Synaptic Targets of the Intrinsic Axon Collaterals of Supragranular Pyramidal Neurons in Monkey Prefrontal Cortex

DARLENE S. MELCHITZKY, GUILLERMO GONZÁLEZ-BURGOS, GERMAN BARRIONUEVO, AND DAVID A. LEWIS

1Department of Psychiatry, University of Pittsburgh, Pittsburgh, Pennsylvania 15213
2Department of Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

ABSTRACT

The principal axons of supragranular pyramidal neurons in the cerebral cortex travel through the white matter and terminate in other cortical areas, whereas their intrinsic axon collaterals course through the gray matter and form both local and long-distance connections within a cortical region. In the monkey prefrontal cortex (PFC), horizontally oriented, intrinsic axon collaterals from supragranular pyramidal neurons form a series of stripe-like clusters of axon terminals (Levitt et al. [1993] J Comp Neurol 338:360–376; Pucak et al. [1996] J Comp Neurol 376:614–630). The present study examined the synaptic targets of the intrinsic axon collaterals arising from supragranular pyramidal neurons within the same stripe (local projections). Approximately 50% of the within-stripe axon terminals in monkey PFC area 9 targeted dendritic spines. In contrast, for both the intrinsic axon collaterals that travel between stripes (long-range projections), and the axon terminals that project to other PFC areas (associational projections), over 92% of the postsynaptic structures were dendritic spines (Melchitzky et al. [1998] J Comp Neurol 390:211–224). The other 50% of the within-stripe terminals synapsed with dendritic shafts. Dual-labeling studies confirmed that these within-stripe terminals contacted γ-aminobutyric acid-immunoreactive dendritic shafts, including the subpopulation that contains the calcium-binding protein parvalbumin. The functional significance of the differences in synaptic targets between local and long-range intrinsic axon collaterals was supported by whole-cell, patch clamp recordings in an in vitro slice preparation of monkey PFC. Specifically, the small amplitude responses observed in layer 3 pyramidal neurons during long-range, low-intensity stimulation were exclusively excitatory, whereas local stimulation also evoked di/polysynaptic inhibitory responses. These anatomic and electrophysiological findings suggest that intrinsic connections of the PFC differ from other cortical regions and that within the PFC, feedback (within-stripe) inhibition plays a greater role in regulating the activity of supragranular pyramidal neurons than does feedforward inhibition either between stripes or across regions. J. Comp. Neurol. 430: 209–221, 2001.

© 2001 Wiley-Liss, Inc.

Indexing terms: Gray’s Type I synapse; biotinylated dextran amine; electrophysiology; GABA neuron; parvalbumin

In the primate prefrontal cortex (PFC), supragranular pyramidal neurons play a central role in the flow of information both between and within cortical regions. The principal axons of these neurons travel through the gray matter, providing excitatory projections to other association areas of the cerebral cortex (Selemon and Goldman-Rakic, 1988; Barbas and Pandya, 1989; Pandya and Yeterian, 1990). Supragranular pyramidal neurons also furnish intrinsic axon collaterals that travel horizontally for long distances (up to 3–4 mm) and give rise to discrete clusters of terminal arbors in layers 1–3 (Levitt et al., 1993; Kritzer and Goldman-Rakic, 1995; Pucak et al., 1996). These clusters form a series of elongated stripes.
These findings indicate that nonpyramidal neurons in the monkey PFC receive relatively little input from either associational or between-stripe intrinsic, excitatory connections. Consequently, they raise the question of whether the within-stripe axon collaterals arising from supragranular pyramidal neurons may be an important source of excitatory synapses to γ-aminobutyric acid (GABA)-containing nonpyramidal neurons. In addition, if these within-stripe excitatory synapses are directed to certain populations of GABA neurons (i.e., those that provide inputs proximal to pyramidal cell bodies), then the resulting microcircuity could provide for a local feedback inhibition of pyramidal cell activity within a stripe in contrast to the feedforward excitation that seems to characterize the between-stripe and between-region connectivity. To test this hypothesis, we combined tract-tracing, immunocytochemistry and electron microscopy techniques in anatomic studies, and used an in vitro slice preparation of monkey PFC in electrophysiological studies, to address the following questions: (1) What are the synaptic targets of the within-stripe axon terminals arising from PFC supragranular pyramidal neurons? (2) How do the targets and size of within-stripe axon terminals compare with those of between-stripe and associational axon terminals? (3) Do the within-stripe axon terminals form synaptic contacts with dendritic shafts of nonpyramidal neurons, as revealed by GABA immunoreactivity? (4) If so, do these targets include the subpopulation of GABA neurons that express the calcium-binding protein parvalbumin (PV)? (5) Are the electrophysiological responses evoked by activation of within-stripe axon terminals consistent with the anatomically identified inputs to GABA neurons?

**MATERIALS AND METHODS**

**Surgical procedures and tissue preparation**

Male, adult cynomolgus monkeys (*Macaca fascicularis*) were used in these studies. All animals were treated ac-

---

**Fig. 1.** Schematic drawing of the dorsal view of a cynomolgus monkey (CM) brain showing the approximate locations of the single biotinylated dextran amine injection (symbols) made in each of the five animals and of the tissue blocks (thin dashed lines) taken from other animals for in vitro slice studies. The open symbols denote the animals that received pressure injections and that were perfused with 4% paraformaldehyde. The closed symbols denote the animals that received iontophoretic injections and that were perfused with 4% paraformaldehyde and 0.2% glutaraldehyde. AS, arcuate sulcus, PS, principal sulcus.
The remaining three animals (see Fig. 1) were perfused transcardially with room temperature (29°C) 1% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M PB, pH 7.4, for 5 seconds followed by 4% paraformaldehyde and 0.2% glutaraldehyde (Sesack et al., 1995) in 0.1 M PB, pH 7.4, for 9 minutes at a flow rate of 350–400 ml/min. The brains were then removed, and coronal blocks (5–6 mm thick) containing the injection sites were immersed in cold 4% paraformaldehyde for 2 hours. For all animals, after post-fixation, tissue blocks were washed in 0.1 M PB, pH 7.4, and sectioned coronally on a Vibratome at 50 μm.

**Histochemistry and immunocytochemistry**

For two of the animals (CM175 and CM176), single-label experiments were performed to visualize the BDA as previously described (Melchitzky et al., 1998). For the other three animals (CM214, CM222, and CM223), double-labeling experiments, using a previously described preembedding approach (Sesack et al., 1995, 1998), were performed to visualize the BDA and to immunocytochemically label GABA- or parvalbumin- (PV) containing structures. Tissue sections were treated with 1% sodium borohydride in 0.1 M PB for 30 minutes followed by several washes in 0.1 M PB. Sections were then incubated for 30 minutes in a blocking solution containing 0.2% BSA, 0.04% Triton X-100, 3% normal goat serum, and 5% NHuS in 0.01 M phosphate buffered saline (PBS), pH 7.4. Next, sections were incubated overnight in blocking solution containing Vectastain ABC reagents (Vector Laboratories, Burlingame, CA) and either a polyclonal rabbit anti-GABA antibody (diluted 1:750, DiaSorin, Inc., Stillwater, MN) or a rabbit polyclonal anti-PV antibody (diluted 1:1,000, kindly provided by Dr. Kenneth Baimbridge, University of British Columbia). The following day, sections were rinsed in 0.1 M PB, incubated in 0.1 M PB containing 0.5 mg/ml DAB and 0.006% H2O2 for 5 minutes, and then washed in 0.01 M PBS. Sections were then incubated in 1-nm gold-conjugated goat anti-rabbit IgG (1:50, Amer sham, Arlington Heights, IL) for 3 hours. Bound gold particles were enhanced by treatment with silver solution (American Optics, Stillwater, CA) and then washed in 0.01 M PBS. Fields containing both BDA and either GABA or PV labeling, as well as those containing only BDA labeling, were examined. For all animals, some sections were mounted on gel-coated slides for light microscopy. All other sections were post-fixed in 2% osmium tetroxide for 1 hour, dehydrated in ascending alcohols and embedded in Epon 812 (Sesack et al., 1995; Melchitzky et al., 1998).

**Sampling regions and procedures**

To identify the targets of within-stripe (local), axon collaterals of supragranular pyramidal neurons, areas approximately 300 μm from the core of the BDA injection sites, and within the zone of labeled axon terminals surrounding the injection site, were sampled (Fig. 2). From each animal, 1–2 trapezoid blocks were sectioned on a Leica ultramicrotome at 80 nm and 3–6 ultrathin sections per grid were collected on individual 200-mesh copper grids. The grids were subsequently counterstained with uranyl acetate and lead citrate and were examined on a JEOL 100 CX electron microscope. For each block, 2–3 grids (separated by at least 10 and at most 20 grids) were analyzed. One section per grid was randomly chosen as the starting point for analysis. Within this section, all BDA-labeled axon terminals were identified and photographed at ×19,000. A total of 290,950 μm² of tissue was sampled for within-stripe terminals. All profiles of BDA-labeled axon terminals were followed in limited (≤4) serial sections. For those terminals that had an identifiable synaptic specialization in any of the serial sections, the postsynaptic target was classified. Those terminals without a definite synaptic specialization were considered to be appositions and were excluded from further analyses. The between-stripe and associational terminals were sampled from deep layer 3 from the center of a stripe, as previously described (Melchitzky et al., 1998). The cross-sectional area of the tissue sampled for the between-stripe and associational terminals was 178,475 μm² and 238,975 μm², respectively. For the three types of BDA-labeled terminals (within-stripe, between-stripe, and associational), the proportions targeting different classes of postsynaptic structures were compared with chi-square analysis. For the between-stripe and associational projection, these data have been previously reported (Melchitzky et al., 1998).

The cross-sectional area of all BDA-labeled axon terminals was measured on negatives from the section with the most obvious synaptic specialization. A computer digitizing system (MCID M2, Research Imaging, Inc., St. Cathe rines, Ontario, Canada) was used to trace the contour of labeled terminals as previously described (Melchitzky et al., 1999). Differences in the mean cross-sectional area of the within- and between-stripe and associational axon terminals were compared by using a one-way analysis of variance (ANOVA) with terminal type as the main effect.

For dual-labeled tissue, BDA-labeled axon terminals were considered to be within a field containing specific GABA or PV labeling if both labeled elements were encompassed by an electron photomicrograph at 10,000× magnification (66.5 μm²). To avoid false-negative results in the dual-labeled tissue, we sampled only from areas of the trapezoid blocks that contained the tissue-Epon inter face and we only analyzed fields that contained both specific peroxidase and gold-silver immunolabeling (Sesack et al., 1995, 1998). The amount of dual-labeled tissue sampled was 274,419 μm² for GABA-containing fields and 254,581 μm² for PV-containing fields.

**Definition of neuronal and synaptic elements**

Neuronal elements of relevance to this study were identified using the criteria of Peters et al. (1991). Axon terminals were generally greater than 0.2 μm in diameter and contained synaptic vesicles and often mitochondria. Dendritic shafts were characterized by the presence of mitochondria, numerous microtubules, and neurofilaments, as well as synaptic specializations. Dendritic spines were identified by the absence of both organelles and microtubules and by the presence of a spine apparatus (in optimal planes of section). Gray’s Type I synapses (Gray, 1959) were characterized by widened and parallel spacing of apposed plasmalemmal surfaces, and a thick postsynaptic density. Furthermore, the axon terminals forming these synapses contained round synaptic vesicles. In contrast, Gray’s Type II (Gray, 1959) synapses had thin postsynaptic densities, intercell filaments, and synaptic vesicles that were usually pleomorphic in shape.
Fig. 2. A: Schematic diagram illustrating a biotinylated dextran amine (BDA) injection site and the spatial arrangement of the stripe-like clusters of axons and terminals that arise from it. Surrounding the dense core (black circle) of the injection site is an elongated area (shaded area) of labeled axons and terminals. The flanking shaded zones represent stripes of axon terminals furnished by either horizontally oriented intrinsic axon collaterals, or the principal axon, arising from pyramidal neurons in the vicinity of the injection site. The horizontal line denotes the rostral to caudal level of the coronal section depicted in B. B: Schematic diagram illustrating the three types of axon terminals furnished by supragranular pyramidal neurons, and how they were sampled for electron microscopic analysis. BDA-labeled axons of pyramidal neurons terminate nearby (within-stripe) or after traveling horizontally through the gray matter (between-stripe) or through the white matter (associational) to form stripes of labeled terminals. The within-stripe terminals were sampled in layer 3 from areas approximately 300 μm from the injection site. Note that terminals within this area may arise from either pyramidal or nonpyramidal neurons and, thus, may form either Gray's Type I or II synapses. The between-stripe and associational terminals were sampled in middle to deep layer 3 from the center of a stripe.
In vitro electrophysiological recordings

Slice preparation. PFC tissue slices, prepared as part of another study, were obtained from young adult, male cynomolgus monkeys (González-Burgos et al., 2000). Briefly, small blocks of tissue containing portions of dorsal area 9 and, in some cases, the medial bank of the principal sulcus (area 46; see Fig. 1) were obtained after a craniotomy was performed over the dorsal PFC in deeply anesthetized animals. The block of tissue was immediately placed in a cold solution (in mM: NaCl 125, KCl 2, NaHPO4 1.2, NaHCO3 33, MgCl2 6, CaCl2 0.5, glucose 10, kynurenic acid 2, pH 7.3–7.4) bubbled with 95%/5% O2/CO2. Whole-cell voltage clamp recordings were obtained from layer 3 pyramidal neurons that were sated and had values between 8 and 25 MΩ resistance. Access resistance was not compensated (González-Burgos et al., 2000). Electrodes (3–7 MΩ resistance) were filled with the following internal solution (in mM): CsF 120; CsCl 10; HEPES 10, EGTA 5; pH 7.3–7.4 when bubbled at room temperature with 95%/5% O2/CO2. Whole-cell voltage clamp recordings were obtained from layer 3 pyramidal neurons that were visually identified through differential interference contrast and infrared video microscopy as previously described (González-Burgos et al., 2000). Electrodes (3–7 MΩ resistance) were filled with the following internal solution (in mM): CsF 120; CsCl 10; HEPES 10, EGTA 5; pH 7.2-7.3. Recordings were made with an Axopatch 1C amplifier (Axon Instruments, Foster City, CA) operating in voltage-clamp mode. Access resistance was not compensated and had values between 8 and 25 MΩ. Data were filtered at 5 kHz, digitized at 10 kHz, and stored on disk for off-line analysis. Acquisition and analysis were performed by using LabView (National Instruments, Austin, TX).

Stimulation was applied by means of a multielectrode stimulation array as described previously (González-Burgos et al., 2000). The arrays consisted of 24–30 nichrome wires glued together at inter-electrode distances of 60–90 μm. The stimulation was applied with an array aligned parallel to the slice pial surface in superficial layer 3. Bipolar square current pulses (20–200 μs, 20–200 μA) were applied by using a constant current stimulus isolation unit attached to adjacent wires, with the cathode always closer to the cell. Single stimulation shocks were delivered every 10 seconds.

RESULTS

Regional and laminar location of injection sites

Each of the five BDA injections were located in layers 2–3 of Walker’s (1940) area 9 (see Fig. 1). Similar to previous studies (Pucak et al., 1996; Melchitzky et al., 1998), these injection sites consisted of a dense core of dark reaction product, which was considered to be the area of effective uptake. A zone containing labeled terminals and neurons immediately surrounded this core, with the distribution of labeled axon terminals extending in an elongated, stripe-like manner under the cortical surface (Fig. 2). In this zone, tissue blocks were taken in layer 3 for analysis of within-stripe axon terminals (Figs. 2B, 3). These injections also labeled horizontally oriented, intrinsic axon collaterals, which produced distinct stripe-like clusters of branching, varicose axons confined to layers 1–3, and the injections also resulted in similar stripe-like clusters of labeled axons and terminals that spanned all six cortical layers in other PFC regions (Levitt et al., 1993; Pucak et al., 1996). In this study, the axon terminals in these clusters are referred to as between-stripe and associational terminals, respectively (Fig. 2B).

Fig. 3. Darkfield photomicrograph of a stripe-like cluster (viewed in cross-section) of labeled axons extending from a biotinylated dextran amine injection in the supragranular layers of monkey prefrontal cortex. Note that the stripes formed by the intrinsic axon collaterals of pyramidal neurons are restricted to layers 1–3. The asterisk indicates the approximate center of the zone where within-stripe axon terminals were sampled in middle to deep layer 3. Scale bar = 100 μm.

Targets of BDA-labeled, within-stripe axon terminals

At the ultrastructural level, BDA label surrounding the injection sites was present in axon terminals, preterminal axons, myelinated axons, and occasionally, dendritic spines and shafts. The majority of the within-stripe BDA-labeled axon terminals contained round synaptic vesicles and formed Gray’s Type I synapses. Some BDA-labeled axon terminals formed Gray’s Type II synapses (Fig. 4A). These terminals likely represent those of local circuit, GABA neurons and, thus, were excluded from subsequent analyses. In each of the five animals examined, approximately half (47–54%) of the within-stripe terminals forming Type I synapses contacted dendritic spines (Fig. 4B), and the remaining terminals contacted dendritic shafts (Fig. 4C; Table 1). In addition, the proportions of synapses targeting spines did not differ ($\chi^2 = 0.014, P = 0.91$) between tissue prepared for single-label (22 of 43) or dual-
In contrast to the finding that within-stripe terminals target dendritic spines and dendritic shafts in the same proportions, the between-stripe and associational axon terminals arising from these injections were predominantly directed to dendritic spines. Specifically, 96% of the between-stripe and 92% of the associational terminals contacted dendritic spines (Fig. 5). These proportions of synaptic targets differed significantly ($\chi^2 = 85.3, P < 0.0001$) from that of the within-stripe axon terminals. The mean cross-sectional area of all axon terminals forming Gray’s Type I synapses, or of those terminals that targeted dendritic spines, did not significantly differ among the within-stripe, between-stripe, and associational terminals (Table 2).

### Within-stripe axon terminals contact GABA- and PV-labeled dendritic shafts

Some of the dendritic shafts receiving synapses from within-stripe terminals had the distinctive morphologic characteristics of local circuit, GABA neurons (McGuire et al., 1991; Smiley and Goldman-Rakic, 1993; Sesack et al., 1995). Specifically, these dendritic shafts had a varicose morphology and received a high density of asymmetric synapses (Fig. 4C). However, the majority of the dendritic shafts contacted were cut in cross-section; thus, it was not possible to determine their source (pyramidal vs. local circuit neuron). However, in tissue containing both peroxidase, BDA label and immunogold, GABA label, GABA-labeled dendritic shafts (Fig. 6) were a common target of BDA-labeled, within-stripe axon terminals. Specifically, 56% (9 of 16) of BDA-labeled within-stripe terminals formed synapses onto GABA-labeled dendritic shafts, whereas 13% synapsed with unlabeled dendritic shafts and the remaining 31% targeted unlabeled dendritic spines.

---

**TABLE 1. Synaptic Targets of Within-Stripe Axon Terminals**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Number of BDA-labeled terminals</th>
<th>Synaptic target</th>
<th>Dendritic shaft</th>
<th>Dendritic spine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>CM175</td>
<td>13</td>
<td></td>
<td>7</td>
<td>54</td>
</tr>
<tr>
<td>CM176</td>
<td>30</td>
<td></td>
<td>14</td>
<td>47</td>
</tr>
<tr>
<td>CM214</td>
<td>27</td>
<td></td>
<td>13</td>
<td>48</td>
</tr>
<tr>
<td>CM222</td>
<td>17</td>
<td></td>
<td>9</td>
<td>53</td>
</tr>
<tr>
<td>CM223</td>
<td>24</td>
<td></td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
<td></td>
<td>55</td>
<td>49.5</td>
</tr>
</tbody>
</table>

1BDA, biotinylated dextran amine.
Dendritic spine 0.41 0.39 0.41 0.39
Dendritic shaft 0.14 0.15 0.15 0.15

Fig. 5. Relative proportions of dendritic shafts and spines as targets of BDA-labeled axon terminals arising from within-stripe, between-stripe, and associational axon terminals forming Gray's Type I synapses in monkey PFC. Note that the targets of the within-stripe terminals differ significantly ($\chi^2 = 85.3; P < 0.0001$) from those of the between-stripe and associational terminals.

TABLE 2. Cross-Sectional Area of Within-Stripe, Between-Stripe, and Associational Axon Terminals

<table>
<thead>
<tr>
<th>Synaptic target</th>
<th>Within-stripe</th>
<th>Between-stripe</th>
<th>Associational</th>
<th>F, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All targets</td>
<td>0.38 ± 0.14</td>
<td>0.39 ± 0.14</td>
<td>0.39 ± 0.14</td>
<td>F&lt;sub&gt;2,92&lt;/sub&gt; = 0.11; 0.89</td>
</tr>
<tr>
<td>Dendritic spine</td>
<td>0.41 ± 0.15</td>
<td>0.39 ± 0.14</td>
<td>0.39 ± 0.14</td>
<td>F&lt;sub&gt;2,146&lt;/sub&gt; = 0.17; 0.85</td>
</tr>
</tbody>
</table>

Similarly, BDA-labeled, within-stripe axon terminals targeted the subpopulation of GABA neurons that express the calcium-binding protein, PV (Fig. 7). Within fields that contained both PV and BDA labeling, 53% (8 of 15) of within-stripe axon terminals formed synapses onto PV-labeled dendritic shafts. Unlabeled dendritic shafts received 7% of the within-stripe input and the remaining 40% of within-stripe terminals synapsed onto dendritic spines. The relative proportions of within-stripe terminals that synapsed onto GABA-labeled or PV-labeled dendritic shafts vs. other targets were strikingly similar ($\chi^2 = 0.03, P = 0.87$).

Electrophysiological observations

These anatomic data predict that long-range (between-stripe) horizontal excitatory connections would have primarily a direct excitatory effect on supragranular pyramidal neurons, whereas activation of local (within-stripe) excitatory connections might also elicit feedforward disinhibition. As an initial test of this hypothesis, we obtained whole-cell patch clamp recordings from single layer 3 pyramidal neurons visually identified in an in vitro slice preparation of monkey PFC. Postsynaptic currents (PSC) were evoked by constant, low stimulation intensity applied to both short- (300 µm) and long-distance (900 µm) stimulation sites, by using a multielectrode stimulation array placed in layer 3, lateral to the recorded neuron (González-Burgos et al., 2000). Only PSCs with a latency that was constant from trial to trial and followed a reduction with distance consistent with monosynaptic events were studied (González-Burgos et al., 2000). Stimulation parameters were found at which short- and long-distance stimulation evoked inward PSCs of similar peak amplitudes when recorded at -70 mV (Fig. 8). Subsequently, the soma was voltage-clamped at potentials positive to the reversal potential for the GABA<sub>A</sub>-mediated inhibitory postsynaptic currents (IPSCs) but negative or near to the reversal for the glutamate-mediated excitatory postsynaptic currents (EPSCs). In cortical pyramidal neurons, the GABA<sub>A</sub>-activated IPSCs are mainly chloride currents (McCormick, 1989), whereas glutamate-activated EPSCs are mixed (Na<sup>+</sup> and K<sup>+</sup>) cationic currents (Hestrin et al., 1990). In our experimental conditions (see Materials and Methods section), the IPSCs and EPSCs should reverse from inward to outward at potentials positive to -65 mV and 0 mV, respectively. As illustrated in Figure 8A, recording at -40 and 0 mV revealed that, in the responses to short-distance stimulation, an early EPSC was followed by a noticeable IPSC that peaked later, approximately 20–30 msec after the EPSC. In contrast, when stimulation was applied at a long-distance from the same neuron, outward currents (i.e., as those expected from a reversed IPSC) were not observed at 0 mV holding potential (Fig. 8B). Similar results were observed in 3 of 3 pyramidal neurons recorded in layer 3. Previously, we showed that the outward currents evoked by short distance stimulation were abolished by intracellular application of the GABA<sub>A</sub>-receptor chloride channel blocker 4,4′-disothiocyanostilbene-2,2′-disulfonic acid (DIDS) (González-Burgos et al., 2000), thus confirming that they are GABA<sub>A</sub>-activated IPSCs.

DISCUSSION

The functional consequences of the output from supragranular pyramidal neurons in monkey PFC depends, in part, on the synaptic targets of each type of axon projection furnished by these cells. The present study demonstrates that local, within-stripe axon collaterals from these neurons contact dendritic spines and dendritic shafts with equal frequency, that most of these dendritic shafts belong to GABA-containing, local circuit neurons, and that the latter includes the neuronal subclasses that express PV. In contrast, both the long-range, between-stripe, intrinsic projections and the associational projections of supragranular pyramidal neurons almost exclusively target the dendritic spines of other pyramidal cells. Furthermore, these differences in the synaptic targets of the within- and between-stripe intrinsic axon collaterals of supragranular pyramidal neurons are supported by electrophysiological findings.

Differential targeting of axon collaterals arising from supragranular pyramidal neurons

The differences in the proportions of dendritic spines and shafts contacted by within-stripe, as opposed to between-stripe and associational, terminals suggest that different types of axonal projections from the same pyramidal neurons have distinct postsynaptic targets. Furthermore, the relative proportions of axospinous and axo-
dendritic synapses for the within-stripe, between-stripe, and associational terminals were all significantly different ($\chi^2 = 21.3, P < 0.001$; $\chi^2 = 24.5, P < 0.0001$; and $\chi^2 = 9.2, P < 0.003$, respectively) from the general PFC neuropil where 75% of all terminals forming Gray's Type I synapses contact dendritic spines (Melchitzky et al., 1998). These comparisons provide further evidence for the target specificity of each of these three types of axonal projections. However, additional studies are needed to determine the target specificity of axon terminals that are within the same stripe but at distances greater than 300 $\mu$m from the injection site.

It is possible that the differences in synaptic targets across within-stripe, between-stripe, and associational projections could reflect their origin from different populations of supragranular pyramidal neurons. However, several lines of evidence suggest that they arise from the same population of pyramidal neurons. First, intracellular fills of pyramidal neurons have generally revealed labeling of both local and long-range intrinsic axon collaterals, and of principal axons projecting into the white matter (Kisvárday et al., 1986; McGuire et al., 1991), supporting the assertion that the three types of axonal projections examined in the present study originate from the same neurons. In particular, individual supragranular pyramidal neurons in monkey PFC have been shown to give rise to these three types of axonal projections (González-Burgos, unpublished observations; González-Burgos et al.,

Fig. 6. A–C: Electron photomicrographs of biotinylated dextran amine (BDA) -labeled, within-stripe axon terminals in layer 3 of monkey prefrontal cortex (PFC) forming Gray's Type I synapses (curved arrows) onto GABA-labeled dendritic shafts ($G_d$). Note that B and C are from adjacent sections. Scale bar = 0.40 $\mu$m for A–C.
Second, by using dual-label, retrograde transport techniques, we previously demonstrated that supragranular PFC pyramidal neurons furnishing associational projections also provide intrinsic axon collaterals that form stripes (Melchitzky et al., 1998). Third, we found that associational, between-stripe and within-stripe terminals were virtually identical in size (see Table 2). In contrast, axon terminals arising from different populations of neu-

Fig. 7. Electron photomicrographs of biotinylated dextran amine (BDA) -labeled, within-stripe axon terminals in layer 3 of monkey prefrontal cortex (PFC) forming Gray's Type I synapses (curved arrows) onto parvalbumin (PV) -labeled dendritic shafts (PVd). A: The PV-immunoreactive (IR) dendritic shaft is heavily labeled with immunogold particles. B–D: In contrast, the PV-IR dendritic shaft shown serially contains fewer immunogold particles but they are present in multiple sections. The varicose shape and presence of another synaptic contact (arrowhead) from an unlabeled axon terminal provide additional morphologic evidence that this PV-IR shaft is from a local circuit neuron. However, the apparent absence of PV labeling in D illustrates the potential for underestimating the contacts onto labeled shafts if serial section analysis is not used. Scale bar = 0.40 μm for A–D.

Fig. 8. A,B: Postsynaptic currents (PSCs) evoked by short- and long-distance stimulation recorded in layer 3 pyramidal neurons in a monkey prefrontal cortex (PFC) brain slice preparation. During whole-cell patch clamp recording at a holding potential of −70 mV, stimulation parameters were found at which either short- (~300 μm; A) or long-distance (~900 μm; B), stimulation evoked inward PSCs of similar peak amplitude. Identical stimulation parameters were used at each stimulation site. The left side of A and B show example traces of the recordings made at different holding potentials. Down- and up-going deflections represent inward and outward currents, respectively. Note that outward currents are observed at −40 mV and 0 mV, only in the short-distance response (A). On the right side of A and B, the currents measured at the times corresponding to the peak inward currents (open circles) and peak outward currents (filled circles) observed in the short-distance PSC, were plotted vs. holding potential. Only the current-voltage plot in A exhibits currents reversal from inward to outward at the more positive potentials, consistent with the presence and absence of an inhibitory PSC in the short- and long-distance responses, respectively.

2000). Second, by using dual-label, retrograde transport techniques, we previously demonstrated that supragranular PFC pyramidal neurons furnishing associational projections also provide intrinsic axon collaterals that form stripes (Melchitzky et al., 1998). Third, we found that associational, between-stripe and within-stripe terminals were virtually identical in size (see Table 2). In contrast, axon terminals arising from different populations of neu-
rons in the same cortical regions may differ in size. For example, in layer 4 of cat visual cortex, synaptic boutons arising from layer 6 pyramidal neurons were significantly smaller than those originating from layer 4 spiny stellate cells (Ahmed et al., 1997). Furthermore, corticothalamic axon terminals originating from layer 5 pyramidal cells in cat primary auditory or visual cortices were larger than those arising from layer 6 neurons in these same cortical areas (Ojima, 1994; Ojima et al., 1996). Thus, the similarities in mean cross-sectional areas of the within-stripe, between-stripe, and associational terminals are consistent with the idea that they all arise from the same population of supragranular pyramidal neurons.

The target specificity of these different types of axon terminals, especially for the within- and between-stripe terminals, may represent a unique feature of the monkey PFC. For example, in cat and monkey striate visual cortex, both the local and long-range intrinsic axon collaterals of intracellularly filled, layer 3 pyramidal neurons preferentially target dendritic spines (Kisvárday et al., 1986; McGuire et al., 1991). Interestingly, the proportions of both local and long-range terminals in the monkey visual cortex that target spines (72% and 79%, respectively) tend to be intermediate to those of the within- and between-stripe (50% and 96%, respectively) axon terminals in the monkey PFC. In contrast, the local axon collaterals of supragranular pyramidal neurons in cat motor cortex target dendritic spines with a greater frequency (68%) than do the long-range collaterals (58%) (Keller and Asanuma, 1993). Although differences in species and methodologic approaches must be kept in mind, together these observations suggest that the intrinsic axon collaterals of supragranular pyramidal neurons have regional differences in the target specificity of their local and long-distance branches that may reflect the unique information processing demands of each region.

**Functional significance of within-stripe inputs to GABA neurons**

Our results also indicate that the dendritic shafts of GABA-containing neurons are a major target of the within-stripe, local axon collaterals of PFC supragranular pyramidal neurons. However, currently available immunocytochemical methods do not permit a determination of the absolute frequency of these synaptic connections. In addition, some of the methodologic parameters used in the present study may have contributed to an underestimation of such inputs. For example, the fixation required for adequate ultrastructural preservation most likely resulted in reduced GABA immunostaining and, thus, fewer GABA-labeled structures. In addition, the immunogold method is less sensitive than immunoperoxidase staining (Chan et al., 1990), although the preembedding immunogold technique used in this study provides greater sensitivity than postembedding immunogold methods (Chan et al., 1990; Pickel et al., 1993). Thus, although the relative incidence of BDA-labeled synapses onto GABA-labeled dendritic shafts was probably underestimated, the most sensitive combination of available immunolabels was used. On the other hand, our sampling method, which required the presence of both BDA and GABA labeling in the same field, was designed to reduce false negatives. Although this approach could have contributed to an overestimation of the proportion of synapses onto GABA-labeled dendritic shafts, the incidence of BDA-labeled synapses with GABA-labeled dendritic shafts (56%) was very similar to that of BDA-labeled synapses with dendritic shafts (50%) in the single label study. This sampling method may have also contributed to the apparent lower incidence of within-stripe terminals forming synapses with dendritic spines in the dual-label than in the single-label tissue. However, when all within-stripe synaptic contacts examined (n = 142) were considered together, 53% of BDA-labeled axon terminals contacted dendritic shafts and 47% contacted dendritic spines. Thus, both the single- and dual-label approaches are consistent in indicating that the dendritic shafts of local circuit neurons are a major synaptic target of within-stripe axon terminals.

Our preliminary electrophysiological findings also indicate that the activation of within-stripe excitatory pathways is much more likely to elicit inhibition than is activation of between-stripe inputs. The delayed nature of the IPSCs in layer 3 pyramidal neurons resulting from local stimulation, relative to that of the EPSCs in these cells, suggests that the IPSCs were evoked at least disynaptically. Disynaptic IPSCs were observed even when using very low stimulation intensities that produced small amplitude responses, which were unlikely to elicit the pyramidal neuron firing required to evoke feedback inhibition. Therefore, the results suggest that the recorded disynaptic IPSCs were evoked by means of a feedforward pathway, implicating the firing of an intercalated GABAergic interneuron.

The functional significance of these within-stripe excitatory inputs to GABA neurons depends, at least in part, on the subclass(es) of GABA cells that are targeted. In the primate PFC, different subclasses of GABA cells can be distinguished by their axonal and dendritic morphologies, laminar locations, and the expression of different calcium binding proteins, such as PV (Lund and Lewis, 1993; Gabbott and Bacon, 1996; DeFelipe, 1997). The similar frequency with which within-stripe terminals target PV-immunoreactive (-IR) dendritic shafts (53%) and GABA-IR dendritic shafts (56%) suggests that PV-containing local circuit neurons are the principal, and possibly the sole, subclass of GABA neurons to receive this input, at least in layer 3 of the PFC. Interestingly, this apparent target specificity of the within-stripe axon terminals for pyramidal neurons and PV-containing interneurons is consistent with the recent report that pyramidal neurons and fast-spiking interneurons (which contain PV [Kawaguchi and Kubota, 1993]) in layers 2–3 of rat visual cortex share the same source of intrinsic excitatory input, whereas other electrophysiologically defined classes of interneurons receive excitatory synapses from other sources (Dantzker and Callaway, 2000). In the monkey PFC, approximately 25% of GABA neurons are PV-containing (Condé et al., 1994; Gabbott and Bacon, 1996), and these neurons include the wide-arbor and chandelier cell subtypes. Interestingly, these two cell subtypes provide the most proximal inhibitory input to pyramidal neurons. For example, wide-arbor neurons, large basket-like cells most commonly found in layers 3 and 5 of monkey PFC (Lund and Lewis, 1993), form Gray’s Type II synapses onto the cell bodies of pyramidal neurons, as well as proximal dendritic shafts and spines (Freund et al., 1983; Williams et al., 1992), whereas the axon terminals of chandelier cells synapse exclusively onto the axon initial segments of pyramidal cells (Freund et al., 1983; DeFelipe et al., 1985). Thus, our findings suggest that the output of within-stripe pro-
jections from supragranular pyramidal neurons may be directed to the subclasses of inhibitory neurons that exert substantial control over pyramidal cell output (Fig. 9). The sustained firing of PFC neurons during the delay period of delayed response tasks (Funahashi et al., 1989, 1990) is thought to represent the neural substrate for the temporary maintenance of information in working memory (Goldman-Rakic, 1995; Fuster, 1997). This sustained neuronal activity has been suggested to be subserved, at least in part, by reverberating excitatory circuits (Funahashi and Kubota, 1994; Fuster, 1997; Tanaka, 1999), such as those formed by the intrinsic connections among layer 3 pyramidal neurons (Lewis and Anderson, 1995; Pucak et al., 1996). Thus, the connections between- and within-stripes have been proposed to link PFC pyramidal cells that share common memory fields (Goldman-Rakic, 1995, 1996). Recent studies indicate that neighboring regular-spiking and fast-spiking units, which seem to represent pyramidal and PV-containing GABA neurons, respectively, show very similar response properties (e.g., isodirectional tuning) during the delay phase of visual spatial working memory tasks (Rao et al., 1999). In this context, the within-stripe excitation of PV-containing neurons is likely to have a potent inhibitory effect on PFC pyramidal neurons whose activity mediates working memory processes. However, the impact of excitatory inputs to wide-arbor vs. chandelier neurons will differ in terms of the recruited synaptic inhibition. For example, the axonal arbors of wide-arbor neurons typically extend 900–1,000 μm in diameter (Lund and Lewis, 1993), making it possible for these cells to provide inhibitory synaptic input to pyramidal cells located in the zones or gaps between an interconnected group of stripes (Pucak et al., 1996). This architecture suggests that the within-stripe excitatory input to wide-arbor neurons would inhibit the activity of pyramidal neurons in the adjacent gap region (Fig. 9). On the other hand, the spread (250–300 μm) of the axonal arbor of chandelier cells (Lund and Lewis, 1993) is similar to the average width of a stripe (Pucak et al., 1996), suggesting that within-stripe excitatory input to chandelier cells may be specialized to regulate the output of pyramidal neurons within the same stripe. Other interneurons, such as the calretinin- or calbindin-containing double bouquet cells, also have axonal arbors that are similar to or smaller than the width of individual stripes (Lund and Lewis, 1993). However, the results of the present study suggest that within-stripe excitatory input to these cells is minor, although direct investigations of these classes of interneurons is required.

Fig. 9. Schematic diagram illustrating the connections formed by the axon terminals from within-stripe, between-stripe, and associational projections of supragranular pyramidal neurons in monkey prefrontal cortex (PFC). Although each of these types of projections originate from supragranular pyramidal cells, it is not known whether individual cells contribute axon collaterals to each of these pathways (dashed lines). Pyramidal neurons in the supragranular layers are clustered into stripe-like arrays that are reciprocally connected by means of axon collaterals. The associational and between-stripe terminals predominantly target dendritic spines of other pyramidal neurons, whereas the remaining terminals target dendritic shafts, at least some of which have the morphologic characteristics of γ-aminobutyric acid (GABA; G) neurons. Our previous studies have shown that there are reciprocal connections between stripes (Pucak et al., 1996; Melchitzky et al., 1998), but the proportions of synaptic targets could not be determined due to technical limitations (Melchitzky et al., 1998). In contrast, the within-stripe axon terminals contact dendritic spines and dendritic shafts with equal frequency. The majority of dendritic shafts contacted by within-stripe axon terminals belong to GABA-containing, local circuit neurons. Furthermore, the parvalbumin-containing chandelier cell (CH) and wide-arbor (WA) subclasses of local circuit neurons appear to be preferentially contacted.
Interestingly, the efficacy of excitatory synaptic connections made by pyramidal neurons onto cortical GABA neurons has been shown to exhibit frequency-dependent, short-term depression or facilitation in a target-specific manner (Thomson, 1997; Ali et al., 1998; Markham et al., 1998; Reyes et al., 1998). These data, and the results of the present study, suggest that a differential short-term plasticity in the efficacy of excitatory transmission onto the chandelier and wide-axor classes of PV-positive GABA neurons could be important in shaping sustained activity of PFC pyramidal neurons. Specifically, we suggest the hypothesis that the synaptic efficacy is high at connections made by within-stripe excitatory inputs onto chandelier cells, but decreases during high frequency presynaptic activity. In contrast, synaptic efficacy is low if the postsynaptic target is a wide-axor neuron, but then increases as the presynaptic pyramidal cell fires at a high rate (Lewis and González-Burgos, 2000). Thus, when PFC pyramidal cells have a low level of activation, such as may occur for neurons that do not encode the “correct” information for a given working memory task, their within-stripe collaterals will tend to recruit chandelier neurons, thereby inhibiting pyramidal cells within the same stripe, reducing the activation and inhibiting the output from PFC modules encoding information that is not relevant to the task at hand. In contrast, with an increase in firing rate during the delay period of working memory tasks, within-stripe excitatory inputs recruit mainly wide-axor cells, resulting in inhibition of pyramidal cells located in the adjacent gaps. This spatial pattern of inhibition would help tune the activity of PFC pyramidal cells during the delay period, after a cross-module inhibition model similar to the cross-orientation inhibition in visual cortex (Das, 1996), but specific to the delay-related activity of PFC cells.

In summary, the findings of the present study contribute to a model of the functional architecture of the supragranular layers of monkey PFC in which the between-stripe and associational excitatory connections furnished by supragranular pyramidal cells are specialized mainly to mediate recurrent excitation among pyramidal cells in different stripes of the same module. In contrast, inhibitory control over the activity of layer 3 pyramidal cells seems to be provided by “feed-back” inhibition within a stripe. The recruitment of chandelier and wide-axor neurons by within-stripe axon collaterals may provide critical inhibitory constraints needed for proper working memory function.

ACKNOWLEDGMENTS

The authors thank Mrs. Mary Brady and Mr. Colin Stebbins for excellent assistance with the graphics.

LITERATURE CITED


Lund JS, Lewis DA. 1993. Local circuit neurons of developing and mature