Selective Staining of a Subset of GABAergic Neurons in Cat Visual Cortex by Monoclonal Antibody VC1.1

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VC1.1 is a monoclonal antibody generated against cat area 17, which selectively outlines subsets of cortical neurons (Arimatsu et al., 1987). This study was conducted to determine the ultrastructural distribution of the VC1.1 antigen and to identify the particular subclasses of cortical neurons that were labeled.

In the light microscope, VC1.1 delineated the surfaces of neurons located mainly in layer IV but also in other layers. The staining surrounded neuronal cell bodies and dendrites in a periodic or meshwork pattern but did not label axons. VC1.1-labeled neurons were morphologically heterogeneous and included multipolar, bipolar, and bitufted classes. In the electron microscope, VC1.1 immunoreactivity surrounded presynaptic membranes of terminal boutons and intersynaptic sections of postsynaptic membranes, but was not present within terminal boutons or synaptic clefts. Both asymmetric and symmetric synapses were immunoreactive. Labeling was also observed intracellularly on VC1.1-outlined neurons, associated with perisynaptic portions of plasma membranes.

Tract-tracing methods were used in conjunction with immunocytochemistry to determine whether VC1.1 identified projection neurons, local circuit neurons, or a combination of both types. Layer V and VI corticogeniculate and corticotectal projection neurons were retrogradely labeled with rhodamine fluorescent latex microspheres. In a large sample of retrogradely labeled neurons, none were VC1.1-positive, suggesting that VC1.1 stained a population of local circuit neurons. Additional immunocytochemical double-labeling studies with an antiserum to GABA and VC1.1, revealed that VC1.1-positive neurons were immunoreactive to GABA. These were a major subset of the GABAergic neurons in area 17 and tended to have medium to large cell bodies.

It is concluded that VC1.1 identifies a new, immunologically distinct subset of GABAergic neurons in area 17. The restricted distribution of this antigen on perisynaptic por-

tions of GABA-containing cells and surrounding terminal boutons onto these cells suggests that this antigen may play an important role in inhibitory cortical circuits.

Many of the local circuit neurons in the visual cortex contain the inhibitory neuro-transmitter GABA and its biosynthetic enzyme GAD. Antisera against these substances have made it possible to determine the relative numbers of GABAergic cell bodies and terminals within different cortical layers, and to analyze their synaptic interactions at the electron-microscopic level (Ribak, 1978; Hendrickson et al., 1981; Ribak et al., 1981; Hendry et al., 1983; Somogyi and Soltesz, 1987). GABAergic neurons in the cortex have been further subdivided into a number of distinct subclasses, using dual immunohistochemical localization of neuropeptides and GABA (Hendry et al., 1984b; Somogyi et al., 1984; reviewed by Houser et al., 1984) or Golgi impregnation and intracellular HRP injection combined with GABA immunocytochemistry (Somogyi et al., 1983; Somogyi and Hodgson, 1985; Somogyi, 1986).

These methods provide only partial information about each of the functional classes of local circuit neurons within area 17. In addition to transmitter and neuropeptide heterogeneity, there is increasing evidence that functionally distinct classes of cortical neurons can be distinguished according to their cell surface antigens (Hockfield et al., 1983; Levitt, 1984). A number of novel surface antigens, restricted to subsets of neurons, have been identified in cortical as well as noncortical areas through the application of monoclonal antibody techniques. In both vertebrates and invertebrates, monoclonal antibodies have been produced against antigens restricted to neuronal subsets that have a shared transmitter phenotype (Dodd et al., 1984; Akagawa and Barnstable, 1986, 1987), anatomical location (Levitt, 1984; Barnstable, 1985), or physiological function (Zipser and McKay, 1981; McKay and Hockfield, 1982; Hendry et al., 1984a).

We have focused for the last several years on the production of immunological markers for cell surface antigens that are restricted to functional subclasses of visual cortical neurons. Among the panel of monoclonal antibodies that we have generated, 2 antibodies, VC1.1 and VC5.1, label the cell bodies and dendrites of a subpopulation of nonpyramidal neurons in middle and deep layers of cortical area 17 (Arimatsu et al., 1987).

Despite their similar patterns of labeling in area 17, VC1.1 and VC5.1 have clearly different epitopes, as shown by differences in the quality and extent of cellular labeling and by differences between the biochemical properties of the antigenic determinants (Arimatsu et al., 1987). VC1.1 labeling around cell bodies and proximal dendrites is punctate and discontinuous, whereas VC5.1 labeling is smooth and continuous and

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extends further along the distal portions of dendrites. Subcellular fractionation and extraction studies showed that the VC1.1 antigens are integral membrane proteins, preferentially located in a synaptosomal plasma membrane fraction. In contrast, the VC5.1 antigens are preferentially located in a soluble cytoplasmic or extracellular fraction. Each antibody also labels separate molecular-weight bands on immunoblots.

The irregular, punctate distribution of VC1.1 binding sites around cell bodies and dendrites raised the intriguing possibility that the VC1.1 epitope was selectively expressed on synapses terminating on a functionally unique subset of cortical neurons. The present studies were conducted to determine the distribution of the VC1.1 antigen at the ultrastructural level, as well as to define the relationship between the outlined set and other identified subsets of cortical neurons. A preliminary account of these results has appeared in abstract form (Naegele et al., 1986).

Materials and Methods

Immunohistochemical reagents. The monoclonal antibody VC1.1 was generated by immunization of BALB/c mice with an unfixed homogenate of cat area 17, as described previously (Arimatsu et al., 1987). Antibody VC1.1 was previously identified as an IgM (Arimatsu et al., 1987). For light microscopy, tissue culture supernatants were used. Rabbit antiserum to GABA-BSA was obtained from Immunonuclear, Inc. The specificity of this reagent has been fully characterized and is described elsewhere (Maley and Newton, 1985). The antiserum was used at dilutions ranging from 1:4000 to 1:8000. Commercially available antibodies were produced in horse or goat and included peroxidase-conjugated anti-mouse IgM, rhodamine isothiocyanate (RITC)-conjugated anti-mouse IgM (μ -chain-specific) or fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG + IgM (Cappel or Boehringer Mannheim) diluted 1:50–1:100; biotinylated anti-rabbit IgG and biotinylated anti-mouse IgM (Vector Labs) diluted 1:220. Avidin–DH–peroxidase (ABC), avidin–DH–alkaline phosphatase (ABC-AP), and an alkaline phosphatase substrate, Vector red, were all obtained in kit form (Vector Labs) and used at the recommended dilutions.

Preparation of active, low-molecular-weight IgM fragments. Active fragments of IgM immunoglobulins were prepared in order to aid penetration for electron microscopy, using a modification of the method of Matthew and Reichardt (1982). Ammonium sulfate-precipitated ascites fluid was resuspended in 3–4 ml PBS (pH 7.4, 150 mM NaCl, 8 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2 mM KH_2PO_4) and then dialyzed sequentially against PBS and Tris-Cl (TBS; pH 8.0, 50 mM Tris-HCl, 150 mM NaCl, and 20 mM CaCl_2). The dialysate was spun at $100,000 \times g$ for 1 hr and the protein concentration adjusted to 1 mg/ml in TBS. Samples were then digested with TPCK trypsin (Cooper Biomedical) for 6 hr at 37°C, followed by procedures described previously (Matthew and Reichardt, 1982). BSA (0.1%) and sodium azide (0.05%) were added to prevent loss of activity during longer periods of storage at 4°C. The activity of the fragments was determined over a range of dilutions by immunofluorescence staining of cryostat sections prepared from cat area 17.

Electron-microscopic localization. Three adult cats were used for electron microscopy. The cats were deeply anesthetized with pentobarbital (Nembutal; 40 mg/kg). Prior to the perfusion, 1 ml of 1% sodium nitrate solution was injected intracardially. The perfusion consisted of an initial rinse of 150 mM sucrose, 1 mg/ml lidocaine hydrochloride, and 0.5 U/ml sodium heparin in 0.1 M phosphate buffer, pH 7.4, followed by fixation with 4% paraformaldehyde (EM grade; Polysciences) and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. One hour after the perfusion, the brain was removed from the skull and placed in fresh fixative for 1–5 hr at 4°C. Small blocks were removed from area 17 and washed in several changes of 0.1 M phosphate buffer at 4°C. For enhancement of penetration, some blocks were cryoprotected and subjected to a freeze–thaw cycle in liquid nitrogen according to the method of Eldred et al. (1983). Fifty micron sections were cut on a Vibratome in cold 0.1 M phosphate buffer.

For monoclonal antibody dilution and rinses, 3% normal serum obtained from the host species of the secondary antibody (horse or goat) was prepared in 0.1 M phosphate buffer containing 0.1% BSA. Sections were incubated in this buffer for 1 hr, then in the monoclonal antibody fragment preparation for 18–24 hr at 4°C with gentle agitation. The

sections were then rinsed for 1 hr at room temperature and incubated in biotinylated anti-mouse IgM for 1 hr at room temperature. The tissue was rinsed in 2 additional 30 min washes and incubated in ABC (Vector Labs) for 1 hr at room temperature. Sections were washed 2 additional times in 0.1 M phosphate buffer without normal serum and then reacted for the presence of HRP in 3,3'-diaminobenzidine (0.5 mg/ml) to which 0.003% hydrogen peroxide was added. Sections were reacted for 5–10 min, rinsed thoroughly, and then postfixed from 1 hr to overnight in 3% glutaraldehyde. The following day, the sections were rinsed again, fixed in 1% osmium tetroxide at 4°C for 1 hr, dehydrated in alcohols, infiltrated with Epon araldite resin, flat-embedded between sheets of Aclar, and cured at 60°C for 48 hr.

VC1.1-outlined neurons were located within layers IV–VI in plastic, flat-embedded sections. These areas were cut from the section and glued onto blank Epon lozenges. The blocks were sectioned obliquely or *en face* to the original cut edge of the tissue. Ultrathin sections were cut and lifted onto Formvar-coated, single-slot nickel grids. The grids were lightly counterstained or left unstained and viewed in a Jeol 100 CX-II transmission electron microscope. Because of nonspecific labeling and disruption of ultrastructure at the edge of the section, only immunoreactive profiles internal to this edge were photographed.

Immunohistochemical double-labeling. Three cats were used for dual immunohistochemical staining. One cat received 3 injections of colchicine (6 mg/ml; total of 3.2 μ l) along the medial bank of area 17, 24 hr prior to perfusion. Colchicine treatment did not alter the staining patterns of either VC1.1 or GABAergic antibodies. Animals were deeply anesthetized and perfused through the heart with freshly prepared fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (80 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 20 mM NaH_2PO_4), pH 7.4. Blocks were removed from area 17, cryoprotected and subjected to a freeze–thaw cycle to aid penetration of antibodies (Eldred et al., 1983). Fifty-micron-thick Vibratome sections were cut in cold Tris-HCl (0.1 M, pH 7.4) and incubated in Tris-HCl containing 5% normal goat serum (T-NGS). All reagents were subsequently diluted with T-NGS and rinsed between incubations in T-NGS unless otherwise noted. Sections were incubated in the following sequence: rabbit anti-GABA-BSA for 24–48 hr at 4°C, biotinylated anti-rabbit IgG (1 hr, room temperature), ABC-AP (1 hr, room temperature), Vector red substrate for 3–5 min (kit form; Vector) in Tris-HCl, pH 8.2, containing 1 mM levamisole; incubation was overnight in VC1.1 antibody at 4°C, anti-mouse IgM conjugated to peroxidase, and sections were then reacted with 3,3'-diaminobenzidine HCl in the presence of hydrogen peroxide for 5–10 min. Sections were mounted on chrome–alum gelatin-coated slides, air-dried, dipped in 100% ethanol, defatted briefly in xylene, and cover-slipped in Krystalon.

Immunohistochemical controls. The specificity of the GABA antiserum was checked by diluting the antiserum 1:4000–1:8000 and preincubating it for 24–48 hr with GABA (100 μ g/ml; Sigma). Vibratome or cryostat sections were then immunostained with this mixture instead of the GABA antiserum and reacted using the ABC-AP. There was no specific staining with this method.

Controls were also performed to test for nonspecific IgM immunoreactivity using a mouse monoclonal antibody that reacts with retina but not cortex ("Jones"; Constantine-Paton et al., 1986). Sections were incubated under conditions identical to those used with the VC1.1 antibody and stained using immunoperoxidase histochemistry. In no instance was labeling observed in either white or gray matter of the cortex.

Other controls included incubating sections in 5% NGS instead of the primary antibody and then processing the sections through the full sequence of immunocytochemical steps and reacting them with either Vector red or diaminobenzidine (DAB). Peroxidase reaction end product was observed in red blood cells and represented endogenous peroxidase activity not associated with immunoreactive sites. A faint background of Vector red reaction product was observed in NGS controls; however, no specific cellular staining was observed.

Reconstructions of sections and quantitative measurements. Two-dimensional reconstructions of sections were made using a computer graphics system that included a digitizing tablet (Summagraphics) and a vector graphics scope with an array processor (Megatek), in conjunction with a Minivax computer (Gilbert and Wiesel, 1983). The outlines of each section were charted at low power and labeled neurons were then traced at higher magnifications. For the size analyses of VC1.1 and GABA-immunoreactive cells, 2 different cases were used, in which sections of area 17 had been stained with either VC1.1 antibody alone, VC1.1 antibody and GABA antiserum, or GABA antiserum alone. Only

labeled neurons that contained an obvious nucleus were selected for size measurements ($n = 480$). The perimeters of labeled cells, excluding the dendrites, were drawn with the aid of the digitizing tablet. An in-house software program, AREA, calculated the average diameter and area of each cell. Repeated measurements for the same cell were reproducible within 10%.

Injections of fluorescent latex microspheres. One cat received an injection of rhodamine fluorescent latex microspheres in distilled water at the concentration supplied by the company (LumaFluor, New City, NY). Injections were made into the dorsal lateral geniculate nucleus (dLGN) and optic radiations to retrogradely label neurons in cortical layers V and VI (Katz et al., 1984). To map the area of the visual field penetrated, physiological recordings were made using a tungsten microelectrode. During the recording experiment, the cat was maintained on sodium pentothal anesthesia, paralyzed with succinyl choline, and artificially respired. The animal's electrocardiogram, temperature, and respired CO_2 were monitored on an oscilloscope display. Temperature was maintained with a heating pad at 38°C . The microelectrode was then removed and a micropipette, containing rhodamine latex microspheres, was lowered into 4 separate locations in the LGN and approximately $0.1\text{--}1\ \mu\text{l}$ of the tracer solution was injected throughout ipsi- and contralateral, and into the optic radiations. Injections were made by brief pulses of pressurized air.

The animal was allowed to recover from paralysis, then removed from anesthesia, and perfused 6 d later with 4% paraformaldehyde in $0.1\ \text{M}$ phosphate buffer, pH 7.4. Injection sites in the LGN and optic tract were verified by dissection. Blocks from the appropriate regions of area 17 were sunk in 30% sucrose, sectioned at $10\text{--}15\ \mu\text{m}$ on a cryostat (Hacker Instruments), melted onto slides, dried under vacuum, and stored in the dark at -20°C .

Visualization of VC1.1 and retrogradely transported microspheres in area 17. The presence of retrogradely transported rhodamine latex microspheres in cortical neurons was verified by examining sections in a Zeiss epifluorescence microscope equipped with a combination filter for rhodamine (RITC) and fluorescein (FITC) fluorescence. Sections containing large numbers of labeled neurons were incubated in humidified petri dishes according to the following sequence: first, in 5% normal goat serum overnight at 4°C in VC1.1 antibody, rinsed, then incubated 1 hr in anti-mouse IgG + IgM-FITC, rinsed, and then coverslipped with a polyvinyl alcohol and glycerol mixture containing paraphenylenediamine to retard fading (Valnes and Brandtzaeg, 1985).

Labeled neurons were examined in the microscope and scored first for microspheres and then for VC1.1 immunofluorescence from a 1 in 3 series of 24 well-labeled sections. Adjacent sections were not scored to avoid counting the same cell twice. A total of 824 microsphere-containing neurons in layers V and VI was examined for double-labeling with antibody VC1.1.

Results

Light-microscopic appearance of immunoreactivity

In area 17, the neurons recognized by VC1.1 had pale cytoplasm that contrasted with the darkly stained puncta surrounding the perikarya and proximal dendrites (Fig. 1). Light osmication of the tissue sections produced a pale brown background and allowed a clear identification of unlabeled neurons. In addition to the clearly outlined neurons, weaker staining of neuropil was present in all layers, particularly layers I and IV. This staining was sometimes associated with portions of dendrites, but in many cases formed a more diffuse neuropil stain (Arimatsu et al., 1987).

The shapes of outlined cells were generally nonpyramidal and included multipolar (Fig. 1A), bipolar, or bitufted varieties of nonpyramidal neurons (Fig. 1, B, C). As a rare exception, VC1.1 weakly outlined an occasional pyramidal-shaped cell in layers V or VI.

The labeled dendritic processes of neurons in layers II–V were most often directed vertically. Labeled dendrites in layer VI, however, tended to extend horizontally more often than vertically. VC1.1 did not outline distal dendrites and axons, which

Figure 1. Examples of VC1.1 outlined perikarya and dendrites in area 17 of the cat. Multipolar (A, C) and bipolar (B) forms were commonly observed. Nomarski optics. Scale, $25\ \mu\text{m}$.

limited further identification of the labeled neuronal subclasses using these morphological criteria.

Electron-microscopic appearance of VC1.1 immunoreactivity

VC1.1 immunoreactivity was typically observed along the membranes of presynaptic terminal boutons and adjacent postsynaptic cell membranes. It was not present within synaptic clefts or synaptic boutons (Fig. 2, A–F). Many of the VC1.1-outlined synapses were classified as Gray's type II in that they formed symmetric synaptic contacts without distinct postsynaptic densities and contained numerous mitochondria and flattened, pleomorphic vesicles (Fig. 2, D–F). This symmetric type of immunoreactive profile was commonly associated with a cell body or the proximal portion of a dendrite and made multiple points of synaptic contact (Fig. 2F). Other immunoreactive synapses were classified as Gray's type I, since they contained round vesicles and made asymmetric synaptic contacts with distinct postsynaptic densities (Fig. 2, A, C). Labeled synapses were

Figure 2. Electron micrographs of VC1.1-immunoreactive synapses in cat area 17. Synapses containing round vesicles (*A–C*) and distinct postsynaptic densities (*C*, *open arrow*) were immunoreactive. More commonly, synapses with flattened, pleomorphic vesicles (*D–F*) and no discernible postsynaptic specialization were observed in association with labeled cell bodies and dendrites (*D, F*). *Arrows* point to electron-dense immunoperoxidase reaction product. Scale, 0.5 μ m.

observed on cell bodies, proximal dendrites, and, on a few occasions, dendritic spines. Typically, unlabeled synapses were interspersed among the labeled synapses along cell bodies and dendrites. VC1.1 surface immunoreactivity was also observed at periodic postsynaptic foci, particularly adjacent to labeled synapses (Fig. 2*F*).

In addition to immunoreactivity associated with pre- and post-synaptic membranes, additional labeling was sometimes observed along the intracellular surface of plasma membranes cut open by Vibratome sectioning (Fig. 3). The immunoreactivity was observed along discontinuous segments of plasma membrane, often surrounding zones of synaptic contacts (Fig. 3, arrowheads). It was not obviously associated with any particular intracellular organelle.

Dual VC1.1 immunohistochemistry and retrograde labeling

To investigate whether projection neurons in layers V and VI of area 17 were VC1.1-positive, double-labeling experiments were carried out. Corticotectal and corticogeniculate projection neurons were marked by retrograde transport of rhodamine fluorescent latex microspheres, and the sections were subsequently stained by immunofluorescent procedures to locate VC1.1-positive neurons. Microsphere-filled neurons were found extensively in layer VI and less frequently in layer V. Retrogradely labeled neurons were clearly indicated by the presence of 5 or more punctate fluorescent spheres in the cytoplasm when viewed with rhodamine excitation wavelengths (Fig. 4, *A*, *C*). In these same fields, FITC excitation wavelengths were used to view VC1.1 immunofluorescence. VC1.1-positive cells were scattered among the microsphere-filled neurons, but they never contained microspheres (compare Fig. 4, *A* and *B*, *C* and *D*).

These observations were quantified by examining a series of 8 sections spaced 30–40 μm apart, in which every microsphere-containing cell was viewed under fluorescein epifluorescence and scored as either VC1.1-negative or VC1.1-positive. Out of a sample of 824 microsphere-labeled cells, none were also outlined by the VC1.1 antigen. The VC1.1-positive cells were also examined for the presence of microspheres, with negative results. These results were independently confirmed by a second investigator. These tract-tracing experiments indicated that it was highly unlikely that the VC1.1 antibody labeled a population of projection neurons, at least in deeper cortical layers.

Colocalization of GABA and VC1.1

In order to determine whether VC1.1-positive neurons were a subpopulation of local circuit neurons, we performed double-labeling experiments with the VC1.1 antibody and a polyclonal rabbit antiserum to GABA. Two contrasting chromogens, diaminobenzidine and Vector red, were used to map the distribution of staining for each antibody. The cytoplasm of GABAergic neurons was stained pale pink to deep rose and could be easily distinguished from VC1.1 staining, which appeared as a discontinuous, dark brown ring of peroxidase reaction product surrounding neuronal perikarya and proximal dendrites. We observed that all but a few VC1.1-stained neurons were also immunoreactive for GABA. Examples of the different types of neurons stained with VC1.1 and GABA are shown in Figure 5. The double-labeled neurons varied in shape and size. They were generally medium to large, bipolar or multipolar neurons. Some additional VC1.1-negative, GABA-positive neurons were found in the same fields as double-labeled cells. Some of these GABA-

Figure 3. Three semiserial sections through the perikaryon of a VC1.1-immunoreactive cell in area 17, showing VC1.1 immunoreactivity along the cytoplasmic face of the cell body membrane. Immunoreactivity is associated postsynaptically with a synapse containing flattened, pleomorphic vesicles on the right side of each micrograph (arrows point to reaction product). Scale, 1 μm .

Figure 4. Pairs of immunofluorescence photomicrographs showing layer VI neurons retrogradely labeled with rhodamine latex microspheres from an injection in the dLGN (*A*, *C*) and a nonoverlapping distribution of VC1.1-positive neurons in the same fields (compare *B* with *A*, *D* with *C*). Rhodamine excitation wavelengths (*A*, *C*), fluorescein excitation wavelengths (*B*, *D*).

positive cells can be seen as pinkish neurons, without the distinctive ring of brown puncta imparted by VC1.1 immunoreactivity (Fig. 5).

The laminar positions of GABA-positive/VC1.1-negative ($\text{GABA}^+/\text{VC1.1}^-$) and $\text{GABA}^+/\text{VC1.1}^+$ neurons were analyzed in a closely spaced series of sections, stained with the GABA antiserum, the VC1.1 antibody, or both, as shown in Figure 6. GABA-immunoreactive cell bodies were most numerous in superficial layers of area 17, and their numbers diminished in deeper layers (Fig. 6*A*). By contrast, the subset of double-labeled cells was rare in layer I and more frequent in middle and deeper layers (Fig. 6*B* shows only double-labeled neurons, to simplify comparisons with the adjacent sections). Overall, a majority of the GABA-positive neurons in the middle and deeper layers were also VC1.1-positive. These observations were quantified for the region of area 17 shown in Figure 6. The most numerous population was the $\text{GABA}^+/\text{VC1.1}^-$ (496); the next most numerous was $\text{GABA}^+/\text{VC1.1}^+$ neurons (264); and the least common were $\text{GABA}^-/\text{VC1.1}^+$ (4). The double-labeled subset therefore represented approximately 35% of the total number of GABA-positive cells. These counts were also analyzed by superficial, middle, and deep cortical layers. The single layer containing the most double-labeled cells was layer IV (107). Layers V and VI combined contained 110 double-labeled cells, and layer II + III contained only 47 double-labeled cells.

A majority of the GABAergic cells not outlined by VC1.1 were in layer I and the upper half of II and III. In the section shown in Figure 6*B*, the laminar distribution of $\text{GABA}^+/\text{VC1.1}^-$

neurons was 332 in layers I and II + III, 60 in layer IV, and 104 in layers V and VI.

When the distribution of the double-labeled cells in a section stained with VC1.1 and GABA (Fig. 6*B*) was compared with that of VC1.1-positive cells from a separate section stained with VC1.1 only (Fig. 6*C*), there were similar patterns of labeling. This comparison further supports the finding that all but a few VC1.1-positive cells were GABA-positive.

The differences between the $\text{GABA}^+/\text{VC1.1}^+$ and $\text{GABA}^+/\text{VC1.1}^-$ cells were further analyzed by comparing cell body diameters. GABA-immunoreactive cells in all layers of area 17 ($n = 240$) were measured and compared with a sample of double-labeled cells ($n = 240$). The GABA-immunoreactive population had a broader range of cell diameters than did the subpopulation of double-labeled cells, as shown in Figure 7. The diameters of GABA-stained cells ranged from 10 to 30 μm , with a mean of 15.5 μm . The $\text{GABA}^+/\text{VC1.1}^+$ double-labeled cells had diameters that ranged between 12 and 26 μm , with a mean of 18.0 μm . These results suggested that the VC1.1-positive subset contained mainly the larger-diameter subclasses of GABAergic cells. Statistical comparison of the cell diameter measurements in the 2 samples showed that the difference between the sample means was statistically significant ($p < 0.001$; Student's *t* test).

Discussion

Our results indicate that a major subset of GABAergic neurons in area 17 is identified by the VC1.1 antibody. VC1.1 immunoreactivity surrounded individual neuronal cell bodies and

Figure 5. Colocalization of VC1.1 and GABA immunoreactivity in the same neurons within area 17, showing a range of cell sizes and morphology. VC1.1 immunoperoxidase reaction product appears as *brown punctata* along cell bodies and proximal dendrites. GABA immunoreactivity, demonstrated by the ABC-AP method, using the Vector red chromogen, appears as a *reddish-pink stain* in cytoplasm over nuclei and in terminals. In upper left field, one GABAergic neuron does not exhibit VC1.1 staining. Nomarski optics.

Figure 6. Charts of computer-reconstructed sections showing positions of immunoreactive cell bodies in three different sections through the medial bank of area 17. *Solid dots* indicate the positions of all GABA-immunoreactive cells in a section stained with anti-GABA antiserum (*A*; $n = 400$). In *B*, stained with both VC1.1 and anti-GABA antiserum, all the double-labeled and GABA⁺/VC1.1⁺ cells are shown (GABA⁺/VC1.1⁺ cells, *solid dots*; $n = 264$; GABA⁻/VC1.1⁺ cells, *open circles*; $n = 4$). Quantitative comparison indicated that approximately 35% of GABAergic cells express the VC1.1 antigen. A control section stained only with VC1.1 is shown in *C* ($n = 196$). The 27% increase in the number of VC1.1-immunoreactive cells in *B*, as compared with *C*, may be a result of variability of immunoreactive staining from section to section. Scale, 1 mm.

proximal dendrites in a periodic or meshwork pattern. The surface-reactive neurons were a morphologically diverse collection of nonpyramidal cells, with many displaying bitufted and multipolar dendritic shapes. They were scattered throughout layers II–VI, but most were found within layer IV.

Further evidence that a unique subset of GABAergic cells was

identified by antibody VC1.1 was shown by size differences between the double-labeled subset and the population of GABA cells as a whole. This comparison indicated that antibody VC1.1 demarcated a subset of larger GABAergic neurons. Thirty to fifty percent of all GABAergic neurons was outlined by the VC1.1 antibody. In a recent study, GABAergic cells in the cat

visual cortex were calculated to make up 20% of the total number of neurons (Gabbott and Somogyi, 1986). Together, these findings indicate that 5–10% of the total number of neurons in the visual cortex consists of dual GABA⁺/VC1.1⁺. Our cell counts may slightly overestimate the number of cells that express both GABA and the VC1.1 determinant, since a comparison of our cell size measurements (see Fig. 7) with published data (reviewed in Houser et al., 1984), indicates that the small-diameter GABAergic neurons were not detected very frequently in our material. If these smaller cells were included in our analysis, the actual percentage of VC1.1-outlined GABA cells would be smaller.

Other subclasses of cortical GABAergic cells have been identified by their neuropeptide contents, including cholecystokinin octapeptide (CCK), somatostatin (SRIF), and neuropeptide Y (NPY) (Hendrickson et al., 1981; Emson and Hunt, 1984; Hendry et al., 1984b). The neuronal subclasses that exhibit GABA and CCK-like immunoreactivity are separate from those exhibiting SRIF- or NPY-like immunoreactivity, since these latter 2 neuropeptides coexist in the same cells but do not coexist with CCK (Hendry et al., 1983, 1984b). Since none of these subclasses defined by dual GABA/neuropeptide phenotype has numbers or a laminar distribution similar to that of VC1.1, we conclude that the VC1.1 antibody identifies a group of GABAergic cells that has not been previously defined. Further studies will address the question of whether VC1.1-outlined neurons include one or more of these neuropeptide-containing GABAergic subclasses.

The diversity of shapes of VC1.1-positive neurons clearly indicates that more than one morphologically distinct subset of GABAergic neurons are labeled. Both morphology and size suggest that each of the 3 main types of interneurons in area 17 is outlined by VC1.1, including the multipolar, bitufted, or bipolar forms.

The occurrence of dendritic spines has been used to further distinguish the different types of nonpyramidal cells (Peters and Regidor, 1981). Since dendritic spines were very rarely labeled in our electron-microscopic preparations, it seems likely that the VC1.1-positive subpopulation consists primarily of smooth or sparsely spinous multipolar, bitufted, and bipolar neurons.

Different classes of cortical neurons can also be distinguished on the basis of the types of synaptic contacts made on the cell body. In the electron microscope, VC1.1 reactivity frequently surrounded axosomatic or axodendritic synapses. Most synapses could be identified as Gray's type II (thought to be inhibitory) and contained flattened or pleomorphic vesicles. Labeling of Gray's type I synapses (thought to be excitatory), containing round vesicles and postsynaptic densities, was observed, though less frequently. This may have simply been due to the fact that there are fewer type I synapses on cell bodies and proximal dendrites. That both types of synapses were observed on the cell bodies and proximal dendritic shafts of labeled neurons is evidence that pyramidal or spiny stellate cells were generally not recognized by antibody VC1.1, since these classes receive only type II synapses on their cell bodies and proximal dendrites (Colonnier, 1968; LeVay, 1973).

In recent studies of local circuit neurons in cortical areas 17–19 of the cat, further distinctions have been drawn by correlating axonal arborizations with cell body location and dendritic morphology (Meyer, 1983; Somogyi, 1986). These different classes include chandelier cells, aspiny bitufted cells of layer VI, neurogliaform cells, clutch cells, and large basket cells. The lack of clear axonal labeling given by the VC1.1 antibody, however,

Figure 7. Frequency histograms showing diameters of all GABA⁺ (upper histogram) or the subset of GABA⁺/VC1.1⁺ double-labeled cells (lower histogram) in a series of 4 sections from 2 different cats measured in area 17. Two-hundred and forty stained neurons were measured for each histogram. The shift in the peaks of the histograms is reflected by differences in the average cell diameters (GABA cells averaged 15.5 μm diameter, VC1.1/GABA cells averaged 18 μm diameter). The means are significantly different ($p < 0.001$; Student's *t* test).

does not allow us to assign the GABA⁺/VC1.1⁺ neurons to any of these classes. In order to do so, it will be necessary to combine intracellular dye injections or Golgi impregnation with VC1.1 immunohistochemistry using approaches similar to those described previously (Somogyi et al., 1983; Somogyi, 1986).

Most VC1.1-labeled neurons were found to be immunoreactive for GABA, but, in a few instances, large pyramidal-shaped perikarya were VC1.1-positive and GABA-negative. The tract-tracing experiments were conducted to investigate the possibility that some pyramidal-shaped projection neurons were outlined by VC1.1. In a large sample of retrogradely labeled corticogeniculate and corticotectal neurons in layers V and VI, none were VC1.1-positive. Since VC1.1 also stains neurons scattered in layers II–VI in the rat cortex, we studied other sets of projection neurons using this approach. Preliminary double-labeling experiments in the rat using microsphere injections into the corpus callosum showed that commissural projection neurons were also not VC1.1-positive (unpublished observations). The results were probably not due to a failure to retrogradely label VC1.1-positive projection neurons. A more likely expla-

nation is that some nonprojecting pyramidal neurons that form local connections are also outlined by VC1.1 (Gilbert and Wiesel, 1983; Katz, 1987). Together, these results strongly suggest that the VC1.1 determinant defines a unique subset of local circuit neurons.

A number of other monoclonal antibodies that also stain restricted subpopulations of cortical neurons have been described. We have previously described a mouse IgG monoclonal antibody, VC5.1, which we generated against paraformaldehyde-fixed homogenates of cat visual cortex (Arimatsu et al., 1987). VC5.1 immunofluorescence in areas 17 and 18 resembles VC1.1 immunofluorescence, and both outline subpopulations of nonpyramidal neurons mainly in layer IV but also scattered in layers II–VI (Arimatsu et al., 1987). Double-labeling immunofluorescence experiments in area 17 revealed that all VC1.1-reactive cells were also VC5.1-reactive, and that 83% of VC5.1-reactive cells were VC1.1-reactive, suggesting that the antibodies identify the same subpopulation of cortical cells. In contrast to staining patterns in visual cortex, each antibody stains separate structures in retina and cerebellum (Arimatsu et al., 1987). These previous results, in combination with the findings presented here, suggest that both the VC1.1 and the VC5.1 determinants are expressed by the same or overlapping subsets of GABAergic neurons. As additional molecular markers for cortical interneurons are developed, it may become possible to provide a functional taxonomy based on the selective expression of groups of different antigens by each cell class.

Immunological distinctions between functionally defined classes of neurons were previously demonstrated with an antibody that was generated against cat spinal cord (Hockfield et al., 1983). This reagent, Cat-301, labels subsets of geniculate and visual cortical neurons within the Y-pathway, thought to be involved in visual motion detection (Hockfield et al., 1983). There are some similarities in the cellular staining patterns of VC1.1 and Cat-301 in the visual cortex. Both label the surfaces of neuronal cell bodies and proximal dendrites, but not axons, in a fenestrated pattern (Hockfield et al., 1983; Arimatsu et al., 1987). In the electron microscope, VC1.1 and Cat-301 immunoreactivity surrounds pre- and postsynaptic sides of axosomatic synaptic junctions, and neither labels synaptic clefts (see Fig. 2; Hockfield et al., 1983).

Despite these similarities, VC1.1 and Cat-301 clearly label different subpopulations of cortical neurons. We have shown that VC1.1 labeling is restricted to subsets of local circuit neurons. Cat-301 immunoreactivity, however, outlines both projection and nonprojection neurons (Hendry et al., 1984a). These antibodies also exhibit different laminar patterns of immunoreactivity.

One possibility worth further investigation is that cells that are outlined by antibodies VC1.1 and VC5.1 or Cat-301 may represent nonoverlapping cortical cell populations, each subserving different aspects of local cortical processing. In this regard, when identified X- or Y-type input from the LGN to GABAergic neurons in the visual cortex was studied, it was found that separate subsets of inhibitory neurons were activated by each pathway (Freund et al., 1985). Each of these parallel pathways for visual information-processing may have unique sets of GABAergic neurons that express different antigens and perform different functions within cortical microcircuits.

In the electron microscope, VC1.1 immunoreactivity was encountered periodically along sections of postsynaptic membrane and surrounding, adjacent presynaptic structures. Additional

labeling was also observed on the cytoplasmic face of some perikaryal membranes. At present, the most parsimonious explanation for these findings is that the antigen is produced by a subset of GABAergic neurons and inserted into plasma membranes around sites of synaptic junctions. Ultrastructural immunocytochemistry, as well as colchicine treatment, failed to reveal cytoplasmic staining over Golgi or other organelles, which would be expected if the VC1.1 antigen were produced postsynaptically. It is possible, however, that a precursor molecule is produced by the postsynaptic GABA neurons and then undergoes modifications at the cell surface, allowing the VC1.1 antibody to bind.

Alternatively, the VC1.1 antigen may be produced by presynaptic neurons and transported to terminal endings on GABA-immunoreactive cells. However, VC1.1 immunoreactivity was not detected within synaptic terminals. The VC1.1 antigen could also be transferred from these presynaptic neurons across synaptic junctions, and onto the postsynaptic GABAergic neurons. This seems unlikely, however, since the VC1.1 determinants are probably integral membrane proteins, as shown by extraction from membranes with detergent but not high ionic strength or low or high pH (Arimatsu et al., 1987).

Finally, the VC1.1 antigen may arise from another cell type that maintains a close association with GABA cell bodies and dendrites. Since some artifactual rearrangement of peroxidase reaction product can occur with pre-embedding ultrastructural histochemistry (Novikoff, 1980), it is not at present possible to distinguish between these various alternatives. Postembedding staining methods could be used in combination with particulate markers, such as colloidal gold, to help resolve this issue.

Although the functional significance of the epitope recognized by antibody VC1.1 is not yet known, the restricted distribution of this antigen primarily along the surfaces of a subset of GABA-containing cortical cells and to some synapses onto these cells suggests that the antigen may play an important functional role in inhibitory cortical circuits. By using VC1.1, VC5.1, and other antibodies as links between anatomical and physiological studies, future investigations may address the issue of whether the molecular compositions of different subsets of local circuit neurons correlates with their particular afferent inputs or receptive-field properties.

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