In rat visual cortex neurons that are immunoreactive for the calcium-binding protein calretinin (CR⁺) constitute a distinct family which accounts for 17% of γ-aminobutyric acid (GABA)-expressing cells. It is not clear, however, (i) whether CR is expressed exclusively in GABAergic neurons and (ii) how CR⁺ neurons are incorporated into neuronal circuits of rat visual cortex. To address these questions we studied synaptic relationships of CR⁺ neurons with GABA⁺ and GABA⁻ elements in the neuropil of rat primary visual cortex (area 17). All CR⁺ neurons are nonpyramidal cells with smooth or sparsely spiny and often beaded dendrites. Of all CR⁺ neurons, 56% are located in layers 1 and 2/3. In layer 2/3, most CR⁺ neurons are bipolar-shaped and have vertically oriented dendrites. Many ascending dendritic branches reach layer 1 where they run parallel to pial surface. CR⁺ axons are thin, highly branched near the cell body and often send descending collaterals to layers 5 and 6. Double immunofluorescence labeling revealed GABA in 94% of CR⁺ cell bodies in layer 2/3. Electron microscopic analysis shows that all CR⁺ axon terminals contain elongated vesicles and form symmetric synapses. Postembedding staining shows that 98% of CR⁺ terminals are GABA⁺. GABA-immunoreactivity is also present in somata and thick dendrites of CR⁺ neurons but many thin dendrites are GABA⁻. CR⁺ somata, dendrites and axon terminals are enriched in mitochondria. Somata and thick CR⁺ dendrites are densely innervated. At least 68% of the targets of CR⁺ terminals in layer 2/3 are GABA⁺ and ≥50% of these are other CR⁺ neurons. The remainder (32%) of targets of CR⁺ terminals are thin dendrites of GABA⁻ cells. In contrast, in layers 5 and 6, 60% of CR⁺ terminals form synapses with GABA⁺ somatic profiles. The preferential interactions of layer 2/3 CR⁺ neurons with GABAergic neurons, and with CR⁺ neurons in particular, suggests that these cells play a role in the inhibition of inhibitory neurons of the same layer. Through these interactions CR⁺ cells may reduce inhibition of pyramidal cells in layers 2/3, 5 and 6 and thus disinhibit a column of neurons.

Introduction
When a bar of light falls on the retina, neurons in the visual cortex respond with increased firing of action potentials. The initial excitation in spiny neurons is typically followed by opposing inhibition from smooth γ-aminobutyric acid (GABA)-ergic interneurons (Ferster and Jagadeesh, 1992). This inhibition sharpens the selectivity of visual responses (Somers et al., 1995) and prevents excessive firing (Benardo and Wong, 1995). However, smooth neurons in the visual cortex are not only connected to spiny neurons but are also innervated by GABAergic neurons (Freund et al., 1983; Kisvárday et al., 1985, 1993; Somogyi and Soltesz, 1986; Wouterlood et al., 1995; Meskenaite, 1997; Staiger et al., 1997; Gonchar and Burkhalt, 1999). The function of these connections is to inhibit inhibitory neurons (Tamás et al., 1998) and in turn to disinhibit spiny neurons. One effect of disinhibition is to synchronize pyramidal cell activity (Jefferys et al., 1996). Since GABAergic neurons are morphologically and physiologically diverse (Fairen et al., 1984; DeFelipe, 1993; Kawaguchi and Kubota, 1997), the effects of disinhibitory interactions on the temporal and spatial patterns of activity must depend on the cell type(s) involved. It is, therefore, important to know how different types of GABAergic neurons are interconnected.

GABAergic neurons in rat visual cortex fall into at least three distinct families that are distinguished by the expression of parvalbumin (PV⁺), somatostatin (SOM⁺) and calretinin (CR⁺) (Gonchar and Burkhalt, 1997). CR⁺ neurons account for ~17% of GABAergic neurons and form intracolumnar connections between different layers (Meskenaite, 1997). In rat hippocampus and upper layers of monkey visual cortex, CR⁺ neurons innervate mainly GABAergic neurons (Gulyás et al., 1996; Meskenaite, 1997). Among these, hippocampal CR⁺ neurons favor interactions with CR⁺ cells. CR⁺ neurons in rat visual cortex may have a similar preference, which may provide a system for disinhibiting pyramidal cells. Although in cerebral cortex >90% of CR⁺ neurons express GABA (Kubota et al., 1994; Gonchar and Burkhalt, 1997), the transmitter phenotype of the remaining CR⁺ neurons is unknown. Several studies have found that a fraction of CR⁺ neurons does not stain with antibodies against GABA (Rogers, 1992; Gonchar and Burkhalt, 1997; Miettinen et al., 1997) and that a subset of CR⁺ axon terminals form asymmetric synapses (Lüth et al., 1993; Del Rio and DeFelipe, 1997). This suggests that CR⁺ neurons might be both inhibitory and excitatory. To resolve this issue we studied whether CR⁺ axon terminals contain GABA. The second goal was to determine the synaptic relationships of CR⁺ neurons with other elements in the neuropil and to delineate how CR⁺ neurons are incorporated into the visual cortical circuitry. To address these questions we combined pre-embedding immunostaining for CR with postembedding immunogold staining for GABA, and analyzed the colocalization of GABA in CR⁺ neurons in the electron microscope.

Materials and Methods
Eleven adult rats, housed and treated in accordance with institutional and NIH regulations, were anesthetized with sodium pentobarbital and perfused through the aorta with phosphate-buffered saline followed by three different fixatives: (i) 4% paraformaldehyde, 1.5% glutaraldehyde and 0.1 picric acid (four animals); (ii) 4% paraformaldehyde 0.5% glutaraldehyde and 0.1 picric acid (four animals); and (iii) 4% paraformaldehyde and 0.25% glutaraldehyde (three animals). The brains were postfixed in the same fixative and coronal sections of visual cortex were cut at 25 µm on a Vibratome.

Pre-embedding Immunocytochemistry
To reduce free aldehydes, increase antibody penetration and inactivate endogenous peroxidases the sections were treated with (a) 1% hydrogen peroxide followed by 60% ethanol and 0.1% H₂O₂. After preincubation in 10% fish gelatin sections were transferred to anti-CR antiserum (rabbit-anti-CR, 1:2000, Swant, Bellinzona, 1993). Two different secondary antibodies were used to detect the antibody reaction products: (i) 1:200 biotinylated sheep-anti-rabbit IgG (Kirkeby et al., 1987) followed by 1:1000 avidin-biotinylated peroxidase complex (ABC) and 0.02% diaminobenzidine tetrachloride (DAB) in 0.05 M Tris-0.1 M NaCl buffer pH 7.5 (Somogyi et al., 1985). (ii) 1:400 swine-anti-rabbit IgG (Kirkeby et al., 1987) followed by 1:200 swine-anti-sheep IgG and 0.02% DAB. When necessary for the visualization of small numbers of CR⁺ neurons, sections were processed for pre-embedding Immunocytochemistry by exposure to 0.02% DAB in a buffer similar to that used for postembedding immunocytochemistry. This method is called peroxidase-antiperoxidase or PAP, and allows the visualization of very small amounts of CR⁺ staining in contrast to the much more intensive staining with ABC.

Switzerland) for 20–60 h. Sections were then incubated in biotinylated goat-anti-rabbit antibody (1:200, Chemicon, Temecula, CA) and treated with avidin and biotinylated horseradish peroxidase (HRP; Vectastain ABC Kit, Vector, Burlingame, CA). To visualize HRP activity for light microscopy every third section was pretreated with 0.4% Ni(NH₄)₂ and 0.5% CoCl₂ (Adams, 1981) and reacted with 0.005% H₂O₂ in the presence of 0.05% diaminobenzidine tetrahydrochloride (DAB). Sections were mounted on slides and coverslipped using DPX mountant.

Double Immunofluorescence of GABA and CR

Fluorescence immunolabeling for GABA and CR was performed as described previously (Gonchar and Burkhalter, 1997). In brief, sections of brains fixed with 4% paraformaldehyde and 0.25% glutaraldehyde were incubated in a mixture of goat and horse normal sera (5%/5%), followed by overnight incubation in rabbit-anti-CR antibody and mouse-anti-GABA antibody (Chemicon). The next day sections were transferred to fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit IgG (1:200; Chemicon) followed by incubations in biotinylated horse-anti-mouse IgG (1:200; Vector) and avidin–neutralite–Texas Red (1:1000, Molecular Probes, Eugene, OR). Sections were mounted, coverslipped and viewed under a fluorescence microscope equipped with rhodamine, fluorescein and ultraviolet optics.
Electron Microscopy

Pre-embedding CR/Postembedding GABA Double Immunolabeling

Every fifth 25 µm thick section of area 17 fixed with 4% paraformaldehyde, 1.5% glutaraldehyde and 0.2% picric acid was selected. Sections were immunostained for CR using DAB histochemistry, omitting Ni/Co intensification. Next, sections were rinsed in maleate buffer (MB, pH 7.2), incubated in 1% tannic acid in MB, and stained in maleate buffered 1% uranyl acetate. This was followed by dehydration, flat-embedding in Durcupan (Fluka, Ronkonkoma, NY), cutting 50 nm ultrathin sections, and staining with uranyl acetate and lead citrate. Because CR-immunostaining was confined to the superficial 5 µm of a 25 µm thick section, thin sections from this tier were used for post-embedding staining for GABA. Incubations were performed on drops of filtered solutions in a humidified chamber. Sections were rinsed in Tris-buffered saline (TBS, pH 7.6), incubated in 1% bovine serum albumin (BSA) containing 0.02% Triton X-100 (TBST) and treated for ∼18 h with a monoclonal mouse-anti-GABA antibody (Chemicon, 1:10 000, 0.1% BSA). The next day, sections were rinsed in TBST containing 1% BSA (pH 7.6), washed in TBST (pH 8.2) and incubated in goat-anti-mouse IgG conjugated to 15 nm gold particles (Amersham, Arlington Heights, IL, 1:25 in TBST, pH 8.2). Sections were rinsed, dried and stained with uranyl acetate and lead citrate. Profiles were considered GABA+ if the density of gold particles was ≥2× that over axon terminals forming asymmetric synapses.

Figure 2. Partial reconstructions of dendritic trees of CR+ neurons in layers 1 and 2/3. CR+ neurons have nonpyramidal somata and smooth dendrites (a–f). Spine-like appendages are rare (g). Layer 1 contains cells associated with the pial surface (h), multipolar cells (i) and Cajal–Retzius cells (j). Cells in layer 2/3 have a variety of morphologies that include bipolar (b) and multipolar (g) cells at opposite ends of the spectrum. Scale bar = 100 µm.

Figure 3. Fluorescence photomicrographs of CR+ neurons in layer 2/3 of area 17 stained with an FITC-labeled secondary antibody. (A) Four CR+ boutons in close apposition to soma of bipolar-shaped CR+ neuron, suggesting synaptic contacts. (B) Arrows point to CR+ boutons in contact with CR+ cell body and CR+ proximal dendrites layer 2/3. Arrowhead indicates the nucleolus. Magnification in (A) and (B) is the same. Scale bar = 25 µm.
To control for the preservation of tissue fixed without osmium, selected CR-immunostained sections were additionally fixed with 1% osmium, embedded and used for postembedding GABA staining.

Quantitative Analysis
Area 17 and its layers were identified by counterstaining sections with bisbenzimide (0.005% in PB). To determine the proportion of GABAergic neurons expressing CR and the percentage of CR+ cells expressing GABA, area 17 was analyzed under the fluorescence microscope in systematically randomly selected 25 µm sections spaced 100 µm apart. Cell counts were performed at ×625 in 41 µm wide strips oriented perpendicular to the pial surface, extending through all cortical layers. Only cells whose nuclei were contained within the section were scored.

Numerical densities of CR+ neurons in area 17 and the lateromedial area (LM) (Coogan and Burkhalter, 1993) were determined employing the optical dissector method (Sterio, 1984; West, 1993; Coggeshall and Lecan, 1996). Counts were restricted to neurons whose nucleoli were in focus between 5 and 20 µm below the surface of the section. Densities of CR+ cells in each layer were calculated from neuronal counts performed in 60 vertical strips from sections of two animals. Numerical densities were corrected for an estimated 6% linear or 17% volume shrinkage.

Thin sections were studied under a Jeol-100 electron microscope. To determine the proportions of asymmetric synaptic profiles formed on CR+ neurons of the total number of asymmetric synapses and to estimate the percentage of CR+ axon terminals of the total number of terminals, photographs of the neuropil were taken at ×14 000 in systematic random order (Weibel, 1979; Coggeshall and Lecan, 1996). To increase the sample of CR synaptic contacts the neuropil was photographed at ×20 000. Similarly, photographs (×20 000) of CR+, somatic profiles cut through the center of the nucleus, were used to study distribution and anatomy of synaptic inputs to CR+ somata. The specimens were studied with the axis...
Figure 5. Electron micrograph of somatic profile of CR+, GABA-immunogold labeled (black particles) neuron in layer 2/3, observed in osmium-free tissue. Nucleus (N), whose membrane shows deep infoldings, is more darkly stained than the cytoplasm of the perikaryon (P) and dendrite (d). The cytoplasm is rich in mitochondria (M), contains lysosomes (L), cisternae of rough endoplasmic reticulum (asterisks) and a Golgi apparatus (G). Synapses on the somatic membrane are formed by GABA⁻ (single arrow), GABA⁺ (two arrows) and CR⁺ (three arrows) axon terminals. Notice that the resin-filled blood vessel is devoid of immunogold particles, indicating a very low level of background staining. Scale bar = 1 µm.
of the electron beam oriented perpendicular to the plane of section. Determinations of synaptic contacts were made on photographs at a final magnification of ×50,000. Measurements of profile lengths and areas were made on prints using a digitizing tablet and NIH Image software. The t-test was used to determine significant differences (P ≤ 0.05). Averages are represented as mean ± SD.

Results

Light Microscopy

Morphology

CR-immunoreactivity is present in neuronal cell bodies, dendrites and axons, and reveals the detailed morphology of individual neurons. Without exception CR+ neurons have non-pyramidal morphologies. Dendrites are mostly smooth with occasional spine-like appendages (Figs 1A, B and 2). Many CR+ dendrites in layers 1–3 show swellings, particularly in processes that run parallel to the pial surface (Fig. 1B). CR+ cell bodies are distributed throughout all layers, but most (56%, 113/203) are located in layers 1 and 2/3. CR+ staining of the neuropil is weakest in layer 4, and densest in layer 1 and superficial layer 5. Intermediate levels of neuropil staining are found in layer 2/3, deep layer 5 and layer 6.

Layer 1 contains few CR+ cells (Fig. 1C) which fall into three classes. The most conspicuous type is found immediately below the pial surface. It shows a round cell body that gives rise to a single dendritic tuft which descends and branches within layer 1 (Fig. 2f). The two other types include multipolar neurons with short dendrites (Figs 2i) and Cajal–Retzius cells with long, horizontal dendrites extending for >1 mm within layer 1 (Fig. 2j). Most axons of layer 1 CR+ neurons remain within the layer of origin.

CR+ neurons are most abundant in layer 2/3 (Fig. 1C). They form ~500 µm wide clusters in which GABAergic cells of the PV or SOM families (Gonchar and Burkhalter, 1997) are sparse. Most CR+ somata are oval and, similar to bipolar cells (Peters, 1984), give rise to individual vertically ascending and descending primary dendrites (Figs 1A, B and 2a–c). Many neurons issue an additional horizontal dendrite from the cell body (Fig. 2e). Other CR+ cells have primary dendrites that arborize close to the cell body (Fig. 2d–f). As a group, CR+ neurons show features that vary between bipolar and multipolar morphologies (Fig. 2g) at opposite ends of the spectrum. All layer 2/3 CR+ neurons send dendrites to layer 1 where they extend for >1 mm parallel to the pial surface.

CR+ neurons in layer 2/3 issue single thin axons (diameter <1 µm) from the cell body or a primary dendrite. Most axons branch within layer 2/3 and form small en passant and terminal boutons which are only slightly larger than the parent axon. Most axons send descending collaterals to layers 5 and 6, giving off short branches along the way. In layer 5, and to a lesser extent in layer 6, CR+ axons form terminals which surround CR+ somatic profiles resembling pyramidal cells. Ascending axons to layer 1 are rare.

CR+ dendrites in layer 2/3 form vertical bundles of 3–5 main trunks which appear to make dendro-dendritic contacts (Fig. 1B). Contacts are also formed by CR+ axons which terminate near CR+ somata and dendrites (Figs 2b and 3A,B).

Besides intensely stained CR+ cells in layer 2/3 there is a small population of weakly CR+ neurons. Most of these have bipolar morphology. Their presence throughout the depth of the section suggests that weak staining is a distinct phenotype and is unrelated to antibody penetration.

Neurochemistry

Analysis of sections stained with antibodies against CR and GABA shows that 17.9% (190/1059) of GABA+ cells in area 17 are CR+. Most (94%, 190/203) CR+ neurons examined are GABA+. Typically, GABA staining is intense in CR+ somata and thick CR+ proximal dendrites, but is absent in thin CR+ dendrites. In layer 1, every CR+ neuron is GABA+ (69/69). Layer 2/3 differs in that it contains ~10% (13/134) GABA+/CR+ cells. As a rule CR staining is weak in GABA+/CR+ neurons.

Electron Microscopy

Ultrastructure of CR+ Neurons

Ultrastructurally, CR+ cell bodies in layer 2/3 are heterogeneous. Most strikingly some neurons show CR immunostaining in both nucleus and cytoplasm (Fig. 5), whereas in others CR immunoreactivity is confined to the cytoplasm. All CR+ neurons show deep infoldings of the nuclear membrane (Fig. 5), their cytoplasm is rich in organelles, contains an elaborate Golgi apparatus, numerous cisternae of rough endoplasmic reticulum, and lysosomes. CR+ cells contain a large number of mitochondria which occupy up to 40% of the cytoplasm (Figs 5 and 6, Table 3). In the few CR+ cells in which the density of mitochondria is low GABA staining is weak (Fig. 6A).

Layer 2/3 contains a large number of CR+ dendrites. Most of these profiles are small and are among the thinnest processes in the neuropil (Fig. 4A). The innervation density of thin dendritic profiles is low. In contrast, the less abundant thick CR+ dendrites are more densely innervated (Fig. 7B).

The vast majority of CR+ axons are unmyelinated and most of them are thin. Unlike CR+ somata and dendrites the ultrastructural features of CR+ axon terminals observed in osmium-fixed and osmium-free material differ. In osmium-fixed material the majority of CR+ terminals contain mostly elongated vesicles and lack a conspicuous postsynaptic density (Fig. 4C). The same material also reveals occasional CR+ terminals that contain large, round synaptic vesicles and form asymmetric synapses (Fig. 4A,B). In contrast, in osmium-free material all CR+ terminals uniformly contain mostly elongated vesicles and form exclusively symmetric synapses (Figs 6B and 8B,D,E).

Postembedding staining for GABA shows that the majority of CR+ somata (92%, 56/61), most (88%, 73/83) CR+ dendrites with profile areas ≥0.5 µm2 and almost all (98%, 124/127) CR+ axonal terminals are GABA+ (Fig. 8A). As a rule, GABA-staining in CR+ dendrites is less intense than in CR+ axonal terminals (Fig. 7A). In thin CR+ dendritic profiles (profile area ≤0.5 µm2) GABA immunogold particle density is generally low (Figs 6C and 8C).

Synaptic Innervation of CR+ Neurons

Of all asymmetric synapses in layer 2/3, 6.5% (61/943) form synapses with CR+ neurons (Table 1). Most of these (97%, 59/61) are located on CR+ dendrites and few (3%, 2/61) are found on CR+ cell bodies.

Table 1

<table>
<thead>
<tr>
<th>Total area studied</th>
<th>All axon terminals</th>
<th>Asymmetric synapses total on CR+ profiles</th>
<th>Asymmetric synapses on CR+ axon terminals/synapses</th>
<th>CR+ dendritic profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>2189 µm²</td>
<td>1746</td>
<td>943</td>
<td>61</td>
<td>44/15</td>
</tr>
</tbody>
</table>
Figure 6. Axon terminals in direct contact with cell bodies of CR+ neurons, observed in osmium-free tissue. (A) GABA- axon terminal (AT) forming asymmetric synaptic contact with CR+ soma. Nearby CR+/GABA- axon terminal (asterisk) is in direct contact with the same cell body. The density of gold particles in CR+ terminal is higher than in the perikaryon (P). (B) Two CR+/GABA+ axon terminals (asterisks) forming contacts with CR+/GABA+ perikaryon (P). Both terminals contain elongated vesicles. The larger terminal forms synaptic contact with the soma (arrows). Nearby dendrite (arrowhead) is GABA-. Magnification in (A) and (B) is the same. Scale bar shown in (B) = 0.5 μm. (C) CR+/GABA+ perikaryon (P), containing numerous mitochondria, receiving synaptic input from large GABA+ axon terminal (arrows) with clear cytoplasm. Asymmetric synapses formed by GABA- axon terminals with thin CR+/GABA- (arrowhead) and thicker GABA+/CR+ dendrites (asterisk). Scale bar = 0.5 μm.

Table 2
CR+ axon terminals and their targets in layer 2/3 of rat area 17

<table>
<thead>
<tr>
<th>Total no. of CR+ terminal profiles</th>
<th>CR+ terminal profiles which do not form synaptic contacts of these GABA+</th>
<th>CR+ terminal profiles forming symmetric synaptic contacts</th>
<th>Targets of CR+ terminal profiles forming symmetric synaptic contacts</th>
<th>CR+ terminal profiles putatively forming symmetric synaptic contacts of these GABA+</th>
<th>Targets of CR+ terminal profiles in putatively symmetric synaptic contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR+ profiles</td>
<td>GABA+/CR+ profiles</td>
<td>GABA+ profiles</td>
<td>CR+ profiles</td>
<td>GABA+/CR+ profiles</td>
<td>GABA+ profiles</td>
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<tr>
<td>390 (100%)/379 (97.2%)</td>
<td>201 (100%)/195 (97.0%)</td>
<td>127 (100%)/124 (97.6%)</td>
<td>53 (41.7%)</td>
<td>33 (26.0%)</td>
<td>41 (32.3%)</td>
</tr>
<tr>
<td>(D: 47; Som: 6)</td>
<td>(D: 31; Som: 2)</td>
<td>(D: 35; Sp: 6)</td>
<td>62 (100%)/61 (98.4%)</td>
<td>31 (50.0%)</td>
<td>15 (24.2%)</td>
</tr>
<tr>
<td>(D: 29; Som: 2)</td>
<td>(D: 15)</td>
<td>(D: 16; Sp: 1)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

D, dendritic profiles; Som, somatic profiles; Sp, profiles showing the spine apparatus.
In layer 2/3 the majority (90%, 172/191) of synapses on CR+ dendrites are asymmetric and are formed by GABA– axon terminals (Figs 6C and 7A, B). In contrast, 72% (70/97) of synapses on CR+ layer 2/3 cell bodies are formed by GABA+ terminals and are symmetric (Fig. 6B, C).

Because synapses on CR+ profiles are rare and each part of the CR+ neuron receives a different set of GABA+ and GABA– synapses, we sought out CR+ somata and dendrites to study synaptic interactions with these elements. Of a total of 11 GABA+/CR+ profiles, representing the equator of the cell body, we found 97 axonal profiles that formed synaptic contacts (Table 3). Individual somatic profiles are contacted by 3–13 (mean contacts/profile = 8.8 ± 3.2) axon terminals which are either GABA+/CR+, GABA+/CR– or GABA–. Nine somatic profiles receive contacts from each of the three types of synapses. By far the largest proportion of synapses are formed by GABA+/CR– axon terminals (63%, 61/97; mean contacts/somatic profile = 5.4 ± 1.8). In this group the most conspicuous are large axon

### Table 3

<table>
<thead>
<tr>
<th>Average area (µm²)</th>
<th>Average area (µm²)</th>
<th>No. of synapses on 11 CR+ somatic profiles</th>
<th>No. of mitochondria in the perikaryon</th>
</tr>
</thead>
<tbody>
<tr>
<td>of somatic profile</td>
<td>of nuclear profile</td>
<td>Total GABA+ (asymmetric)</td>
<td>GABA+/CR–</td>
</tr>
<tr>
<td>88.5 ± 17.2</td>
<td>32.3 ± 9.5</td>
<td>97</td>
<td>27</td>
</tr>
</tbody>
</table>

This table contains the actual measurements of the profile areas. The linear post-perfusion shrinkage of the tissue was 9.2%. Therefore the areas before fixation are 1.18 × of those presented in the table. Average values represent mean ± SD.

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terminals with clear axoplasm, elongated, dispersed vesicles and presynaptic membranes that conform to the shape of CR+ somata (Figs 5 and 6C). Another group of GABA+/CR- terminal profiles are smaller and contain darker axoplasm. As a rule, both types contain one or more mitochondria. The remainder of GABA+/CR- terminals lack distinctive features. All GABA+/CR- axon terminals form symmetric synapses.

CR+ inputs to layer 2/3 CR+ cell bodies are surprisingly rare and account for only 9% (9/97, mean contacts/profile = 1.0 ± 0.8; Table 3 and Figs 5 and 6B) of axosomatic synapses. Although in the light microscope we observed numerous appositions of CR+ boutons on CR+ cell bodies (Fig. 3), in the electron microscope such contacts are surprisingly rare. Of the CR+ terminals that do form synapses on CR+ somata, all are GABA+, contain mostly elongated synaptic vesicles and form symmetric contacts (Fig. 6B). Typically, CR+ somatic profiles receive less than three CR+ synaptic inputs.

Each of the CR+ somatic profiles examined in layer 2/3 is contacted by at least a single asymmetric synapse (1.4/profile, mean = 2.1 ± 1.0) formed by a GABA- axon terminal that contains round vesicles (Figs 5 and 6A, Table 1). These synapses account for 28% (27/97) of all synapses on CR+ cell bodies.

CR+ dendrites receive symmetric GABA+/CR+, GABA+/CR+ and asymmetric GABA- synapses. Unlike inputs to somata, the majority of synapses on both thick (88%, 96/109) and thin (93%, 76/82) CR+ dendritic profiles are formed by GABA- axon terminals. Profiles of GABA- synapses on thick dendrites, many of which are primary dendrites of CR+ neurons, are formed by CR+ (6%, 6/109) and CR- (6%, 7/109) synapses. Most GABAergic inputs to small dendritic profiles derive from CR- terminals (7%, 6/82). A summary of synaptic inputs to CR+ neurons is provided in Figure 9A.

 Targets of CR+ Axon Terminals
In layer 2/3 CR+ axon terminal profiles account for 2.5% (44/1746) of terminals in the neuropil (Table 1). Of the 390 systematically, randomly selected CR+ terminal profiles found in GABA-stained, osmium-free tissue (Table 2), all are enriched in elongated synaptic vesicles (Figs 6B and 6B-E). Of these, 201 CR+ terminals do not form synapses in the plane of section. Sixty-two terminals show specializations that suggest putative synaptic contacts with adjacent profiles. One hundred and twenty-seven CR+ terminals form synaptic contacts which show a narrow synaptic cleft and an inconspicuous postsynaptic density.
density (Figs 6B and 8D,E). Most (98%, 124/127) CR+ terminals forming such symmetric synapses are GABA+ (Figs 5 and 6A,7). Eighty-six percent (109/127) of terminals contain at least one mitochondrion (mean mitochondria/profile = 1.35 ± 0.6). In contrast, mitochondria are rare in CR− terminal profiles (mean mitochondria/profile = 0.2 ± 0.1, n = 152).

In layer 2/3 most (42%, 53/127) CR+ terminals form synaptic contacts with GABA+ CR+ profiles (Fig. 9B). GABA+ CR+ profiles account for 26% (33/127) of targets (Fig. 8B,C). Altogether 68% of CR+ axon terminals form synapses with GABA+ neurons. Interestingly, GABA+ CR+ terminals form synapses with dendrites which often receive nearby inputs from GABA+ CR+ axon terminals. Synapses on GABA+ profiles account for 32% of targets of CR+ axons. Some of these targets resemble apical dendrites of pyramidal neurons. CR+ contacts with spines are rare.

Similar to layer 2/3, CR+ terminals in layer 5 are GABA+ and form symmetric contacts. Unlike in layer 2/3, in layer 5 60% (36/60) of CR+ terminals form synapses with GABA+ targets. These include spines (11.7%, 7/60), dendrites (41.6%, 25/60) and somata (6.7%, 4/60) of putative pyramidal neurons. GABA+ targets include dendritic profiles, of which 25% (15/60) are CR+ and 15% (9/60) are CR− (Fig. 9B).

In 67% (263/390) of CR+ terminal profiles synaptic contact was ambiguous (Figs 4A,B, 7A and 8A, Table 2). Because synaptic contacts were seen in all of the eight serially reconstructed CR+ terminals, the low yield might be due to the small size of CR+ synapses. By contrast, synaptic contacts by CR− terminals were more abundant and were observed in 54% (943/1746) of profiles analyzed.

Discussion

Previous studies in rat neocortex have shown that the calcium-binding protein CR is expressed in nonpyramidal neurons with smooth dendrites (Rogers, 1992; Lüth et al., 1993; Kubota et al., 1994; Fonseca et al., 1995; Gabbott et al., 1997; Gonchar and Burkhalter, 1997). Most nonpyramidal cells, including the majority of CR+ neurons, use the inhibitory neurotransmitter GABA (Houser et al., 1984; Peters and Jones, 1984; Gonchar and Burkhalter, 1997). However, a fraction of CR+ cell bodies do not stain for GABA (Rogers, 1992; Miettinen et al., 1997; Gonchar and Burkhalter, 1997)(see also this study) and some CR+ terminals form asymmetric (type 1) synapses (Lüth et al., 1993; Del Rio and DeFelipe, 1997), which are characteristic of excitatory nerve endings (Gray, 1959; Colonnier, 1968; Ribak, 1978; Houser et al., 1984; Peters and Jones, 1984; Beaulieu et al., 1994). Such putative excitatory terminals might arise from GABA bipolar cells (Peters, 1984; Meinecke and Peters, 1987). Since 98% of CR+ terminals in rat (this study) and 95% in monkey visual cortex (Meskenaite, 1997) are GABA+, most of these synapses must be inhibitory. This agrees with observations showing elongated vesicles in CR+ terminals forming symmetric (type 2) synaptic contacts (Meskenaite, 1997) (see also this study). Importantly, asymmetric (type 1) CR+ synapses containing round vesicles (Lüth et al., 1993) are rare and are only seen in osmium-fixed material (this study). Therefore, it seems likely that they are a fixation artifact. If excitatory CR+GABA+ terminals do indeed exist, they may originate from cholinergic bipolar cells which express vasoactive intestinal polypeptide (VIP) (Kosaka et al., 1988; Peters and Harriman, 1988; McCormick, 1992; Cauli et al., 1997) and which show similarities to CR+ nerve endings (Mrzljak et al., 1995). They are certainly not glutamatergic since glutamate is only present in spiny neurons (Conti and Minelli, 1996).

Inputs to CR+ Neurons

CR+ neurons account for 3.5% (2512/72 000) of all neurons contained <<1 mm3 of primary visual cortex (Beaulieu, 1993; Gonchar and Burkhalter, 1997). The proportion of CR+ cells is slightly higher in layer 2/3 (4.8%; 2912/60 000) (Gabbott and Stewart, 1987; Beaulieu, 1993; Gonchar and Burkhalter, 1997). Similar percentages of CR+ neurons (3.8–4.3%) were found in rat medial prefrontal cortex (Gabbott et al., 1997). Of all asymmetric synaptic profiles in layer 2/3, 6.8% are in contact with CR+ neurons. This is a surprisingly large percentage, considering that the proportion of CR+ neurons is low and the surface area of these aspynic cells is small. This suggests that the innervation density of CR+ neurons is high and corresponds to that seen in the population of cortical interneurons (Colonnier, 1981; McGuire et al., 1991).

Most CR+ axons terminate on thick dendrites, suggesting a preference for synaptic contacts close to the cell body. We have ruled out that these contacts are on varicosities of distal dendrites by showing in reconstructions from serial sections that CR-recipient, CR+ profiles represent proximal dendrites.

The origin of asymmetric GABA+ synapses on CR+ terminals in layer 2/3 is unknown. Considering their location, CR+ dendrites may receive inputs from local collaterals of layer 2/3 pyramidal neurons. In addition, they may receive inputs from cells in layers 4, 5 and 6, and the thalamus (Martin, 1984; Burkhalter, 1989; White, 1989; Miller et al., 1993; Lund et al., 1993; Meskenaite, 1997). At present, we can only rule out input from the secondary visual area LM (Y. Gonchar, unpublished observations). Thus, CR+ cells in layer 2/3 differ from PV+GABA+ neurons in that they are not a target of interareal cortical projections (Gonchar and Burkhalter, 1999). Many dendrites of layer 2/3 CR+ neurons ascend to layer 1 where they extend horizontally. Axons from other cortical areas and subcortical nuclei terminate in layer 1 (Levitt and Moore, 1979; Bear et al., 1985; Herkenham, 1986; Burkhalter, 1989; Vogt, 1991; Coogan and Burkhalter, 1993; Casagrande and Kaas, 1994; Marin-Padilla, 1998). Thus, similar to pyramidal cell dendrites (Caulier and Connors, 1994; Yuste et al., 1994), CR+ dendrites may receive excitatory synaptic inputs from afferents in layer 1.

CR+GABA+ terminals which innervate CR+ somata (Fig. 9A) resemble VIP+ synapses on pyramidal cells (Peters and Harriman, 1992), and presumably originate from neurons that coexpress CR and VIP (Kubota et al., 1994). Since most CR+ neurons are VIP+ (Kubota et al., 1994) and PV+GABA+ neurons strongly innervate VIP+ cells (Staiger et al., 1997), CR+GABA+ terminals on CR+ somata (Fig. 9A) presumably originate from PV+ neurons. Indeed, CR+GABA+ terminals look like ‘medium size’ nerve endings on pyramidal cell somata (Peters and Harriman, 1992) which resemble PV+ terminals (Wouterlood et al., 1995; Czeiger and White, 1997; Gonchar and Burkhalter, 1999), and which account for most inhibitory axosomatic synapses in the cortex (Hendry et al., 1989; Ren et al., 1992; DeFelipe, 1993; Jones, 1993; Kisvárday et al., 1993; Kubota et al., 1994; Wouterlood et al., 1995; Gonchar and Burkhalter, 1999). Large GABA+ terminals that form synapses on CR+ somata show similarities to ‘large’ terminals found on pyramidal neurons (Peters and Harriman, 1992), but they differ from PV+ and CR+ axon terminals.

Direct evidence that somata of CR+ neurons receive inputs from GABA+ neurons is lacking. SOM+ neurons include double bouquet and Martinotti cells which preferentially synapse on thin dendrites and spines of pyramidal neurons (Hendry et al., 1983; De Lima and Morrison, 1989; Somogyi and
Cowey, 1984; Hendry et al., 1989; DeFelipe et al., 1990, Condé et al., 1994; Peters and Sehares, 1997) and express substance P (SP) (Jones et al., 1988). Many CR+ neurons express SP receptors (Acsády et al., 1997). Thus, it is possible that CR+ cell bodies are innervated by SP/GABA+ terminals which form symmetric synapses on nonpyramidal cells (Jakab et al., 1997).

The majority of GABAergic synapses on CR+ neurons are located on dendrites (Fig. 9B). Of these synapses, ~50% are formed by CR+ nerve endings which preferentially terminate on thick dendrites. Both the magnitude and location of this input indicates that CR+ neurons may be able to inhibit each other. The identity of the remaining 50% of CR+/GABA+ synapses on thin CR+ dendrites remains unknown.

**Targets of CR+ Terminals**

In layer 2/3 of area 17, 68% of CR+ terminals form synapses with GABA+ profiles (Fig. 9B). This proportion might be even higher because inputs on distal dendrites were hard to see due to weak GABA staining. A similar preference of CR+ axons for different types of GABA+ neurons was found in rat hippocampus (Gulyás et al., 1996) and in supragranular layers of monkey visual cortex, where 81% of targets are GABA+ (Meskenaite, 1997). In fact, the interconnectivity of CR+ neurons with other GABAergic neurons is ~5 times greater than that of PV+ cells (Gonchar and Burkhalter, 1999). Most interestingly, CR+ neurons show a strong preference to interact with other CR+ neurons.

Because CR+ neurons are concentrated in layer 2/3 and the density of CR+ axons in this layer is high, the main source of CR+ input to layer 2/3 CR+ neurons may be neighboring CR+ cells or self-innervating autapses (Tamás et al., 1997, 1998). Additional inputs may arise from CR+ neurons in layers 1, 5, and 6. However, these inputs are much less prominent since most layer 1 axons...
terminate locally (Hestrin and Armstrong, 1996; Zhou and Hablitz, 1996) and CR+ projections from deep layers are rare (Gonchar and Burkhalter, 1997). There are no known major extrinsic sources of CR+ axons innervating area 17.

Unlike in layer 2/3, in layers 5 and 6 ~60% of targets of CR+ axons are GABA- (Fig. 9B). The triangular arrays of CR+ boutons surrounding these GABA- profiles suggests that these are pyramidal cells (Meskenaite, 1997) (see also this study) which are innervated by descending CR+ axons (Meskenaite, 1997) (Y. Gonchar, unpublished observations). This striking laminar difference in target selection by single CR+ neurons suggests that during development the specificity of synaptic connections might be determined by cues from the postsynaptic target (Davis and Goodman, 1998).

**Functional Significance**

Our finding that CR+ axon terminals are GABA+ and preferentially innervate GABA- neurons suggests that CR+ neurons form a network for disinhibiting pyramidal neurons. A tentative scheme of the underlying circuit is shown in Figure 9C. CR+ neurons in upper layers are connected to somata and proximal dendrites of both CR+/GABA- and CR-/GABA+ neurons. In addition, CR+ neurons form connections with distal dendrites of pyramidal cells. CR+ synapses on distal pyramidal cell dendrites presumably regulate the frequency and timing of dendritic spikes (Kim et al., 1995) and have little effect on neuronal firing (Tsubokawa and Ross, 1996). In contrast, perisomatic CR+ inputs to CR+/GABA- and CR-/GABA+ neurons may strongly inhibit spike generation (Miles et al., 1996). This may relieve layer 2/3 and deep layer pyramidal cells from inhibition by intralaminar CR-/GABA- and interlaminar CR+/GABA+ projections respectively (Gonchar and Burkhalter, 1999).

CR+ neurons are presumably coupled electrically through dendro-dendritic gap junctions (Fig. 1B) (Gulyás et al., 1996). This may synchronize the activity in groups of 3–5 CR+ neurons and provide a coherent disinhibitory signal to deep layer pyramidal cells. Thus, the net effect of excitation of layer 2/3 CR+ neurons may be to increase the excitability in columns of pyramidal cells and in turn to increase firing in postsynaptic target neurons in cortex, thalamus and superior colliculus (Burkhalter and Charles, 1990; Coogan and Burkhalter, 1993). Since CR+ neurons may be visually driven by geniculocortical inputs and be innervated by the lateral posterior thalamic nucleus (pulvinar) and feedback connections from higher cortical areas via afferents to layer 1 (Herkenham, 1986; Coogan and Burkhalter, 1993), it is conceivable that CR+ neurons are part of a circuit that provides for attentional modulation of visual responses (Olschhausen et al., 1993).

Stressing a role of CR+ neurons in cortical disinhibition might be an oversimplification. CR+ neurons may be reciprocally connected and may be innervated by autapses. Such connections may form a network that synchronizes activity in hippocampal and neocortical pyramidal neurons (Cobb et al., 1995; Jefferys et al., 1996).

**Notes**

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