Axo-Axonic Synapses Formed by Somatostatin-Expressing GABAergic Neurons in Rat and Monkey Visual Cortex

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ABSTRACT
In cerebral cortex of rat and monkey, the neuropeptide somatostatin (SOM) marks a population of nonpyramidal cells (McDonald et al. [1982] J. Neurocytol. 11:809–824; Hendry et al. [1984] J. Neurosci. 4:2497–2517; Laemle and Feldman [1985] J. Comp. Neurol. 233:452–462; Meineke and Peters [1986] J. Neurocytol. 15:121–136; DeLima and Morrison [1989] J. Comp. Neurol. 283:212–227) that represent a distinct type of γ-aminobutyric acid (GABA) -ergic neuron (Gonchar and Burkhalter [1997] Cereb. Cortex 7:347–358; Kawaguchi and Kubota [1997] Cereb. Cortex 7:476–486) whose synaptic connections are incompletely understood. The organization of inhibitory inputs to the axon initial segment are of particular interest because of their role in the suppression of action potentials (Miles et al. [1996] Neuron 16:815:823). Synapses on axon initial segments are morphologically heterogeneous (Peters and Harriman [1990] J. Neurocytol. 19:154–174), and some terminals lack parvalbumin (PV) and contain calbindin (Del Rio and DeFelipe [1997] J. Comp. Neurol. 342:389–408), that is also expressed by many SOM-immunoreactive neurons (Kubota et al. [1994] Brain Res. 649:159–173; Gonchar and Burkhalter [1997] Cereb. Cortex 7:347–358). We studied the innervation of pyramidal neurons by SOM neurons in rat and monkey visual cortex and examined putative contacts by confocal microscopy and determined synaptic connections in the electron microscope. Through the confocal microscope, SOM-positive boutons were observed to form close appositions with somata, dendrites, and spines of intracortically projecting pyramidal neurons of rat area 17 and pyramidal cells in monkey striate cortex. In addition, in rat and monkey, SOM boutons were found to be associated with axon initial segments of pyramidal neurons. SOM axon terminals that were apposed to axon initial segments of pyramidal neurons lacked PV, which was shown previously to label axo-axonic terminals provided by chandelier cells (DeFelipe et al. [1989] Proc. Natl. Acad. Sci. USA 86:2093–2097; Gonchar and Burkhalter [1999a] J. Comp. Neurol. 406:346–360). Electron microscopic examination directly demonstrated that SOM axon terminals form symmetric synapses with the initial segments of pyramidal cells in supragranular layers of rat and monkey primary visual cortex. These SOM synapses differed ultrastructurally from the more numerous unlabeled symmetric synapses found on initial segments. Postembedding immunostaining revealed that all SOM axon terminals contained GABA. Unlike PV-expressing chandelier cell axons that innervate exclusively initial segments of pyramidal cell axons, SOM-immunoreactive neurons innervate somata, dendrites, spines, and initial segments, that are one of their targets. Thus, SOM neurons may influence synaptic excitation of pyramidal neurons at the level of synaptic inputs to dendrites as well as at the initiation site of action potential output. J. Comp. Neurol. 443:1–14, 2002.

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Cortical circuits are formed by the connections of pyramidal and nonpyramidal cells (Douglas and Martin, 1991). Nonpyramidal cells differ from pyramidal cells by their distinctive dendritic trees and the scarcity of spines (Fairen et al., 1984). Most nonpyramidal cells synthesize or contain γ-aminobutyric acid (GABA) (Ribak, 1978; Somogyi and Hodgson, 1985), which they use as neurotransmitter in axon terminals that contain flattened vesicles and form symmetric synapses (Somogyi and Soltesz, 1986;
Thomson et al., 1996). Many GABAergic synapses make connections with pyramidal cells (Johnson and Burkhalter, 1996), and when activated, they suppress excitation in postsynaptic neurons (Ferster and Jagadeesh, 1992). The inhibitory effects may involve hyperpolarization and blockade of action potential firing (Miles et al., 1996) or may lead to changes in membrane conductance that reduce the amplitude of excitatory responses (Borg-Graham et al., 1998). Although, it is known for some time that interneurons are morphologically heterogeneous (Fairen et al., 1984), the identification of different types of GABAergic neurons with distinct intrinsic membrane properties (Kawaguchi, 1995), discrete molecular characteristics (DeFelipe, 1993; Weiser et al., 1995; Kawaguchi and Kubota, 1996; Cauli et al., 1997) and diverse synaptic dynamics (Thomson et al., 1996; Reyes et al., 1998; Gupta et al., 2000) suggests that different neurons may produce different postsynaptic effects.

In rat visual cortex, immunostaining for PV, SOM, and calretinin (CR) revealed three separate families of GABAergic neurons (Gonchar and Burkhalter, 1997). PV is expressed in basket cells that preferentially innervate somata and proximal dendrites and, in addition, is contained in chandelier cells that provide synaptic inputs to axon initial segments of pyramidal cells (Somogyi, 1977; Peters et al., 1982; Somogyi et al., 1982; DeFelipe et al., 1988; Kisvarday et al., 1993; Gonchar and Burkhalter, 1999a; DeFelipe, 1999). CR neurons predominantly innervate other inhibitory nonpyramidal neurons (Gulyas et al., 1996; Meskenaite, 1997; Gonchar and Burkhalter, 1999b), whereas SOM neurons mainly innervate thin dendritic shafts and spines of pyramidal cells (Hendry et al., 1984; Meinecke and Peters, 1986; DeLima and Morrison, 1989; Kawaguchi and Kubota, 1997). Although different inhibitory neurons have dissimilar preferences for innervating pyramidal and nonpyramidal cells, most known types of nonpyramidal cells form synapses on pyramidal cell somata and dendrites (Kawaguchi and Kubota, 1997). One exception is that of PV-containing chandelier cells that are thought to form exclusively connections with axon initial segments of pyramidal cells (Somogyi, 1977; DeFelipe et al., 1989; Williams et al., 1992). Based on these observations, it was proposed that all inputs to axon initial segments originate from chandelier cells (Somogyi, 1977). Several reports, however, suggest that axo-axonic synapses in rat and monkey cerebral cortex are morphologically heterogeneous and that subpopulations of axon terminals lack PV and express corticotropin releasing factor or calbindin (DeFelipe et al., 1985; Lewis and Lund, 1990; Peters and Harriman, 1990; Del Rio and DeFelipe, 1997), raising the possibility that in addition to inputs from PV neurons axon initial segments may be innervated by other cell types. Synapses at the axon initial segment, the site where action potentials are generated (Stuart and Sakmann, 1994), are in a privileged position to control the output of pyramidal cells. Because different interneurons have diverse physiological properties (Kawaguchi and Kubota, 1996), it is important to find out which type(s) of GABAergic neuron(s) contribute to the innervation of the axon initial segment.

In human temporal cortex a subset of terminals that form axo-axonic synapses contain calbindin (Del Rio and DeFelipe, 1997). Studies in rat cerebral cortex have demonstrated that calbindin is expressed in pyramidal cells and GABAergic neurons. Some of these GABAergic neurons contain SOM (Kubota et al., 1994; Gonchar and Burkhalter, 1997). Therefore, SOM neurons might be involved in the innervation of the axon initial segment of pyramidal neurons. To test this hypothesis, we labeled SOM terminals and studied the distribution of synaptic inputs to axon initial segments of pyramidal neurons by using confocal and electron microscopy. The results show that SOM neurons innervate axon initial segments, suggesting that SOM axo-axonic synapses participate in a facilitating form of local inhibition (Thomson and Deutchars, 1997; Reyes et al., 1998; Gibson et al., 1999).

### MATERIALS AND METHODS

Experiments were performed on sixteen 6- to 8-week-old Long-Evans rats (150–200 g body weight). Tissue from primate visual cortex was obtained from perfusion fixed brains of three adult monkeys (Macaca fascicularis) that were used in experiments that were unrelated to the present study. All experimental protocols were approved by the Animals Studies Committee of Washington University and conformed to NIH guidelines and regulations.

**Identification of pyramidal neurons**

**Retrograde labeling of pyramidal neurons.** Rats were anesthetized with ketamine (87 mg/kg body weight) and xylazine (14 mg/kg) and secured in a headholder. For retrograde tracing of projection neurons, glass micropipets (tip diameter, 15 μm) were lowered through a small hole in the skull 1 mm deep into the lateromedial area (LM). Biotinylated dextran amine (10%, 10,000 molecular weight, BDA [Molecular Probes, Eugene, OR] in 0.1 mM phosphate buffer, pH 7.4, 20 nl) was injected by applying puffs of pressurized air to the back of the pipet, by using a Picospritzer (General Valve, Fairfield, NJ). To maximize retrograde filling with BDA, tracer injections were preceded by an injection of 50 nl of N-methyl-D-aspartic acid (10 mM NMDA) at the same site (Jiang et al., 1993). After 48 hours, survival rats were perfused through the left ventricle with heparinized (100 units/ml) phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed from the skull and post-fixed for 2 hours at 4°C in the same fixative. Sections of the visual cortex were cut on a Vibratome at 25 μm and collected in PBS (pH 7.4). The tissue was then treated with sodium borohydride (1% in PBS, 1 hour), H₂O₂ (0.1%, 30 minutes), ethanol (50%, 30 minutes), and transferred for overnight incubation in avidin-neutralite-Texas Red (1:300; Molecular Probes). A complete series of sections was mounted on gelatinized slides, air-dried, dehydrated in ethanol, cleared in citrus based clearing agent, and cover-slipped in Krystallon (Harleco, EM Science, Gibbstown, NJ).

**Immunofluorescence staining for SMI-32.** Pyramidal cells in monkey visual cortex were visualized with an antibody against nonphosphorylated neurofilaments (Campbell and Morrison, 1989; Hof et al., 1996). For this purpose, tissue blocks from occipital cortex of monkeys perfused with PBS followed by 4% paraformaldehyde and 0.25% glutaraldehyde in PB and post-fixed for 2 hours were sectioned at 40 μm on a Vibratome. Sections were pretreated with borohydride, H₂O₂ and ethanol as described above and incubated for 36 hours in SMI-32 monoclonal antibody (1:1,000; Sternberger Monoclonals, Balti-
more, MD). The sections were then incubated for 2 hours in Cy-3-labeled goat anti-mouse secondary antibody (1:400, Chemicon, Temecula, CA).

Confocal microscopy

Inputs to axon initial segments of pyramidal cells. Sections from rat and monkey visual cortex that contained retrogradedly BDA-labeled neurons or SMI-32-immunoreactive pyramidal cells, respectively, were used for additional immunofluorescence staining with antibodies against SOM, PV, or both. For this purpose, rat sections were treated in 10% fish gelatin and later incubated for 48 hours in a mixture of rabbit anti-SOM antibody (1:500, gift from Dr. Robert Benoit, McGill University, Montreal/Canada; Morrison et al., 1983) and mouse anti-PV (1:1,000, Swant, Bellinzona/Switzerland). Rabbit IgGs were visualized with a Cy2-labeled goat anti-rabbit secondary antibody (1:400 in 2% fish gelatin; Amersham, Arlington Heights, IL), whereas mouse IgGs were stained with a Cy5-labeled donkey anti-mouse secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA).

Monkey sections were treated with 10% fish gelatin and incubated for 48 hours in a mixture of rat anti-SOM (1:200, Chemicon) and rabbit anti-PV (1:1,000, Swant). The rat IgGs were visualized with a Cy2-labeled goat anti-rabbit (1:200, Jackson) secondary antibody, whereas the rabbit IgGs were stained with a Cy5-labeled donkey anti-rabbit secondary antibody (1:200, Jackson). Sections were mounted on gelatinized slides and cover-slipped in Permount (Fisher, Fair Lawn, NJ).

Types of interneurons. To determine whether in monkey cortex SOM neurons are distinct from PV and CR containing neurons as was shown in rat visual cortex (Gonchar and Burkhalter, 1997), triple-labeling experiments were performed by using 40-μm Vibratome sections of monkey visual cortex. Sections were pretreated successively with sodium borohydride, H2O2 and ethanol as described above, immersed in 10% fish gelatin, and incubated (48 hours, 4°C) in a mixture of rat anti-SOM (1:200, Chemicon), mouse anti-PV (1:1,000, Swant, Bellinzona/Switzerland) and rabbit anti-CR (1:1,000, Swant Bellinzona/Switzerland) antibodies. Staining with secondary antibodies involved goat anti-rat Cy2 (1:200), donkey anti-mouse Cy5 (1:200) and goat-anti-rabbit Cy3 (1:200, Chemicon) to reveal SOM, PV, and CR immunofluorescence, respectively.

Analysis. A multiphoton confocal microscope (Bio-Rad MRC-1024 ES) was used to study and compare the distributions of SOM- and PV-immunoreactive axon terminals on initial axon segments of BDA or SMI-32-labeled pyramidal cells. The green (Cy2), red (Texas Red, Cy3), and infrared (Cy5) fluorochromes were excited with 488-, 568-, and 647-nm emission lines of an argon-krypton laser, respectively. Appropriate barrier filters were used to split the emitted light into three distinct channels that were detected by separate photomultipliers. Digitized images (1,024 × 1,024 pixels, 8 bits/pixel) were displayed on the screen in three separate windows and superimposed in the fourth window. Stacks of images (10 stack, optical thickness 0.5μm each) of retrogradely filled neurons were acquired with a low-magnification 10× objective to document the location of a neuron. High-magnification images (10×stack, optical thickness 0.2μm each) were acquired with a 1.4 numerical aperture 60× objective to determine whether SOM and PV are colocalized in boutons and to study the relationships of SOM and PV terminals with axon initial segments in three dimensions. Close encounters of SOM- and PV-immunoreactive boutons with axon initial segments were scored as appositions if (1) SOM- and PV-positive terminal boutons were located in the same focal plane as the target structure, and (2) three-dimensional images (Laser Sharp and Confocal Assistant software, Bio-Rad) viewed at different angles showed no gap between boutons and axon initial segments.

Electron microscopy

Preembedding staining for somatostatin. For ultrastructural analyses rats were perfused with heparinized PBS, followed by a phosphate buffered solution containing 4% paraformaldehyde, 0.5% of glutaraldehyde, and 0.1% of picric acid (pH 7.6, 4°C). The brains were removed and post-fixed for 2 hours in the same fixative at 4°C. Sections were cut at 30 μm on a Vibratome, pretreated sequentially in ice-cold 1% sodium borohydride (1 hour), 0.1% H2O2 (30 minutes) and 10% fish gelatin (30 minutes) and then incubated in rabbit anti-somatostatin antibody (gift of Dr. R. Benoit, McGill University, Montreal/Canada, 1:500, 60 hours, 4°C). Later sections were washed in 2% fish gelatin (30 minutes) and treated in biotinylated goat anti-rabbit IgG (Chemicon, 1:200) followed by avidin and biotinylated horseradish peroxidase (HRP, Vectastain ABC kit, Vector, Burlingame, CA). The HRP reaction was performed in the presence of diaminobenzidine tetrahydrochloride (0.05% DAB) and H2O2 (0.005%). The staining intensity was titrated to reveal immunolabeled structures without masking cellular organelles. Sections were fixed for 40 minutes with OsO4 (0.5% in PBS), dehydrated for 10 minutes in ethanol of increasing concentrations, infiltrated with propylene oxide, and flat-embedded in Durcupan (Fluka, Ronkonkoma, NY).

Resin-embedded sections from rat primary visual cortex that showed SOM-labeled cell bodies, dendrites, and axon terminals were further processed for ultrastructural analyses in the electron microscope. Small samples of layer 2/3 were identified using a Reichert Ultracut E ultramicrotome equipped with a diamond knife (Diatome, Fort Washington, PA). The sections were mounted on 200-mesh nickel grids, additionally stained with 1% uranyl acetate and lead citrate and studied in a JEOL 100 electron microscope. To control for artifactual staining, in selected sections, contrasting with lead citrate was omitted (Fig. 5). SOM-immunoreactive profiles were identified by the presence of electron dense, amorphous DAB reaction product. Pyramidal neurons and their initial axon segments were identified in serial sections, by using structural features described by Peters et al. (1991). Photomicrographs were taken at 5,000× and 20,000×. For prints, the negatives were enlarged 2.5×. The distances of SOM synapses on axon initial segments from the cell body and the cross-sectional areas of pyramidal cell profiles were measured on prints by using a digitizing tablet and NIH image software (http://rsb.info.nih.gov/nih-image/).

Postembedding immunostaining for GABA. Thin sections of SOM immunoperoxidase stained tissue were mounted on 300-mesh nickel grids and subsequently stained on drops of filtered solutions in a humidified chamber. Sections were rinsed in Tris-buffered saline (TBS, pH 7.6), incubated for 30 minutes in 1% a bovine serum albumin (BSA) in TBS containing 0.02% Triton X-100 (TBST), and treated for 18 hours with mouse anti-
GABA monoclonal antibody (Chemicon, 1:8,000 in TBST containing 0.1% BSA, pH 7.6). The next day, sections were thoroughly rinsed in TBST containing 1% BSA (pH 7.6), rinsed in TBST (pH 8.2), and then incubated for 2 hours in goat anti mouse-IgG conjugated to 15-nm gold particles (Amersham, 1:25 in TBST, pH 8.2). Sections were rinsed in TBS (pH 8.2), followed by rinses in TBS (pH 7.6) and in distilled water. Finally, sections were stained with 1% uranyl acetate (40 minutes) and Reinold’s lead citrate (10 minutes).

Preparation of figures

Electron photomicrographs were printed from negatives by using Kodak Polycontrast paper, and/or scanned at 1,200 dpi resolution by using a flatbed scanner (UMAX Power Look III). Adobe Photoshop 5.0 software was used to make linear adjustments of brightness and contrast in the scanned images until the electronic version most closely matched that of the photomicrograph. Final figures were printed on a Phaser 440 (Tektronix) digital printer.

RESULTS

Confocal microscopy

Confocal microscopy enabled the simultaneous observation of two to three fluorochromes representing retrogradely BDA-filled (red) or SMI-32 (red) immunolabeled pyramidal cells, immunolabeled SOM-containing (green) and PV-containing neurons (infrared, false-colored blue) (Figs. 1–3).

In the rat, BDA injections into LM retrogradely labeled projection neurons in layer two to six in ipsilateral and contralateral area 17 (Figs. 1A,B, 3A). In many cases, labeling was extensive and filled large parts of the basal and apical dendritic arbors that resembled those of typical pyramidal cells (Fig. 1A; Feldman, 1984). Well-labeled cells showed a single axon that exited at the base of the soma and descended toward white matter (Fig. 1A,B).

SMI-32 staining of monkey visual cortex revealed labeled neurons in layers 2, 3, 4B and 6 (Fig. 2A). The vast majority of labeled neurons showed pyramidal cell morphologies. The staining consisted of a mesh of neurofilaments that extended throughout somata and dendrites.
Fig. 2. Confocal images of SMI-32 stained pyramidal cells and GABAergic neurons in supragranular layers of monkey primary visual cortex. A: Low-power image of SMI-32 immunostained pyramidal neurons in layers 2, 3, and 4. B: Low-power image of the distribution of parvalbumin (PV; blue, Cy5), calretinin (CR; red, Texas Red), and of somatostatin (SOM; green, Cy2) -immunoreactive neurons in layers 1–4B of monkey primary visual cortex. Boxed area is shown at higher magnification in C. Magnification in A and B is the same. C: PV (blue), CR (red), and SOM (green) are expressed in different nonpyramidal neurons. D: SMI-32–immunoreactive pyramidal neuron in layer 3. Staining extends throughout the basal and apical dendritic arbor and includes the initial segment of the axon. Numerous SOM-immunofluorescent fibers and puncta (green) are apposed to dendrites and axon of pyramidal cells. Optical thickness, 2 μm. E: High-power image of pyramidal neuron shown in D. Clusters of SOM boutons (arrows) are apposed to the axon initial segment. Optical thickness, 2 μm. Scale bars = 100 μm in B (applies to A,B), 10 μm in C, 20 μm in D,E.
Labeling of axons was most prominent in the axon hillock and the axon initial segment (Figs. 2D,E, 3B).

SOM immunostaining in rat primary visual cortex was expressed in multipolar, bipolar, and bitufted nonpyramidal neurons and was absent from pyramidal cells. The dendrites of SOM-immunoreactive cells were often beaded and lacked spines. SOM neurons were present in layers 2–6 (Fig. 1A). Their distribution was nonuniform and consisted of small clusters of three to five neurons (Fig. 1A). Layer 1 was devoid of SOM-stained somata but contained labeled axons and dendrites.

SOM-labeled cells in monkey primary visual cortex included multipolar, bipolar, and bitufted nonpyramidal neurons with spine-free dendrites. Similar to the rat, SOM neurons in monkey visual cortex were scattered throughout layers 2–6 (Fig. 2B,C). Triple-labeling experiments with antibodies against SOM, PV, and CR revealed that SOM neurons in supragranular layers on monkey visual cortex lacked PV and CR staining (Fig. 2B,C).

The neuropil in both rat and monkey primary visual cortex contained a network of SOM-immunoreactive axons that formed terminal boutons and boutons of passage (Figs. 1B, 2D,E, 3A,B). In layers 2 and 3, axons of variable lengths criss-crossed the neuropil in all different directions. This pattern of staining differed from the pericellular baskets and vertically oriented rows of boutons seen in sections of monkey visual cortex labeled with antibodies against PV (DeFelipe et al., 1989). The analyses of the innervation of pyramidal neurons by SOM axon terminals revealed that in both species SOM-positive puncta formed close appositions with pyramidal cell somata, and primary and higher order branches of apical and basal dendrites (Figs. 1B, 2D,E, 3A,B). Most appositions were formed on shafts of basal and apical dendrites where they were often but not exclusively located at branch points (Figs. 1B, 2D,E). Thick dendrites were frequently decorated by short rows of SOM boutons (Fig. 3A). Occasionally, vertically oriented rows of SOM boutons were associated with dendrites but never with axon initial segments.

Three-dimensional reconstruction of high resolution confocal images indicated that in primary visual cortex of both rat and monkey, SOM-immunoreactive axons approached axon initial segments at oblique angles relative to the main vertical axis of the pyramidal cell axon and formed one to three contacts (Fig. 3B). On fortuitous occasions, we observed individual SOM fibers with boutons of passage that formed appositions with the axon initial segment, the somata, and/or a basal dendrite of the same pyramidal cell.
In three cases, SOM fibers were traced from the point of the axo-axonic apposition back to the nonpyramidal parent cell bodies and were unequivocally identified as axons. The dendrites of SOM neurons were thick and were easily distinguished from the much thinner, cylindrical axons, which makes it unlikely that dendrites adjacent to axon initial segments were mistaken for axo-axon appositions.

Electron microscopy

Although the analysis in the confocal microscope strongly suggested that SOM-expressing axon terminals provide axo-axonic inputs to pyramidal neurons, ultrastructural studies were performed to determine whether these inputs represent synaptic connections. In the electron microscope, pyramidal cells were easily identified by round nuclei surrounded by unfolded membranes, by the presence of thick apical dendrites, and by exclusively symmetric synapses on the somata (Fig. 4A; Peters and Jones, 1984; Peters et al., 1991). Initial axon segments of pyramidal cells were identified by their origin from the base of the soma, by the presence of dense axolemmal undocking, fasciculated microtubules, clusters of ribosomes, and cisternal organelles (Fig. 4B; Peters et al., 1991).

A total of 11 SOM-immunoreactive axon terminals were identified that formed synaptic contacts with axon initial segments of layer 2/3 pyramidal neurons of rat area 17. Three of these were found by reconstructing eight axon initial segments from serial sections. In each of these reconstructions, the axon initial segment was contacted by a single SOM axon terminal that was located 4–9 μm distal to the axon hillock (Fig. 4B). Contacts between SOM-immunoreactive dendrites and axon initial segments of pyramidal neurons were not observed.

To control for lead contrasting artifacts in SOM-immunolabeled tissue, staining with lead citrate was omitted. As expected, the contrast of the images was suboptimal. Despite this, unambiguous staining with SOM immunoperoxidase reaction product was observed in axon terminals that both contained elongated vesicles and formed symmetric synapses with axon initial segments (Fig. 5A,B). Interestingly, two of these terminals formed additional synaptic contacts, one with a dendrite and the other with a cell body (Fig. 5A,B).

SOM-immunoreactive terminals differed from unlabeled terminals that formed synaptic contacts with initial segments. Unlike unlabeled, oval-shaped terminals with smooth outlines, SOM terminals were often elongated and had highly irregular outlines (Figs. 4B, 6A). In addition, most unlabeled terminals were flattened against the initial segment with the long axis parallel to the postsynaptic axon (Fig. 4B), whereas the long axes of SOM-labeled terminal profiles formed oblique angles with axon initial segments (Figs. 4B, 6A). Similar to unlabeled terminals in contact with initial segments most SOM terminals contained one or more mitochondria. SOM-negative synapses on initial segments always outnumbered synapses formed by SOM-positive axon terminals. In addition, SOM-negative terminals in axon initial segments formed prominent clusters, whereas SOM-positive boutons tended to be more dispersed (Fig. 3A,B).

Double-labeling experiments with antibodies against SOM and GABA were performed to examine whether SOM terminals contain GABA. The experiments revealed that all (57 of 57) SOM-stained terminals studied in the double-labeled tissue were GABA-immunogold positive, including three terminals that formed synapses with axon initial segments (Fig. 6A–C).

Numbers of SOM synapses on initial segment

Reassured by the findings in the electron microscope that SOM boutons formed synaptic connections with axon initial segments, we used three-dimensional images acquired in the confocal microscope, which we inspected from different viewing angles, to examine close encounters of SOM boutons with axon initial segments. The analysis revealed that, in rat, the majority (21 of 24) of axon initial segments of forward projecting layer 2/3 pyramidal neurons in area 17 (Figs. 1B, 4A) and of most (10 of 11) callosally projecting neurons in upper layers were closely apposed by SOM-labeled boutons. The number of appositions counted as putative synapses varied from one to four per initial segment and was approximately the same for neurons with ipsilateral and contralateral projections (Table 1). Interestingly, each of the three forward projecting neurons encountered in layer 6 lacked SOM boutons associated with the axon initial segment.

Observations in monkey primary visual cortex revealed close encounters of SOM axon terminals and pyramidal neurons similar to those found in rat. In all cases, SOM-immunoreactive axons approached initial segments from the side and formed one to two appositions (Fig. 3B). To rule out that SOM boutons contain markers that are found in other types of GABAergic neurons, SMI-32 and SOM double-labeled sections were in addition stained for PV or CR. Qualitative observations in triple-labeled tissue from rat and monkey visual cortex showed that SOM boutons on initial segments of layer 2/3 pyramidal neurons did not coexpress PV (Fig. 3A,B), suggesting that SOM boutons derive from a separate population of GABAergic neurons. These results extend previous observations of cell body staining in rat visual cortex that showed that SOM, PV, and CR neurons represent distinct types of GABAergic neurons (Gonçchar and Burkhalter, 1997). Quantitative analysis of random samples from supragranular layers of monkey visual cortex, however, showed coexpression of PV and SOM in 3–9% of boutons of which most represent putative inputs to dendrites and somata and only few may correspond to inputs on axon initial segments (Table 2).

To compare the distribution of SOM inputs with axon initial segments of layer 2/3 pyramidal neurons in rat area 17 seen in confocal and in electron microscopes, the data were pooled. This procedure revealed comparable distributions of synaptic contacts and two peaks of inputs at 2–4 μm and 8–10 μm distal to the cell body (Fig. 7A). Approximately 60% of SOM contacts were located at distances >8 μm from the cell body. The distribution of SOM boutons along the axon initial segment of upper layer pyramidal neurons in monkey striate cortex resembled that in rat, except that ~76% of contacts occurred distal to 8μm from the cell body (Fig. 7A; Table 1). In both monkey and rat, PV terminals were far more numerous than SOM terminals. In both species, the distribution of PV terminals appeared broader and included more proximal and more distal parts of the initial segment than those occupied by SOM terminals (Fig. 3A,B).
Other targets of SOM axon terminals

Although the present study was focused mainly on axo-axonic SOM synapses our observations clearly showed that axon initial segments of pyramidal neurons were not the primary targets of SOM axon terminals. In fact, of a total of 163 SOM synapses only 1.2% were on axon initial segments of pyramidal neurons in layer 2/3 of rat primary visual cortex. 

Fig. 4. Electron photomicrographs of somatostatin (SOM) axon terminals forming synapses with axon initial segment of pyramidal neuron in layer 2/3 of rat primary visual cortex. A: Low-power picture of the profile of a pyramidal neuron. The nucleus (Nu) is round, without infoldings. A thick apical dendrite (AD) is oriented toward the pial surface. An axon exits at the base of the cell. The boxed area contains a portion of the axon initial segment (IS). B: Magnified image of boxed area shown in A. SOM-labeled axon terminal (SOM⁺). Immunoperoxidase reaction product is accumulated in mitochondria and synaptic vesicles lighter staining extends throughout the cytoplasm. The intensity of the immunoperoxidase reaction product was titrated for optimal visualization of organelles. SOM⁺ axon terminal is characterized by irregular outlines, contains elongated synaptic vesicles and forms symmetric synapse with the axon IS of pyramidal neuron. Axolemmal undercoating (arrows) and cisternal organelles (CO) are typical features of axon initial segments. Unlabeled (SOM⁻) axon terminal filled with elongated vesicles forms symmetric axo-axonic synapse distal to the SOM⁺ terminal. Scale bars = 1 μm in A, 0.5 μm in B.
segments. The majority of SOM synapses contacted dendritic shafts (68.2%), 25.1% were on spines (necks: 14.7%, heads: 10.4%), and 5.5% on somata (Fig. 6B,C). These targets included GABAergic and non-GABAergic structures. Among the GABA-positive targets we encountered both SOM-positive and SOM-negative somata and dendrites.

**DISCUSSION**

It is known for a long time that the axon initial segment of cortical pyramidal neurons receives synaptic input (Palay et al., 1968; Peters et al., 1968). The synapses on this stretch of axon located between the axon hillock and the beginning of the myelin sheath are symmetric and are formed by axon terminals that contain elongated vesicles, glutamic acid decarboxylase, and GABA (Somogyi, 1977; Peters et al., 1982; Somogyi et al., 1985). Recordings in hippocampus have shown that GABA release from synapses on axon initial segments leads to fast hyperpolarization of postsynaptic pyramidal neurons and that this effect depends on the activation of GABA_A receptors (Buhl et al., 1994). GABAergic synapses on the axon initial segment are, therefore, considered to be inhibitory. The unique location of these inhibitory synapses near the Na^+ channel-rich axon hillock (Wollner and Caterall, 1986) has attracted considerable attention of anatomists (Palay et al., 1968; Peters et al., 1968, 1982; Jones and Powell, 1969; Somogyi, 1977; Sloper and Powell, 1979; Kosaka, 1980; DeFelipe et al., 1989), who predicted that these synapses play an important role in the suppression of action potentials. More recently, this hypothesis received considerable support from recordings in hippocampal pyramidal neurons (Miles et al., 1996).

In rat, cat, and monkey axo-axonic synapses are formed by a distinct type of nonpyramidal cell that is dedicated to the innervation of axon initial segment (Somogyi, 1977; Peters et al., 1982; Somogyi et al., 1982). Hence, the neurons became known as axo-axonic cells (Somogyi, 1977; Somogyi et al., 1982) or chandelier cells (Peters et al., 1982; Peters, 1984; DeFelipe, 1999). Historically, however, “chandelier cell” referred to nonpyramidal neurons innervating apical dendrites of pyramidal cells (Szentagothai and Arbib, 1974). Although axo-axonic or chandelier cells are the main source of synaptic input to the initial segment of pyramidal neurons, the discovery of morphologic differences among synapses on this part of the cell raised the possibility that they originate from more than a single cell type (Peters and Fairen, 1978;
Fig. 6. Electron photomicrographs of synapses formed by γ-aminobutyric acid (GABA)ergic somatostatin (SOM) -immuno-reactive axon terminals. A: SOM immunoperoxidase-stained, GABA immunogold-labeled (black particles) axon terminal (SOM⁺) forming synapse with axon initial segment (IS). B: SOM-positive immunoperoxidase-stained, GABA-positive (black particles) axon terminal (SOM⁺) forming synapses with a spine (Sp) and a GABA-negative dendrite (D). C: Immunoperoxidase-labeled SOM-containing axon terminal (large arrow) that colocalizes GABA immunogold particles (black particles) and forms a symmetric synapse with a GABA-negative perikaryon (P). Notice, that the outlines of the SOM terminal are highly irregular. Nearby SOM-negative, GABA-positive terminal forms symmetric axo-somatic synapse (small arrow) with the perikaryon (P1). Scale bars = 0.5 μm in A,B, 1 μm in C.
Lund, 1990), use different types of GABAA receptors (Kawaguchi and Kubota, 1996; Gonchar and Burkhalter, 1994), express corticotropin releasing factor (Lewis and DeFelipe, 1994; Kawaguchi and Kubota, 1996; Gonchar and Burkhalter, 1997). In rat, calbindin is expressed by nonpyramidal somata (Hendry et al., 1984; Meinecke and Peters, 1986; DeLima and Morrison, 1989). The present studies suggest that GABA is the main transmitter used by SOM neurons, it is possible that additional substances are involved in synaptic transmission. The presence of nitric oxide synthase in a subset of SOM neurons suggests that nitric oxide may play a role (Kubota et al., 1994; Gonchar and Burkhalter, 1997). Somatostatin itself may activate postsynaptic K⁺ channels through somatostatin receptors and counteract the subthreshold depolarization of pyramidal cells mediated by excitatory synapses (Hicks et al., 1998).

Reconstruction of SOM immunolabeled axon arbors revealed that at least some axo-axonic synapses found on area 17 pyramidal neurons originate from neurons located in primary visual cortex. This finding is consistent with observations from lesioning experiments, showing that SOM axons arborize locally and make connections within the area that contains the cell body (Morrison et al., 1985). SOM neurons in both rat and monkey were shown to innervate primarily dendrites, spines, and somata of pyramidal neurons and rarely contact aspiny dendrites and nonpyramidal somata (Hendry et al., 1984; Meinecke and Peters, 1986; DeLima and Morrison, 1989). The present study confirms these observations. Similar to results in monkey cerebral cortex (DeLima and Morrison, 1989), SOM terminals in rat have a slight preference for spine necks over spine head. Unlike other GABAergic terminals, SOM axon terminals tend to form synapses near dendritic branch points. Thus, SOM terminals are well-positioned to influence subthreshold and suprathreshold activity of pyramidal neurons at different levels. The axon initial segment is the last point at which SOM neurons are able to inhibit postsynaptic pyramidal cells and conceivably control their action potential outputs.

Compared with the massive innervation of dendrites, soma, and axon initial segment by PV neurons (Kisvarday et al., 1993; Gonchar and Burkhalter, 1999a; this study), innervation of pyramidal cells by SOM neurons is much

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**TABLE 1. Number of SOM Boutons Forming Contacts with Axon Initial Segments (IS) of Pyramidal Neurons in Primary Visual Cortex of Rat and Monkey.**

<table>
<thead>
<tr>
<th>Type of pyramidal neuron</th>
<th>Total number of neurons reconstructed</th>
<th>Area of the somatic profile, μm² mean ± SD (range)</th>
<th>Total number of SOM boutons apposed to IS</th>
<th>Number of IS apposed by number of SOM bouton</th>
<th>Average number of SOM bouton per IS, mean ± SD (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Projecting to area LM (rat), layer 2/3</td>
<td>24</td>
<td>137.0 ± 34.9 (64–190)</td>
<td>36</td>
<td>3 : 0 ; 10 : 1 ; 8 : 2 ; 2 : 3 ; 1 : 4 ; 1.5 ± 1.0 (0.2)</td>
<td></td>
</tr>
<tr>
<td>Projecting to area LM (rat), layer 6</td>
<td>3</td>
<td>112.7 ± 8.0 (97–123)</td>
<td>0</td>
<td>3 : 0</td>
<td>—</td>
</tr>
<tr>
<td>Forming calisal projecting (rat), layer 2/3</td>
<td>7</td>
<td>164.0 ± 26.3 (137–203)</td>
<td>11</td>
<td>1 : 0 ; 2 : 1 ; 3 : 1 ; 1 : 3</td>
<td>1.6 ± 1.0 (0.4)</td>
</tr>
<tr>
<td>SOM-stained in layer 3 (monkey)</td>
<td>11</td>
<td>159.2 ± 34.2 (113–221)</td>
<td>21</td>
<td>3 : 0 ; 2 : 1 ; 2 : 2 ; 3 : 1 ; 4 : 1 ; 5</td>
<td>1.9 ± 1.7 (0.5)</td>
</tr>
</tbody>
</table>

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**TABLE 2. Colocalization of PV, CR and SOM Immunoreactivity in Boutons of Layers 2 and 3 of Monkey Primary Visual Cortex. The Numbers Are Derived from the Analysis of Immunofluorescence in the Confocal Microscope. The Optical Thickness of the Images Used for Quantification of Labeled Boutons was 2 μm.**

<table>
<thead>
<tr>
<th></th>
<th>PV</th>
<th>CR</th>
<th>SOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV</td>
<td>—</td>
<td>392/0 (0%)</td>
<td>526/15 (2.9%)</td>
</tr>
<tr>
<td>CR</td>
<td>344/0 (0%)</td>
<td>—</td>
<td>372/0 (0%)</td>
</tr>
<tr>
<td>SOM</td>
<td>164/15 (8.1%)</td>
<td>191/0 (0%)</td>
<td>—</td>
</tr>
</tbody>
</table>

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Somogyi et al., 1982; Peters and Harriman, 1990; DeFelipe and Farinas, 1992).

Recent studies, which increasingly relied on neurochemical attributes to distinguish different types of nonpyramidal neurons and their dendritic and axonal processes, revealed that chandelier cell axon terminals in rat, monkey, and human cerebral cortex contain PV (Hendry et al., 1989; DelFelipe, 1993; Conde et al., 1994; Del Rio and Felipe, 1994; Kawaguchi and Kubota, 1996; Gonchar and Burkhalter, 1997). In rat, calbindin is expressed by nonpyramidal somata (Hendry et al., 1984; Meinecke and Peters, 1986; DeLima and Morrison, 1989). The present studies suggest that GABA is the main transmitter used by SOM neurons, it is possible that additional substances are involved in synaptic transmission. The presence of nitric oxide synthase in a subset of SOM neurons suggests that nitric oxide may play a role (Kubota et al., 1994; Gonchar and Burkhalter, 1997). Somatostatin itself may activate postsynaptic K⁺ channels through somatostatin receptors and counteract the subthreshold depolarization of pyramidal cells mediated by excitatory synapses (Hicks et al., 1998).

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Compared with the massive innervation of dendrites, soma, and axon initial segment by PV neurons (Kisvarday et al., 1993; Gonchar and Burkhalter, 1999a; this study), innervation of pyramidal cells by SOM neurons is much...
weaker. In addition, the pronounced spike frequency adaptation during sustained activation (Kawaguchi and Kubota, 1996) may render inhibition of pyramidal cell firing by SOM neurons less effective than by fast-spiking PV neurons (Kawaguchi and Kubota, 1996; Miles et al., 1996). On the other hand, trains of pyramidal cell inputs are known to facilitate excitation of SOM neurons (Thomson et al., 1996; Reyes et al., 1998). In the course of a spike train, this synaptic enhancement may lead to progressively stronger inhibition of pyramidal cells by SOM neurons. SOM neurons presumably do not receive direct thalamocortical inputs, and most of their excitatory drive derives from axon collaterals of pyramidal cells (Beierlein et al., 2000; Gonchar and Burkhalter, unpublished observations). Therefore, it is attractive to speculate that inhibition from SOM neurons is preferentially directed against recurrent cortical excitation (Douglas et al., 1995) and that effective control of this activity relies in part on axo-axonic synapses from SOM neurons.

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