOS labeled neurons (Table 2). None of the NOS positive cells examined coexpressed CR and PV immunoreactivities (Table 2).

Discussion

Our experiments in rat primary visual cortex have revealed three distinct populations of GABAergic neurons that can be identified by the expression of PV, CR and SOM (Fig. 8). Together, these neurons account for 84.7% of GABAergic neurons across all cortical layers. This is the first evidence for distinct, non-overlapping populations of GABAergic cells in a sensory cortical area. Our findings confirm results obtained in rat frontal cortex (Kubota *et al.*, 1994) and suggest that in all of the cerebral cortex the expression of PV, CR and SOM can be used as markers of distinct, nonoverlapping populations of GABAergic neurons.

To interpret our findings it is important to know whether we have successfully stained all GABAergic neurons. Based on the average neuronal density in rat visual cortex (Peters *et al.*, 1985; Beaulieu, 1993) we estimate that 14.6–22.8% of neurons are GABAergic. This is within the published range of the 15–25% GABAergic neurons in rat visual and somatosensory cortex (Meinecke and Peters, 1987; Ren *et al.*, 1992; Beaulieu, 1993). Because our estimates include cells which express low levels of GABA, such as found in SOM immunoreactive neurons, we believe that the true precentage of GABAergic neurons is closer to the upper limit of the range and is well represented by the 25% estimate obtained by a stereological method (Ren *et al.*, 1992). Although it is important to know the overall percentage of GABAergic neurons, it should be stressed that it does not affect our conclusion that GABAergic neurons contain distinct families of PV, CR and SOM neurons. The relative size of these families in different layers was: PV ~37–73%, CR ~9–21% and SOM ~14–21%. The values for PV (~37–73%) and CR (~9–21%) match those found in motor cortex (PV: 43–61%, CR: 18–25%; Kubota *et al.*, 1994). Only the proportion of SOM immunoreactive cell was smaller in visual than in motor cortex (26–45%; Kubota *et al.*, 1994). This may be a case of areal diversity.

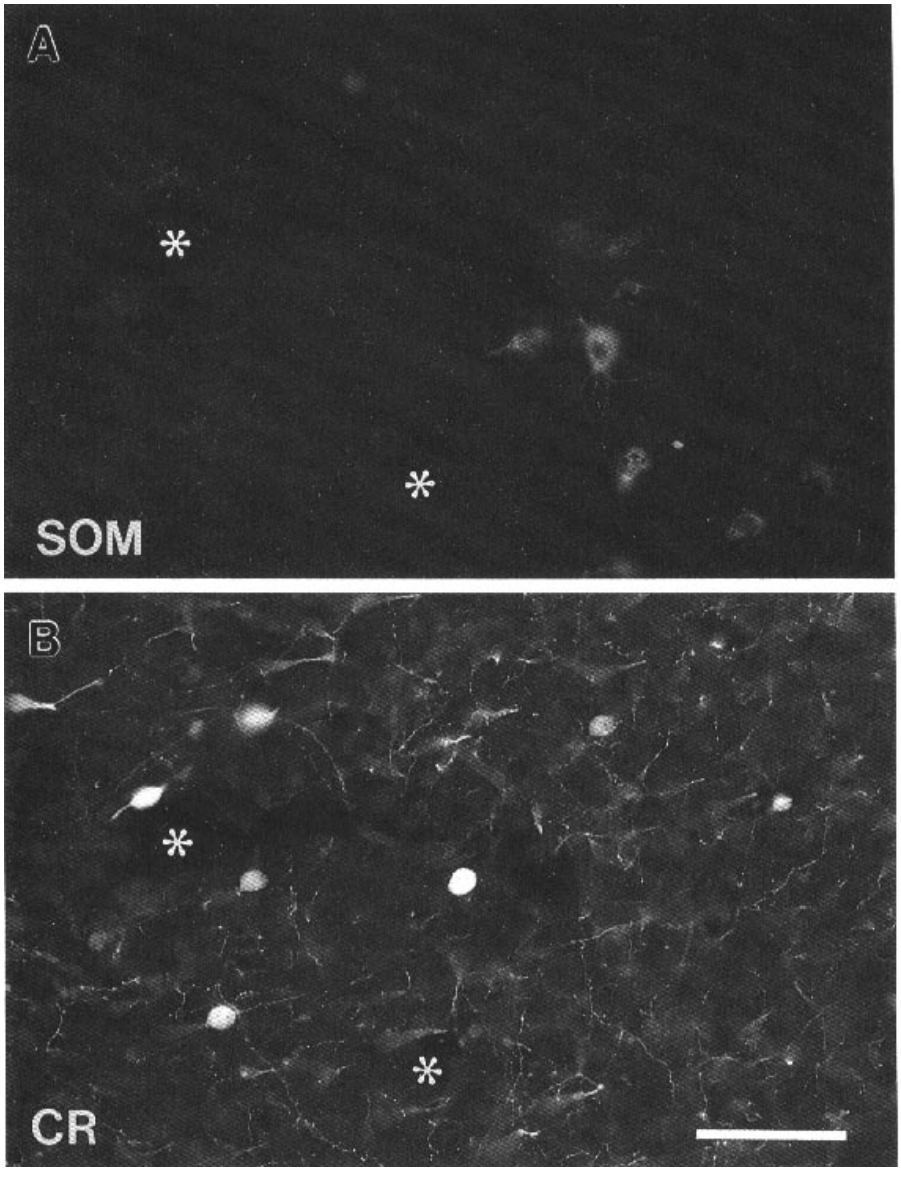

Figure 7. Double immunolabeling of somatostatin (SOM) and calretinin (CR) in rat area 17. (*A*) Cluster of layer 2/3 neurons labeled with antibodies against SOM as seen under fluorescein illumination. (*B*) CR expressing neurons observed in the same field shown in (*A*), revealed under rhodamine illumination. None of the CR cells coexpress SOM. Asterisks mark matching landmarks. Scale bar: 50 μm.

Markers of GABAergic Neurons

Parvalbumin

Our finding that all PV immunoreactive neurons in rat visual cortex intensely stain with antibodies to GABA further supports previous results from a variety of cortical areas in rat (Celio, 1986; Kubota *et al.*, 1994), cat (Demeulemeester *et al.*, 1988, 1989) and monkey (Hendry *et al.*, 1989; DeFelipe, 1993; Jones, 1993) that all of these cells are GABAergic. Most importantly we found that PV immunoreactive cells do not stain with antibodies to CR, SOM, NOS and CR, which leads us to conclude, in agreement with Kubota *et al.* (1994), that PV neurons constitute a distinct group of GABAergic neurons.

Calretinin

There is general agreement that many CR immunoreactive neurons coexpress GABA. Reports differ, however, on the degree of overlap. Our finding that ~94% of CR neurons are GABAergic is similar to the ~100% reported by Kubota *et al.* (1994). These observations stand in contrast to the ~25% reported by Rogers (1992). We attribute these discrepancies to the relatively low level of GABA expression in many CR cells and the resulting ambiguities for identifying double labeled cells. However, Meinecke and Peters (1987) have found non-GABAergic bipolar cells which resemble CR neurons. Thus it seems doubtful that the overlap is 100%. Reports of CR immunoreactive asymmetric synapses in rat cortex support this conclusion (Lüth *et al.*, 1993). Nevertheless, these observations

do not affect our findings that GABA immunoreactive CR neurons constitute a distinct group of GABAergic neurons in rat visual cortex. Similar results have been reported in rat frontal cortex (Kubota *et al.*, 1994).

Somatostatin

Previous investigations have shown that all of SOM and NOS expressing neurons are GABAergic (Somogyi *et al.*, 1984; Valtschanoff *et al.*, 1993). Our results in visual cortex show a slightly smaller overlap with GABA in ~90% of SOM and ~88% of NOS expressing neurons. This difference is presumably because we have not counted SOM cells with very low expression of GABA. We therefore believe that SOM expression in rat visual cortex is comparable to other cortical areas in cat and rat. However, the important finding of our study is that NOS immunoreactive neurons constitute a subpopulation of the SOM expressing neurons and that, similar to motor cortex (Kubota *et al.*, 1994), SOM positive cells in visual cortex constitute a distinct family of GABAergic neurons.

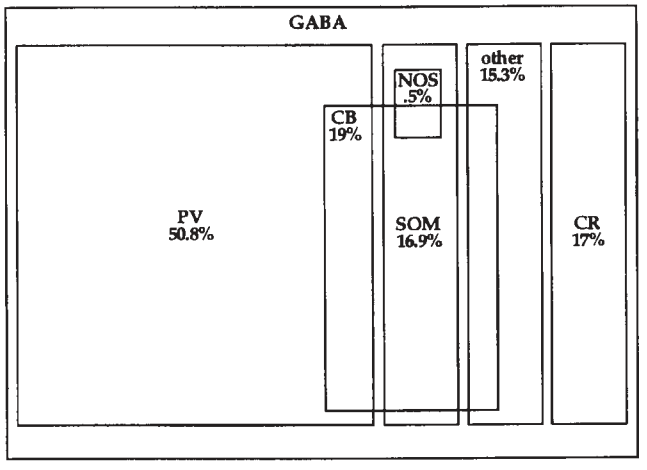

Figure 8. Summary showing relative size and overlap between PV, CR, SOM, CB and NOS expressing families of GABAergic neurons in rat primary visual cortex. CB is coexpressed in PV, SOM and NOS immunoreactive neurons. Neurons lumped in 'other' include cells that might express VIP,NPY, CCK or other unidentified substances.

Calbindin

Although it is known from our results and previous work that CB stains a large subset of GABAergic neurons (see DeFelipe, 1993), our data show that CB expression is not a property that is unique to a distinct family of inhibitory neurons. Unlike PV, CR and SOM, which are expressed only in nonpyramidal cells, CB is present also in pyramidal cells, albeit in much lower concentrations (Hendry *et al.*, 1989; Kubota *et al.*, 1994; this study). More importantly, we have found that a substantial proportion of SOM (86.3%), NOS (63.7%) and PV (5.3%) immunoreactive nonpyramidal cells express CB. Together, SOM and PV immunoreactive neurons account for 82.5% of intensely stained CB neurons. Other authors also reported the presence of CB in cells that coexpress SOM, NOS, PV and CR (Hendry *et al.*, 1989; Van Brederode *et al.*, 1990; Van Brederode *et al.*, 1991; Rogers, 1992; Rogers and Résibois, 1992; Jones, 1993; Dun *et al.*, 1994; Kubota *et al.*, 1994; Bertini *et al.*, 1996; this study; but see Demeulemeester *et al.*, 1991). Together with our results, these findings indicate that CB is a promiscuous molecule which, in the rat visual cortex, is not expressed by a distinct group of GABAergic neurons.

Laminar Distribution

Different layers of rodent visual cortex have different inputs and outputs and distinct response properties (see Burkhalter, 1989). Here we show that different layers also contain different proportions of the PV, CR and SOM families of GABAergic

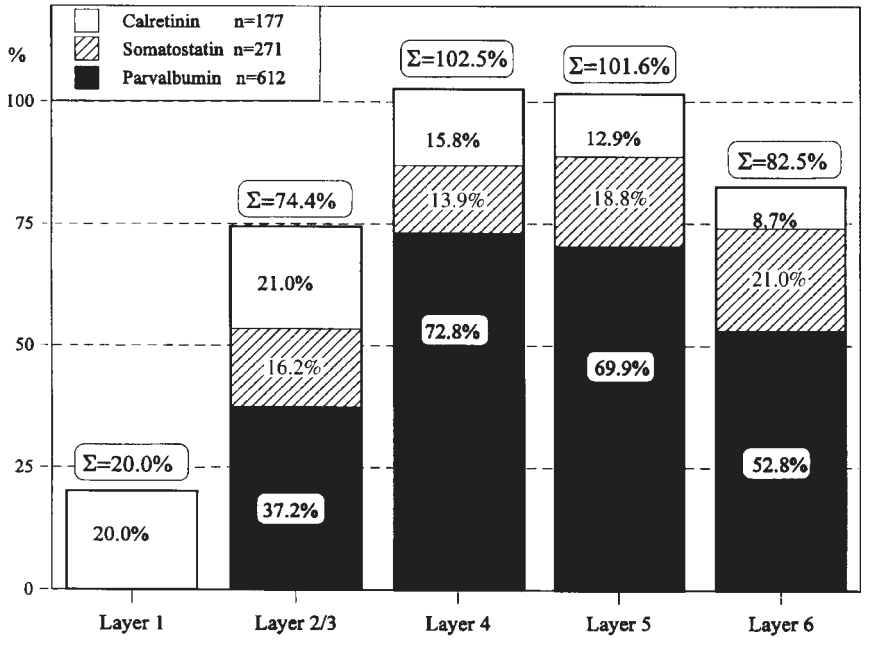

Figure 9. Summed proportions of PV, CR and SOM immunoreactive neurons in different layers of rat area 17. Values >100% in layers 4 and 5 indicate that we have slightly underestimated the number of GABAergic neurons in the overall population.

neurons. In layers 4 and 5 these families account for ~100% of GABAergic cells; in layers 2/3 and 6 the three families comprise 74.4 and 82.5% respectively. In layer 1 we found only a small representation of the CR family (Weisenhorn *et al.*, 1994), and the remaining 80% of GABAergic neurons expressed none of the substances tested. Since visual responses of cortical neurons are influenced by GABAergic inhibition (Sillito, 1984), our results suggest that the selectivity of receptive fields is in part determined by the composition of different types of inhibitory neurons.

Uniformity and Diversity within PV, CR and SOM Families

A common feature of PV immunorecative neurons is that they express high levels of GABA. They all are fast spiking (Kawaguchi, 1995) and evoke fast, $GABA_A$ receptor mediated inhibitiory postsynaptic potentials (Buhl *et al.*, 1994, 1995). However, they are morphologically diverse and make axonal connections which are typical for basket and chandelier cells (Somogyi, 1977; Peters *et al.,* 1982; Martin *et al.*, 1983; Somogyi *et al.*, 1983; Kisvárday *et al.*, 1985, 1987, 1993; DeFelipe *et al.*, 1989a; this study).

The CR family seems more diverse than the PV family. A number of studies have shown that it contains cells whose morphologies resemble double bouquet cells, bipolar cells and arcade neurons (Somogyi and Cowey, 1984; DeFelipe *et al.*, 1990; Kawaguchi and Kubota, 1996; this study). Physiologically they exhibit late spiking, burst firing and regular spiking properties (Kawaguchi and Kubota, 1996). However, CR cells presumably act via $GABA_B$ receptors (Benardo, 1994; Kang *et al.*, 1994) and make columnar projections to targets in deep layers of cortex (DeFelipe *et al.*, 1989b; Lüth *et al.*, 1993; Gulyás *et al.*, 1996; Kawaguchi and Kubota, 1996).

SOM immunoreactive neurons have in common a low level of GABA expression. Although they are morphologically heterogeneous and include bitufted, multipolar and Martinotti cells, they all make ascending connections and synapse on dendrites in superficial cortical layers (Hendry *et al.*, 1984; Kawaguchi and Kubota, 1996). However, SOM neurons differ in the coexpression of transmitter markers such as NPY, CCK and NOS (Rogers, 1992; Kubota *et al.*, 1994). A similar diversity was seen in the spike firing patterns, which are different from the late spiking and fast spiking properties found in CR and PV cells respectively (Kawaguchi abd Kubota, 1996).

Conclusion

Although the members of the PV, CR and SOM families show diverse phenotypes, cells in each family express a unifying set of molecular, morphological and physiological features that support the proposal to subdivide GABAergic cells into at least three non-overlapping groups of inhibitory neurons. Lineage analysis of cortical neurons expressing PV, CR and CB suggest that the different phenotypes are not inherited from clonally related nonpyramidal cells but are induced by environmental cues (Mione *et al.*, 1994). It is plausible that such cues derive from different inputs which may play a role in determining the phenotype of postsynaptic neurons (cf. Mione *et al.*, 1994). Since different areas of rat visual cortex have different inputs and outputs (Herkenham, 1980; Coogan and Burkhalter, 1993), it is conceivable that the composition of inhibitory neurons is different in forward and feedback circuits which interconnect different cortical areas (Shao and Burkhalter, 1996).

Notes

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