Recurrent Connection Patterns of Corticostriatal Pyramidal Cells in Frontal Cortex

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Corticostriatal pyramidal cells are heterogeneous in the frontal cortex. Here, we show that subpopulations of corticostriatal neurons in the rat frontal cortex are selectively connected with each other based on their subcortical targets. Using paired recordings of retrogradely labeled cells, we investigated the synaptic connectivity between two projection cell types: those projecting to the pons [corticopontine (CPn) cell], often with collaterals to the striatum, and those projecting to both sides of the striatum but not to the pons [crossed corticostriatal (CCS) cell]. The two types were morphologically differentiated in regard to their apical tufts. The dendritic morphologies of CCS cells were correlated with their somatic depth within the cortex. CCS cells had reciprocal synaptic connections with each other and also provided synaptic input to CPn cells. However, connections from CPn to CCS cells were rarely found, even in pairs showing CCS to CPn connectivity. Additionally, CCS cells preferentially innervated the basal dendrites of other CCS cells but made contacts onto both the basal and apical dendrites of CPn cells. The amplitude of synaptic responses was to some extent correlated with the contact site number. Ratios of the EPSC amplitude to the contact number tended to be larger in the CCS to CCS connection. Therefore, our data demonstrate that these two types of corticostriatal cells distinct in their dendritic morphologies show directional and domain-dependent preferences in their synaptic connectivity.

Key words: pyramidal cell; frontal cortex; striatum; pons; postsynaptic current; apical dendrite

Introduction
In the neocortex, pyramidal cells projecting to the same target areas are aggregated within given cortical layers, with each layer containing several projection types (Jones, 1984; Kasper et al., 1994; Gao and Zheng, 2004). Even within microregions of the same cortical layer, there can exist several projection cell types (Levesque et al., 1996b; Verselli et al., 2004; Gabbott et al., 2005). Pyramidal cells are recurrently connected with each other (Markram, 1997; Markram et al., 1997; Thomson and Deuchars, 1997; Gao et al., 2001), and recurrent excitatory interactions in groups of neurons induce slow rhythmic depolarizations (depolarized “up” states) during sleep or anesthesia (Metherate and Ashe, 1993; Steriade et al., 1993; Stern et al., 1997). Additionally, reverberating excitation by recurrent connections may be important for information processing in cortical circuits (Anderson et al., 2000; Wang, 2001). Considering the interlaminar connectivity of pyramidal cells, the reverberating excitation could occur in individual cortical layers (Thomson and Morris, 2002), perhaps within layer V (Sanchez-Vives and McCormick, 2000). Although the specific synaptic connectivity of cortical projection neurons within the same layer is not yet established, recent data suggest that different classes of cortical neurons within a layer may show selective connectivity (Mercer et al., 2005), a finding that has important implications for our understanding of the function of the cortex.

In the frontal cortex, two classes of corticostriatal pyramidal cells have been identified in layer V based on their axonal projection patterns (Cowan and Wilson, 1994). Crossed corticostriatal (CCS) cells innervate both the ipsilateral and contralateral striatum, whereas other corticostriatal cells selectively innervate the ipsilateral striatum but project also to the brainstem through the pyramidal tract. Some brainstem projecting neurons include cells projecting to the pontine nuclei [corticopontine (CPn) cells]. In the rat frontal cortex, brainstem-projecting layer V neurons also frequently innervate the ipsilateral striatum (Cowan and Wilson, 1994; Levesque et al., 1996a; Levesque and Parent, 1998). These corticostriatal pyramidal cells transmit the slow oscillation generated within the frontal cortex to striatal cells (Wilson and Groves, 1981; Wilson and Kawaguchi, 1996; Stern et al., 1998). It remains to be determined how the oscillation is produced in the individual cortical layers and propagated to the striatal neurons (Stern et al., 1997).

Because different classes of corticostriatal cells innervate distinct populations of striatal cells (Lei et al., 2004) that have opposing effects on the outputs of basal ganglia (Albin et al., 1989; Alexander and Crutcher, 1990), knowledge of the patterns of synaptic connectivity between corticostriatal cells may suggest a physiological substrate for cortical influence on the basal ganglia.
circuitry. To this end, we investigated the synaptic connections between retrograde-labeled CCS and CPn pyramidal cells. Our results demonstrate distinct morphologies and selective patterns of synaptic connectivity between CCS and CPn cells.

Materials and Methods

Dual fluorescent retrograde labeling of CCS and CPn cells. Retrograde labeling experiments were performed on young [postnatal day 19 (P19) to P23] Wistar rats. Rats were anesthetized with ketamine (40 mg/kg, i.m.) and xylazine (4 mg/kg, i.m.). Two different fluorescent retrograde tracers were applied by pressure injection (PV-820; WPI, Sarasota, FL) into the pons and striatum, respectively, of each animal using glass pipettes (tip diameter, 100 μm) (see Fig. 1A). In the case of striatal injection, the cortex, hippocampus, and fimbria just caudal to the striatum were removed by suction, and the tracer was applied obliquely through the lateral ventricle to prevent tracer spilling into the cortex. Alexa Fluor 555-conjugated cholera toxin subunit B (CTB; Invitrogen, San Diego, CA) was injected into the striatum contralateral to the cortex investigated (80–100 nl, 0.8 mm posterior to bregma, 2.5 mm lateral to bregma; depth, 4 mm) (see Fig. 1A1). Fast Blue (7% in distilled water; Illegis Plastics, Breuberg, Germany) or Fluoro gold (4% in distilled water; Fluorochrome, Englewood, NJ) was injected into the pons including the ipsilateral pontine nuclei (80–100 nl, 5.6 mm posterior to bregma, 0.5–1 mm lateral to bregma; depth, 9–9.5 mm) (see Fig. 1A2). After a survival period of 4 d, the animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused transcardially with saline followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1M sodium phosphate buffer (PB). The frontal cortex was obliquely sectioned on a cryostat (Kawaguchi, 1992), and sections were mounted on glass slides and coverslipped in Krystalon mounting medium (EM Science, Fort Washington, PA). The sections were observed by epifluorescence (excitation, 360–370 nm; emission, 420–439 nm) were cut and immersed in a buffered solution containing the following (in mM): 124 NaCl, 3 KCl, 2.4 CaCl2, 1.2 MgCl2, 26 NaHCO3, 1 NaH2PO4, 10 glucose, 4 lactic acid, 0.2 acetic acid. This solution was continuously aerated with a mixture of 95% O2 and 5% CO2. During recording, lactic acid was omitted. Membrane potentials of retrogradely labeled cells in the frontal cortex (medial agranular cortex and anterior cingulate cortex) were recorded in a whole-cell mode at 29°C. Retrogradely labeled cells were identified by epifluorescence (excitation, 520–550 nm; emission, 580), under a 40× water immersion objective.

Electrophysiological recording. The pipette solution for current-clamp recording consisted of the following (in mM): 126 potassium methylsulfate, 6 KCl, 0.6 EGTA, 2 MgCl2, 4 ATP, 0.3 GTP, 10 HEPES, and 0.75% biocytin (Sigma, St. Louis, MO). The pH of the solution was adjusted to 7.3 with KOH, and the osmolarity was ~290 mOsm. Current-clamp recordings were made in a fast current-clamp mode of EPC9/dual (HEKA Elektronik, Lambrecht/Pfalz, Germany).

EPSC analysis. EPSCs were induced by single presynaptic action potentials generated by depolarizing somatic current pulses (duration, 10 ms) in the presynaptic cells and were measured in post synaptic neurons at a voltage clamped at ~60 mV at a sampling rate of 20 kHz. Series resistance of the post synaptic whole-cell recordings was monitored periodically by the delivery of small voltage pulses (~5 mV, 10 ms) to the post synaptic neuron. Recordings were ended when spikes in the presynaptic cell deteriorated or if the series resistance in the post synaptic cell increased. Post synaptic responses to single action potentials were identified from individual current traces and averaged over at least 20 trials. Electrophysiological data were analyzed by IGOR Pro (WaveMetrics, Lake Oswego, OR). To obtain the peak current of each trace, the current amplitudes (time window, 0.2 ms; five points) were averaged around the EPSC maximum after excluding failure current traces. The baseline current was defined as the averaged current in a window (2 ms duration) before application of depolarizing current pulses to the presynaptic cell. The peak EPSC is the peak current minus the baseline. Spontaneous synaptic currents were analyzed by Mini Analysis (Synaptosoft, Decatur, GA).

The EPSC rise time was calculated from 20 to 80% of the peak EPSC amplitude. The onset was defined as the point at which a line extrapolated from the rise time crossed the baseline. The decay time constant was measured from the peak of the presynaptic spike to the EPSC onset. The decay time constant was obtained by fitting a single exponential. To average EPSC traces, the peaks of presynaptic spikes were aligned. EPSC frequency characteristics at 10 Hz were obtained from pairs having EPSCs with mean amplitudes larger than 6 pA. The coefficient of variation (CV) was obtained in the pairs with ~40 EPSCs.

Histology. Tissue slices containing biocytin-loaded cells were fixed by immersion in 4% paraformaldehyde, 1.25% glutaraldehyde, and 0.2% picric acid overnight at 4°C, and followed by a freeze-thawing procedure in sucrose-containing PB using liquid nitrogen twice. Slices were resectioned at a thickness of 50 μm. Sections were incubated with avidin–biotin–peroxidase complex (1:100; Vector Laboratories, Burlingame, CA) in 0.05 M Tris-HCl-buffered saline (TBS) with 0.04% Triton X-100 overnight at 4°C. After washing in TBS, the slices were reacted with 3,3′-diaminobenzidine tetrahydrochloride (0.02%), nickel ammonium sulfate (0.3%), and H2O2 (0.003%) in Tris-HCl buffer. They were then postfixed in

Figure 1. Two projection types of pyramidal cells in layer V of the frontal cortex. A, Schematic of method for identification of two projection neuron types by retrograde tracers. Alexa Fluor 555-conjugated CTB was injected in the contralateral striatum while Fast Blue (FB) or Fluoro gold (FG) was injected into the pontine nuclei. For simultaneous recording from CCS and CPn cells, RLMs were injected in the contralateral striatum, and CTB was injected in the ipsilateral pons. Ag, Agranular; ant., anterior. A1, Injection site of RLMs in the striatum. Top, Epifluorescence view. Bottom, As in above, but using bright-field microscopy. The tracer was applied obliquely through the lateral ventricle after suctioning the overlying cortex to prevent injection into the cortex. A2, Injection site of Fluoro gold in the pons including the ipsilateral pontine nuclei. B, Fluorescence imaging revealed two nonoverlapping populations of CCS and CPn cells. CPn cells were distributed in patchy regions (asterisks) where CCS cells were absent. Right, As in the left but in thionin staining.
1% OsO4 in PB containing 7% glucose, dehydrated, and flat-embedded on silicon-coated glass slides in Epon. After all of the procedures, the tissue shrank to ~90% in length (Karube et al., 2004). The shrinkage was not corrected in the analysis.

Quantitative morphology. Somata, axons, and dendrites of stained cells were reconstructed three-dimensionally using the Neulucida system (MicroBrightField, Williston, VT). Stained cells were drawn for reconstruction with a 60× or 100× objective combined with an additional 1.25× magnification. The apical shaft diameter was obtained by the cross-sectional area of the straight portion 20 μm far from the somatic origin divided by the measured dendritic length. Reconstructed axons and dendrites were composed of serial points with intervals shorter than 1.5 μm (see Fig. 8, inset). Reconstructed neurons were quantitatively analyzed with NeuroExplorer (MicroBrightField, Colchester, VT). Internode intervals are lengths between two successive nodes (branch points) along the dendrite, including those from the soma origin to the first node. Internode tortuosity is the ratio of the internode interval to the direct distance between nodes. The basal field and tuft area were areas

Figure 2. Morphological characteristics of the two projection types of pyramidal cells. A, Simultaneous fluorescent labeling of CCS cells with RLMs (arrowheads) and CPn cells with CTB (arrows). B1, B2, Firing responses of CCS and CPn cells, respectively. Note the initial doublet firing (•) in a CPn cell. C, Dendritic reconstructions of two CCS and two CPn cells, showing the differences in dendritic patterns, especially in their apical tufts. D, Plot of the differences in the apical tuft area and shaft diameter between CCS (open circles) and CPn (filled triangles) cells. E, Differences in the dendritic length of layer I tufts and branch points in layer I between CCS and CPn cells.

**Table 1. Somatic and dendritic comparisons of CCS and CPn cells in layer V**

<table>
<thead>
<tr>
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<th>CCS</th>
<th>CPn</th>
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<tbody>
<tr>
<td>Somata (n)</td>
<td>(n = 24)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Volume (μm³)</td>
<td>5150 ± 1642</td>
<td>5718 ± 1507</td>
</tr>
<tr>
<td>Basal dendrites (n)</td>
<td>(n = 25)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>Primary dendrite number</td>
<td>7.8 ± 1.3</td>
<td>9 ± 2.3</td>
</tr>
<tr>
<td>Nodes (branching points)</td>
<td>24.3 ± 5.9</td>
<td>34.6 ± 13**</td>
</tr>
<tr>
<td>Internode interval (mean, μm)</td>
<td>22.8 ± 5.3</td>
<td>24.8 ± 5</td>
</tr>
<tr>
<td>Internode tortuosity</td>
<td>1.14 ± 0.08</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Area (× 100 μm²)</td>
<td>731 ± 293</td>
<td>650 ± 200</td>
</tr>
<tr>
<td>Endings 6a Order (mean)</td>
<td>3.6 ± 0.5</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>Horizontal direct distances (mean, μm)</td>
<td>110 ± 18</td>
<td>99 ± 15</td>
</tr>
<tr>
<td>Vertical direct distances (mean, μm)</td>
<td>77 ± 17</td>
<td>63 ± 10**</td>
</tr>
<tr>
<td>Apical dendrites (n)</td>
<td>(n = 24)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>Apical origin (distance from pia, μm)</td>
<td>825 ± 200</td>
<td>760 ± 103</td>
</tr>
<tr>
<td>Shaft diameter (at 20 μm from soma, μm)</td>
<td>1.79 ± 0.37</td>
<td>2.34 ± 0.48**</td>
</tr>
<tr>
<td>Oblique branch density (100 μm)</td>
<td>2.3 ± 0.5</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>Apical tufts (n)</td>
<td>(n = 26)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Layer I dendritic length (μm)</td>
<td>878 ± 684</td>
<td>3478 ± 1431**</td>
</tr>
<tr>
<td>Layer I branch points</td>
<td>6.0 ± 5.3</td>
<td>19.8 ± 11.1**</td>
</tr>
<tr>
<td>Tuft area (× 100 μm²)</td>
<td>215 ± 129°</td>
<td>876 ± 330**</td>
</tr>
<tr>
<td>Tuft origin (vertical position from pia, μm)</td>
<td>208 ± 66°</td>
<td>350 ± 110**</td>
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Data are means ± SD. n, Number of cells. **p < 0.01.

6a True endings.

6 White matter side, positive.

6c CCS cells with the tuft structure (n = 20).
of the plane perpendicular to the pia-white matter axis, onto which the basal dendrites and apical tufts were projected, respectively. To evaluate the dendritic extension, we measured direct distances between the soma centroid and true endings, not endings resulting from slice preparation, along horizontal and vertical axes (horizontal and vertical distances, respectively) (see Fig. 3B, inset).

Potential synaptic contacts (contact sites) were identified as a close apposition of an axonal bouton and a postsynaptic dendrite in the same focal plane at 1250× using a 100× objective (numerical aperture, 1.4) (Markram et al., 1997; Feldmeyer et al., 1999). When the presynaptic axons and postsynaptic dendrites came close within 2.5 μm between the centers of neurites, the nearest encounter sites of dendrites were called approach points (approaches) (see Fig. 8, inset). Data are given as mean ± SD. For statistical comparison of the mean measurements between two cell classes, the Mann–Whitney U test was used.

Results

Morphological differences between CCS and CPn cells

To confirm that CCS and CPn cells were distinct cell types, we used two different fluorescent tracers injected into their projection target areas (three rats). CTB was injected into the contralateral striatum (n = 3), and Fast Blue (n = 2) or Fluorogold (n = 1) was injected into the pons, including the ipsilateral pontine nuclei (Wang and McCormick, 1993) and CCS cells labeled with RLMs injected into the contralateral striatum (Fig. 1A). Resting potentials were −66.4 ± 5.4 mV in CCS cells (n = 20) and −62.1 ± 4.1 mV in CPn cells (n = 10), and input resistances were 139.3 ± 60.1 MΩ in CCS and 90.6 ± 82.5 MΩ in CPn cells. Among 11 CPn cells, nine cells showed initial doublet spikes and in response to step depolarization (Fig. 2B2), followed by nonadaptive repetitive firing (Mason and Larkman, 1990; Hefi and Smith, 2000; Christophe et al., 2005). In contrast, CCS cells (n = 28) displayed no initial doublet firing to step depolarizations (Fig. 2B1).

We compared the dendritic and axonal patterns of biocytin-filled CCS and CPn cells. In the basal dendrites, the primary dendrite number and intermediate interval were similar between the two types (Table 1). The apical dendrites of the two neuronal types showed similar branch density along the shaft, but CPn cells had significantly thicker shaft diameters at their base than did CCS cells (measured at ~20 μm from the somatic origin; p < 0.01) (Fig. 2D, Table 1). Additionally, the apical tufts of the two types of neuron were morphologically distinct (Fig. 2C–E, Table 1). CPn cells had larger tuft areas, longer lengths of layer I dendrites, and more branch points in layer I than did CCS cells (p < 0.01). Additionally, the tufts of CPn cells originated from the main apical shafts at a greater depth than in CCS cells (Table 1) (p < 0.01). Both types had axon collaterals around the somata and included cells innervating layer I (data not shown). Horizontal collaterals were observed in both types, but CPn cells seemed...
to extend further than CCS cells. Together, these data demonstrate that CCS and CPn cells are morphologically differentiated in regard to their apical tufts. However, significant heterogeneity was observed among the apical tufts of both CPn and CCS cells (Fig. 2D,E).

**Depth dependence of dendritic patterns in CCS cells**

We next asked whether the morphological diversity observed in CCS and CPn cells might depend on their somatic locations within layer V. To test for this, we aligned dendritic reconstructions of CCS and CPn cells in accordance with somatic depth from the pia (Fig. 3A,B). The dendritic morphologies of CCS cells changed gradually according to their depth within layer V. Some superficial CCS cells had robust apical tufts (tufted CCS cells), whereas others had poorly developed tufts (slender CCS cells). The apical dendrites of deeper CCS cells tended to have a very reduced or absent apical tuft resembling the superficial slender CCS cells. Tuft dendritic lengths in layer I were heterogeneous in neurons with superficial somata but were significantly shorter in neurons with somata in the deeper areas of layer V (Fig. 3C1) (p < 0.01). Additionally, the internode intervals in the basal dendrites were longer in superficial CCS cells and shorter in deeper CCS cells (Fig. 3D1) (p < 0.01). To compare the dendritic spatial spread, we measured horizontal direct distances between soma centroid and true endings. Horizontal dendritic distances were longer in superficial CCS cells (Fig. 3E1) (p < 0.01). Correspondingly, the basal dendritic fields of superficial layer V CCS cells were larger than those of deeper CCS cells [correlation coefficient (c.c.), −0.58; p < 0.01]. These depth-dependent tendencies were absent or much less pronounced in CPn cells (Fig. 3C2–E2) (tuft lengths, p = 0.7); internode intervals, p = 0.22; in horizontal distances, p = 0.31; c.c., −0.24 in basal dendritic field; p = 0.55). Total dendritic length was negatively correlated with the distance between the pia and soma in CCS cells (c.c., −0.44; p < 0.05) but positively in CPn cells (c.c., 0.6; p < 0.01) because of the length increase of apical shaft and their branches in deeper CPn cells. These data demonstrate that although CCS cells are heterogeneous in their dendritic structures, there is a significant correlation between the size and robustness of their dendritic fields and their sublaminal position within layer V.

**EPSC characteristics and connection patterns**

To reveal synaptic connection patterns between CCS and CPn cells, we investigated the EPSC characteristics and connection probability using paired recordings consisting of a CCS cell and another CCS or CPn cell in layer V (Fig. 4A). EPSCs were induced with connection probability of 0.1 in pairs from CCS to CCS (n = 308) and 0.11 in CCS to CPn pairs (n = 98) within 100 μm in distance but were rarely found from CPn to CCS cells. It was only found once in 96 pairs (Fig. 4B). Among 31 connections from CCS to CCS cells, four were reciprocal (connection probability, 0.13). EPSC characteristics were examined in cell pairs in which series resistances of postsynaptic recordings were low. EPSC latencies and amplitudes were similar between CCS to CCS (n = 24) and CCS to CPn pairs (n = 11) (p = 0.17 and 0.94, respectively) (Table 2). The EPSC rise time and decay time constants were also similar (p = 0.12 and 0.78, respectively) (Table 2). Spontaneous EPSCs were also similar in amplitudes between CCS and CPn cells [10.5 ± 1.8 pA in CCS cells (n = 6) and 10.9 ± 2.8 pA in CPn cells (n = 6)] but more variable in CPn cells (CV, 0.4 ± 0.08 in CCS and 0.82 ± 0.55 in CPn cells). Mean amplitudes of evoked unitary EPSCs did not correlate with those of spontaneous EPSCs (c.c., −0.05; p = 0.88; n = 12), suggesting unitary EPSC amplitudes were not affected by the postsynaptic cell condition. To examine short-term synaptic dynamics in these connections, pairs of EPSCs were generated at 100 ms intervals. The paired-pulse ratios of the second EPSC to first EPSC were significantly smaller in CCS cells (p = 0.31; c.c., −0.01) compared to CPn cells (p = 0.01) (Fig. 4A). These depth-dependent tendencies were absent or more variable in CPn cells (CV, 0.46 ± 0.19 in CPn cells) compared to CCS cells (CV, 0.08 in CCS cells) (Table 2). These data show that although the synaptic responses were qualitatively similar regardless of their postsynaptic target. The distances between the somata of connected CCS to CCS

![Figure 4.](image)

**Figure 4.** Synaptic connections among corticostriatal pyramidal cells. A1, Reconstructions of the dendrites of presynaptic (gray) and postsynaptic (black) CCS cells and the axon of the presynaptic CCS cell (red). Inset, A presynaptic action potential (top trace) generates a unitary postsynaptic current (bottom trace). A2, Reconstructions and postsynaptic current of a presynaptic CCS cell (gray) and postsynaptic CPn (black) cells. B, Connection probability between pyramidal cell subtypes. Note that very few connections were found from CPn to CCS cells.

**Table 2. Unitary EPSC characteristics in CCS and CPn cells, induced by CCS cells**

<table>
<thead>
<tr>
<th></th>
<th>CCS (n = 24)</th>
<th>CPn (n = 11)</th>
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<tbody>
<tr>
<td>Latency (ms)</td>
<td>1.6 ± 0.6</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>17.8 ± 15.4</td>
<td>14.7 ± 9.6</td>
</tr>
<tr>
<td>Rise time (ms)</td>
<td>0.9 ± 0.4</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Decay time constant (ms)</td>
<td>6.0 ± 2.0</td>
<td>6.3 ± 2.5</td>
</tr>
<tr>
<td>Paired-pulse ratio (second/first; 10 Hz)</td>
<td>0.77 ± 0.26 ( ^a )</td>
<td>0.93 ± 0.12 ( ^b )</td>
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Data are means ± SD, n, Number of cells.

\( ^a n = 15 \)

\( ^b n = 4 \)
pairs (c.c., 0.84; p < 0.01; slope, 0.82). Because the somata of recorded pairs were typically within 100 \( \mu m \) of each other, it is likely that the depth dependence of dendritic morphology contributes to the morphological resemblance of synaptically connected CCS cell pairs.

### Contact site distributions between connected pairs and their relation to EPSC amplitudes

To test whether target-specific differences exist in synapse formation onto postsynaptic CCS or CPn cells, we reconstructed the axons and dendrites of paired neurons (Fig. 6A1,A2). Contact points between boutons and postsynaptic dendrites were mapped on dendrograms (Fig. 6A1–A3), and their distances from somata were compared (Fig. 6B). Contact sites in both types of pairs were found on dendritic branches within layer V. No significant differences were found when comparing the mean distances of contact sites from the soma (Table 3) or the dendritic order of contact sites (Table 3). However, CCS axons contacted apical branches more frequently in postsynaptic CPn cells than in CCS cells (Figs. 6B, 9A,B). The apical contact ratio (contacts on apical branches/total contacts) was lower in CCS cells than in CPn cells (\( p < 0.05 \)) (Table 3).

To test whether unitary currents were correlated with the morphological distribution of contacts in the postsynaptic cell, we compared mean EPSC amplitudes with the number and position of contacts (Fig. 6B). EPSCs were detected even in the case of a single bouton located 220 \( \mu m \) from the soma (4.5 pA) (Fig. 6B). EPSC amplitudes per contact (see below) did not correlate well with the mean distance of contacts from the soma (c.c., 0.18, \( p = 0.56 \) in CCS to CCS pairs; c.c., −0.57, \( p = 0.2 \) in CCS to CPn pairs). EPSC amplitudes were better correlated with the number of contact sites rather than their spatial distribution (Figs. 6B, 7A). Additionally, this correlation was stronger in CCS to CCS pairs than in CCS to CPn pairs (c.c., 0.83, \( p < 0.01 \) in the former; c.c., 0.35, \( p = 0.46 \) in the latter). As expected, the EPSC CV was inversely correlated with the number of contact sites (Fig. 7B). These data suggest that the contact number reflects the number of synaptic release sites to some extent. When comparing between cell classes, significantly fewer contact sites were observed in CCS cells than were made onto CPn cells (\( p = 0.01 \)) (Table 3). In the pairs for which both presynaptic and postsynaptic cells were reconstructed, mean somatic EPSC amplitudes were similar between CCS and CPn cells (\( p = 0.91 \)) (Table 3). The EPSC amplitude divided by the number of contacts tended to be lower in CCS cells than in CPn cells (\( p = 0.06 \)) (Table 3), suggesting that the efficacy of individual synaptic release sites may be stronger in CCS to CCS cell pairs.

### Contact formation probability between nearby neurites

Given the data above, we hypothesized that CCS and CPn cells show specificity in synapse formation onto postsynaptic den-
drites. To investigate whether CCS cells show preferences in postsynaptic targets, we compared the number of contacts generated by presynaptic axons onto postsynaptic dendrites with the total number of approaches (potential contact sites) in CCS to CCS or CCS to CPn pairs. First, we confirmed that contacts were never observed in pairs in which EPSCs were not detected (Fig. 9B). Two situations could explain this lack of connectivity: (1) if presynaptic axons do not come within range of the second neuron, synaptic formation would be impossible; or (2) presynaptic axons may approach the postsynaptic dendrites (within distances potential for synapse formation) but avoid making synaptic contacts (Fig. 8, inset). To discriminate between these two possibilities, we mapped the dendrites of potential postsynaptic neurons and identified all points (approach points) where the axons of the other recorded neuron approached within 2.5 μm from the dendritic center (Fig. 8). A distance of 2.5 μm was selected, because the average spine length varies from 1.8 to 2.6 μm (Stepanyants et al., 2002). Approach points included contact sites.

In nonreciprocally connected CCS–CCS pairs, the neuron with no observable EPSC had fewer approach points onto its basal dendrites than did the neuron with detectable EPSCs. The mean number of approach points in the nonsynaptically and synthetically connected neurons, respectively, was 7.2 ± 6.7 (n = 6) and 11.1 ± 5 (n = 13; p = 0.1) (Fig. 9B). In the CCS–CPn pairs, neurons with no EPSC detected had approximately half the number of approach points (mean, 7 ± 6.4; n = 7) onto their basal dendrites as did neurons with synaptic responses (mean, 14.6 ± 6.7; n = 7; p = 0.05). In the apical branches of CCS–CCS pairs, the number of approach points in neurons without postsynaptic EPSCs (1 ± 1.6) was approximately one-third of those in neurons with observable EPSCs (3.4 ± 2.6; p = 0.05) (Fig. 9C). Similarly, in CCS–CPn pairs, the number of apical approaches in nonresponding neurons (1.3 ± 1.8) was one-third of those in synaptically responsive neurons (4.7 ± 4.6; p = 0.08). These data demonstrate that opportunities for synaptic connections onto nontargeted neurons exist as evidenced by approach points onto both apical and basal dendrites.

We next compared the dendritic and spatial distribution patterns between approaches and contacts. The dendritic distribution patterns were similar between contacts and all approaches in the basal dendrites of connected pairs from CCS to CCS (Fig. 9A1, Table 4) (Kolmogorov–Smirnov test; p = 0.59 in basal dendrites) or CCS to CPn cells (Fig. 9A2, Table 4) (p = 0.56 in basal dendrites). The vertical spatial distributions of contact sites and approaches were similar in CCS to CPn pairs (Table 4) (p = 0.34). In CCS to CCS pairs, however, contact sites were more skewed toward the white matter side than approaches (Table 4) (p < 0.05), suggesting spatial selectivity during contact formation.

To estimate the probability of contact formation on nearby neurites, we compared the ratio of contacts to approaches (contact ratio) in basal dendrites and apical branches (Fig. 9B). CCS to

Table 3. Morphological contact sites and EPSC characteristics in the reconstructed pairs

<table>
<thead>
<tr>
<th></th>
<th>CCS (n = 13)</th>
<th>CPn (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact site on dendrites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contact site number</td>
<td>3.1 ± 1.4</td>
<td>4.9 ± 1.1*</td>
</tr>
<tr>
<td>Distance from somatic origin (μm)</td>
<td>90.9 ± 55.2</td>
<td>97.8 ± 57.8</td>
</tr>
<tr>
<td>Dendrite order</td>
<td>4.3 ± 1.9</td>
<td>4.3 ± 2.9</td>
</tr>
<tr>
<td>Apical contact ratio (contacts on apical branches/total contacts)</td>
<td>0.06 ± 0.16</td>
<td>0.19 ± 0.18*</td>
</tr>
<tr>
<td>Unitary EPSC amplitude (pA)</td>
<td>20.2 ± 17.9</td>
<td>16.6 ± 10.1</td>
</tr>
<tr>
<td>Unitary EPSC amplitude/contact site number (pA)</td>
<td>6.0 ± 3.2</td>
<td>3.4 ± 1.8</td>
</tr>
</tbody>
</table>

Data are means ± SD, n, Number of cells, *p < 0.05
basal and apical branches. These data suggest that the location of synapse formation between presynaptic CCS cells and nearby pyramidal cells is target specific, and that synapses onto basal dendrites of other CCS cells but show more balanced innervation of the basal and apical dendrites of CPn cells.

In one case, a single bouton, further than 200 μm, was found to generate a unitary CCS to CCS EPSC at the soma. More generally, EPSC amplitudes induced in CCS cells by other CCS cells were correlated with the number of presynaptic boutons to some extent. This suggests that the number of contact sites in CCS to CCS pairs is correlated with the functional synaptic number (Markram et al., 1997; Kalisman et al., 2005). Different ratios of the EPSC amplitude to contact number were found between the two connections, although not significant. There may be target-specific differences in synaptic efficacy or synaptic integration in these two cell types (Feldmeyer and Sakmann, 2000).

In the case of CCS to CCS connections, slender cells tended to form synaptic connections with other slender cells, although tufted cells preferred similarly tufted neurons. These correlations likely result, in part, because CCS cells in the same sublaminar area of layer V tended to have similar dendritic morphologies (Fig. 10), and most synaptically connected neurons were found within 100 μm of each other. These findings suggest that CCS pyramidal cells with similar dendritic morphologies (and therefore to some extent similar afferent input) may be locally clustered within layer V and show preferential synaptic connectivity. This may reflect vertical aggregates of neurons with a similar target during cortical formation (Vercelli et al., 2004).

Corticostriatal cell heterogeneity and their intracortical connections

Pyramidal cells projecting to the striatum are considered to be functionally heterogeneous (Wilson, 2004). To date, two subtypes of corticostriatal cells have been identified. The first identified subtype, demonstrated in both primates (Jones et al., 1977) and rats (Wilson, 1987), are corticostriatal neurons that do not project to the brainstem but that innervate the contralateral striatum. This innervation pattern was later confirmed using intracellular staining of axons (Cowan and Wilson, 1994; Lévesque et al., 1996a,b). A second subtype of CCS cell identified in rats projects to the brainstem (Donoghue and Kitai, 1981; Cowan and Wilson, 1994; Lévesque et al., 1996a). Although it remains to be investigated how often collaterals are issued from axons descending to the brainstem in the primate (Bauswein et al., 1989), in the rat frontal cortex, brainstem-projecting layer V neurons also frequently innervate the ipsilateral striatum (Lévesque et al., 1996a; Lévesque and Parent, 1998). In this study, we confirmed that CCS and CPn cells are mutually exclusive groups using double-fluorescence markers. Apical dendritic tufts are different in size among pyramidal cell subtypes (Hallman et al., 1988; Hübener et al., 1990; Kasper et al., 1994; Gao and Zheng, 2004). In addition to their striking differences in axonal projection, we found significant morphological differences in their apical tuft structures (Fig. 10). Furthermore, CCS cells were heterogeneous with regard to their tuft branching pattern, showing significant correlation be-
 tween somatic depth and the degree of dendritic arborization in layer I.

Because CCS and CPn cells are differentiated in their dendritic structures, synaptic connectivity, and extracortical projection sites, it is possible that they receive distinct types of inputs within the frontal cortex. Information transfer in the frontal cortical circuit is a crucial point for the forebrain neural loop through the cortex, basal ganglia, and thalamus, involved in the context-dependent release of various motor and cognitive circuits (Graybiel et al., 1994; Hikosaka et al., 2002). Therefore, it is important to know how these two types of corticostriatal cells are innervated by afferent fibers from the mediodorsal and parafascicular thalamic nuclei, areas that receive GABAergic inhibition from the basal ganglia (Kuroda et al., 1998; Cebrián et al., 2005). Thalamic fibers distribute in layer I and the deep part of layer II/III (Deschénes et al., 1996; Marini et al., 1996; Jones, 2001). Both CCS and CPn cells have apical branches in the deeper layer II/III, whereas the apical tuft expansions within layer I are distinct between CCS and CPn cells and heterogeneous among CCS cells. It remains to be investigated which subtypes of layer V corticostriatal cells receive thalamic inputs directly at the layer I tufts at or at deeper layer II/III.

Thalamic afferents innervate layer II/III pyramidal cells in addition to layer V cells (Kuroda et al., 1998). Layer II/III pyramidal cells preferentially innervate thick tufted layer V pyramidal cells rather than slender layer V pyramidal cells (Thomson and Bannister, 1998; Thomson and Morris, 2002). Therefore, layer II/III pyramidal cells with direct inputs from the thalamic nuclei may preferentially innervate CPn cells over CCS cells. Additional investigations elucidating the specifics of intracortical wiring between specific classes of cortical neurons will be needed to understand the influence of cortical circuits on striatal output.

Functional differentiation of corticostriatal pathways

In this study, we found that differential axonal projections and apical tuft structures segregate corticostriatal cells into two types, with CCS cells further differentiated according to their depth-dependent differences in dendritic morphology. This suggests that corticostriatal neurons are heterogeneous according to their extracortical target and the laminar location. Similarly, in the striatum, projection cells are heterogeneous from two independent points of view: the extrastriatal target and intrastriatal location.

In terms of extrastriatal targets, they are divided mainly into two groups (Gerfen and Young, 1988; Kawaguchi et al., 1990; Parent et al., 1995). These two types are considered to affect basal ganglia outputs in opposite ways (Albin et al., 1989; Alexander and Crutcher, 1990). One group exclusively projects to the external pallidal segment (GPe-exclusive cells; indirect pathway), whereas another group, while sending axon collaterals to the external pallidal segment, directly projects to output structures in the basal ganglia (direct pathway cells) (Kawaguchi et al., 1990; Lévesque and Parent, 2005). Direct pathway cells are considered to promote desired movements, and GPe-exclusive cells are considered to inhibit unwanted movement (Albin et al., 1989; DeLong, 1990; Lei et al., 2004). Recently, it has been revealed that two types of corticostriatal cells differentially innervate one of the above two striatal output cells (Reiner et al., 2003; Lei et al., 2004). In light of these data, it is likely that CPn cells preferentially innervate GPe-exclusive cells while CCS cells innervate direct pathway cells. Therefore, activity in CPn cells may promote dis-
concrete motor outputs to the brainstem or spinal cord through the pyramidal tract but suppress unnecessary outputs by excitation of GPe-exclusive cells in the striatum. Interestingly, CPn cells that synapse on the indirect-pathway striatal neurons likely receive more excitatory synaptic input because of their enlarged dendritic trees in layer V relative to CCS cells. In addition, CPn cells are excited by CCS cells while not providing significant feedback excitation, and only CCS cells project to the other hemisphere. In view of these connection patterns, CCS cells seem to regulate the activity balance between the direct and indirect pathways or also between both sides of basal ganglia.

The intrastral locations divided striatal projection cells into two groups, independent of the above extrastriatal projections ones. These two groups of neurons are spatially compartmentalized within the striatum, with one group forming irregularly shaped patches within a surrounding matrix composed of neurons of the other class (Gerfen, 1984, 1992; Kawaguchi et al., 1989). Striatal neurons in each compartment receive distinct cortical afferents from specific cortical regions and laminae. Deep layer V corticostriatal neurons project principally to patch neurons, whereas superficial layer V corticostriatal neurons project principally to neurons in the matrix (Gerfen, 1989). The sublaminar differentiation of layer V CCS cells may be related to their relative contribution to patch and matrix innervation. Our data suggest that the different pathways within the basal ganglia are already differentiated within the intracortical circuits. The heterogeneity in dendritic morphology, sublaminar position, and synaptic formation in CCS and CPn cells may correspond to the striatal cell differentiation and compartmentalization.

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