Synaptic connection patterns between pyramidal cell subtypes in layer V of rat frontal cortex

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Abstract

Corticostriatal pyramidal cells are heterogeneous in the frontal cortex. Here we show that subpopulations of corticostriatal neurons in the frontal cortex are selectively connected with each other based on their subcortical targets. Using paired recordings of retrograde-labeled corticostriatal neurons, we investigated the synaptic connectivity between two pyramidal cell types; (1) those projecting to the pontine nuclei with collaterals to the striatum (corticopontine cell, CPn cell) and (2) those projecting to both sides of the striatum (intratelencephalic crossed corticostriatal cell, CCS cell). CCS cells had reciprocal synaptic connections with each other and also provided synaptic input to CPn cells. However, reciprocal connections from CPn neurons to CCS cells were rarely found. CCS cells preferentially innervated the basal dendrites of other CCS cells but made contacts onto both the basal and apical dendrites of CPn neurons. The amplitude of synaptic responses was correlated with the number of contact sites in CCS-CCS pairs but not so in CCS-CPn pairs. Interestingly, interconnected CCS cells often shared similar dendritic morphologies. Further investigation revealed that the dendritic morphologies of CCS neurons were correlated with their somatic depth within the cortex. These findings suggest that the two types of corticostriatal cells are hierarchically organized, and that intratelencephalic corticostriatal cells are segregated in a sublaminar fashion within layer V and often make connections with other CCS neurons sharing morphological similarities.

Introduction

In the cortex, pyramidal cells projecting to the same target are aggregated according to the layer structure, but each layer contains several projection types (Jones, 1984; Kasper et al., 1994; Gao and Zheng, 2004).. Even within micro-regions of the same cortical layer there can exist several projection cell types (Lévesque et al., 1996b; Vercelli et al., 2004; Gabbott et al., 2005). Excitatory pyramidal cells are recurrently connected with each other (Markram, 1997; Markram et al., 1997; Thomson and Deuchars, 1997; Gao et al., 2001). Recurrent excitatory interactions induce slow rhythmic (<1Hz) depolarizations (depolarized "up" states) during sleep or anaesthesia (Steriade et al., 1993; Metherate and Ashe, 1993; Stern et al., 1997). Further, reverberating excitation by recurrent connections may play an important role in the computation of cortical circuits (Anderson et al., 2000; Wang et al., 2001). However, while the specific synaptic connectivity of cortical projections neurons is not yet established, recent data suggest that different classes of cortical neurons show selective connectivity (Mercer et al., 2005), a finding that has important implications for understanding 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, while corticopontine cells (CPn cells) innervate only the ipsilateral striatum but project also to the brainstem including the pontine nuclei. In the rat frontal cortex, the axons of most layer V neurons issue collaterals to the striatum before invading the brainstem (Cowan and Wilson, 1994; Lévesque et al., 1996a; Lévesque and Parent, 1998; Zheng and Wilson, 2002). Cortical inputs to the striatum participate in generating membrane potential fluctuations in striatal projection cells (Wilson and Groves, 1981; Wilson and

Kawaguchi, 1996; Stern et al., 1998). They are also divided into two main classes on the basis of the projection sites (Gerfen and Young, 1988; Kawaguchi et al., 1990; Parent et al., 1995). These two types of projection cells are considered to affect basal ganglia outputs in the opposite way (Alexander and Crutcher, 1990).

Thus, both the frontal cortex and striatum have two main types of projection cells. Furthermore each type of corticostriatal pyramidal cells is intimately related to one of striatal projection types (Lei et al., 2004). Therefore the intracortical connection pattern between the two pathways would affect the final outputs of basal ganglia greatly. To understand the excitatory interactions between these two principal routes in the frontal cortex and basal ganglia, we investigated the synaptic connections between two pyramidal cell subtypes, CCS and CPn cells. The results demonstrate selective synaptic connectivity between pyramidal cell subtypes in the corticostriatal system.

MATERIALS AND METHODS

Dual fluorescent retrograde labeling of CCS and CPn cells.

Experiments were performed on young (19 - 23 days postnatal) Wistar rats. Rats were anesthetized with ketamine (40mg/kg, i.m.) and xylazine (4mg/kg, i.m.). Fluorescence retrograde tracers were applied using glass pipettes (tip diameter, 100 μm) by pressure injection (PV820, WPI). In the case of striatal injection, the cortex, hippocampus and fimbria just caudal to the striatum were removed by suction, and the tracers were applied obliquely through the lateral ventricle to prevent the spilling of tracers into the cortex. Alexa Fluor 555-conjugated cholera toxin subunit B (CTB; Molecular Probes) 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) (Fig. 1A1). Fast Blue (Illing, Germany; 7% in distilled water) or Fluorogold (Fluorochrome; 4% in distilled water) was injected into the pontine nuclei (80 - 100 nl, 5.6 mm posterior to bregma, 0.5 - 1 mm lateral to bregma, depth 9-9.5 mm) (Fig. 1A2). Following a survival period of 4 days, the animals were anesthetized with sodium pentobarbital sodium (60 mg/kg i.p.) and transcardially perfused with saline followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (PB). Frontal cortex was obliquely sectioned at 20 μm at a cryostat (Kawaguchi, 1992), mounted on glass slide and coverslipped in Krystalon mounting medium (EM Science). The sections were observed by epifluorescence (excitation, 360-370 nm; emission, 420- for Fast Blue or Fluorogold; excitation, 545-580 nm; emission, 610- for Alexa Fluor 555-CTB).

For physiological experiments using an *in vitro* slice preparation (see below), rhodamine-labeled latex microspheres (RLMs) (Lumafluor; 80-100 nl) were injected into the contralateral striatum, and CTB (80-100 nl) into pontine nuclei 2-3 days before the experiment

(Fig. 1A). Pyramidal cells labeled with RLMs could be discriminated from those with CTB because the former showed the granular pattern, but the latter the homogeneous staining in the cytosol (Fig. 1C).

Slice preparation.

Animals were deeply anesthetized with isoflurane and decapitated. Brains were quickly removed and submerged into ice cold physiological Ringer's solution. Three hundred μm thick sections of frontal cortex were cut and immersed in a buffered solution containing, in mM: NaCl, 124.0; KCl, 3.0; $CaCl_2$, 2.4; $MgCl_2$, 1.2; $NaHCO_3$, 26.0; NaH_2PO_4, 1.0; glucose, 10.0; Lactic acid, 4.0; Ascorbic acid, 0.2; This solution was continuously aerated with a mixture of 95% O_2 and 5% CO_2. Membrane potentials of cells in the frontal cortex (medial agranular cortex and anterior cingulate cortex) were recorded in a whole-cell mode at 29°C identifying somata by a 40x water immersion objective (Kawaguchi, 1993). Retrogradely-labeled cells were identified by epifluorescence (excitation, 520-550 nm; emission, 580-), under a 40x water immersion objective.

Electrophysiological recording.

The pipette solution for current-clamp recording consisted of (in mM): potassium methylsulfate 120, KCl 5.0, EGTA 0.5, $MgCl_2$ 1.7, ATP 4.0, GTP 0.3, HEPES 8.5 and biocytin 17. 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).

EPSC analysis.

Excitatory postsynaptic currents (EPSCs) were induced by single presynaptic action potentials generated by depolarizing somatic current pulses in the presynaptic cells and were measured in postsynaptic neurons voltage-clamped at -60 mV at sampling of 20 kHz. Series resistance of postsynaptic whole-cell recordings was always less than 25 M? and was monitored periodically by the delivery of small voltage pulses (-5 mV, 10 ms) to the postsynaptic neuron. Recordings were ended when spikes in presynaptic cells deteriorated or if the series resistance in the postsynaptic cells increased beyond 25 M? . Postsynaptic responses to single action potentials were identified from individual current traces and their average of at least 20 trials. To obtain the peak current of each trace, the current amplitudes (time window, 0.2 ms; 5 points) were averaged around the center of the maximum EPSC. 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.

The EPSC rise time was calculated as the time interval for the EPSC from 20 to 80% of the peak amplitude. The onset was defined as the point at which a line extrapolated from the rise time crossed the baseline current. Latency 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 and coefficient of variation (CV) of EPSC amplitudes were obtained from pairs having EPSCs with a mean amplitude larger than 5 pA.

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 phosphate buffer (PB) using liquid nitrogen twice. Slices were resectioned with a thickness of 50 µm. Sections were incubated with avidin-biotin-peroxidase complex (1:100; Vector, Burlingame, CA) in 0.05 M Tris HCl buffered saline (TBS) with 0.04 % Triton X-100 (TX) overnight at 4 °C. After washing in TBS, the slices were reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (0.02%), nickel ammonium sulfate (0.3%) and H_2O_2 (0.003%) in Tris-HCl buffer. They were then postfixed in 1% OsO_4 in PB containing 7% glucose, dehydrated, and flat-embedded on silicon-coated glass slides in Epon.

Quantitative Morphology.

Somata, axons and dendrites of stained cells were reconstructed three-dimensionally, using the Neurolucida system (MicroBrightField, Williston, VT). Stained cells were imaged for reconstruction with a 60x or 100x objective combined with a further 1.25x magnification. The apical shaft diameter was obtained by the cross-sectional area of the straight portion around 20 µm far from the somatic origin divided by the measured dendritic length (~2.5 µm). Reconstructed axons and dendrites were composed of serial points with intervals shorter than 1.5 µm (Fig. 8A). Reconstructed neurons were quantitatively analyzed with NeuroExplorer (MicroBrightField). Internode intervals are lengths between two successive nodes (branch points) along the dendrite including those from the soma origin to the first node. 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 1250x using a 100x objective (N.A., 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 approaching points (approaches; 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 (3 rats). CTB was injected into the contralateral striatum (n = 3), and Fast Blue (n = 2) or Fluorogold (n = 1) into the pons including ipsilateral pontine nuclei (Fig. 1A). When sections of the medial agranular and anterior cingulated cortex were subsequently processed and visualized with epifluorecence. The labeled cells of both types were found mostly in layer V, but CCS cells were sometimes in the lower part of layer II/III (Fig. 1B). To count fluorescent cells, we selected regions where both fluorescences overlapped strongly. We found 2298 fluorescently labeled cells from 3 rats. Of these, 1141 were labeled with CTB (CCS cells) and 1157 were labeled with Fast Blue or Fluorogold (CPn cells). Importantly, double-labeled cells were never observed, indicating that two types belonged to completely separate neuronal populations. In addition, CPn cells distributed in patchy regions where CCS cells were absent (Fig. 1B). The two types of pyramidal cells seemed to occupy complementary spaces in layer V.

To reveal the morphological differentiation of CCS and CPn cells, we compared the dendritic patterns of biocytin-labeled neurons (Fig. 2A) identified by Alexa 555 CTB injection into the pons (CPn cells) and RLMs into the contralateral striatum (CCS cells) (Fig. 1A). The cells labeled by the two tracers could be differentiated because RLMs showed granular staining, but CTB exhibited homogeneous fluorescence except in their nuclei (Fig. 1C). 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 139.3 ± 60.1 MO in CCS and 90.6 ± 82.5 MO in CPn. Among 11 CPn cells, 9 cells showed initial doublet spikes and in response to step depolarization (Fig. 2D2), followed by non-adaptive repetitive firing

(Mason and Larkman, 1990; Hefti and Smith, 2000; Christophe et al., 2005). In contrast, CCS cells (n = 28) displayed no initial doublet firing to step depolarizations (Fig. 2D1).

We compared the dendritic and axonal patterns of biocytin-filled CCS and CPn cells. In the basal dendrites, the primary dendrite number and internode interval were similar between the two types (Table 1). The apical dendrites of the two neuron types showed similar branch density along the shaft, but CPn cells had thicker shaft diameters at their base than did CCS cells (measured at ~20 μm from the somatic origin) ($p < 0.01$; Fig. 2B). Further, the apical tufts of the two types of neuron were morphologically different (Table 1). CPn cells tended to have larger tuft areas, longer length of layer I dendrites, and more branch points in layer I than did CCS cells ($p < 0.01$). Apical tufts originated from the shafts more deeply in CPn (mean origin was 350 ± 110 μm from the pia, n = 10) than CCS cells (mean origin = 208 ± 66 μm, n = 26; $p < 0.01$). Both types had axon collaterals around the somata and included cells innervating layer I (Fig. 3). Horizontally going collaterals were observed in both types, but CPn cells seemed to extend further than CCS cells. These data show that CCS and CPn cells are morphologically differentiated especially in regard to their apical tufts. However, significant heterogeneity was observed among the apical tufts of both CPn and CCS cells (Fig. 2B, C).

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. 4A, 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), while others had poorly developed tufts (slender CCS cells). The apical dendrites of deeper CCS cells tended to have a very reduced or absent apical tufts 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. 4C1; $p<0.01$). Further, the internode intervals in the basal dendrites were longer in superficial CCS cells and shorter in deeper CCS cells (Fig. 4D1; $p<0.01$). To compare the dendritic spatial spread, we measured horizontal direct distances between soma centroid and true endings (Fig. 4, inset). Horizontal dendritic distances were longer in superficial CCS cells (Fig. 4E1; $p<0.01$). 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.4C2-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. = 0.85, $p<0.01$) due to the length increase of apical shaft and their branches in deeper CPn cells. These data demonstrate that while CCS cells are heterogeneous in their dendritic structures, there is a significant correlation between the size and robustness of their dendritic fields and their sublaminar position within layer V.

Connection patterns and EPSC characteristics

To reveal synaptic connection patterns among CCS and CPn cells, we investigated the connection probability and the EPSC characteristics using paired recordings consisting of a CCS

cell and another CCS or CPn cells in layer V (Fig. 5A). EPSCs were induced with probability 0.1 in pairs from CCS to CCS (n = 308) and 0.11 in 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 96pairs (Fig. 5B). Among 31 connections from CCS to CCS cells, 4 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 (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, pairs of EPSCs were generated at 100 ms intervals (Fig. 5C). The paired-pulse ratios of the second EPSC to first one were 0.77 ± 0.26 in CCS cells (n = 15) and 0.93 ± 0.12 in CPn cells (n = 4). These data show that CCS cells are connected with each other and CPn cells, but CPn cells with CCS cells at much lower probability. Further, the postsynaptic currents generated by presynaptic CCS neurons were quantitatively similar regardless of postsynaptic targets.

The distances between the somata of connected CCS to CCS pairs reconstructed were 63 ± 42 μm (22 ± 16 μm in horizontal direction and 54 ± 46 μm in vertical one, n = 19). Those of connected CCS to CPn pairs were 84 ± 53 μm (p = 0.39; 25 ± 25 μm in horizontal direction and 76 ± 53 μm in vertical one, n = 9). These values are consistent with previous data showing that the highest density

of connected pyramidal cells is found within 25 μm in the horizontal direction and 50 μm in the vertical one, and that the connection probability and EPSP amplitude are greatly reduced when the distance between pairs is larger than 100 μm (Holmgren et al, 2003).

Since CCS cells at the same depth within layer V tended to have similar in dendritic patterns (see Figure 4), and most connections were found between neurons with somata within 100 μm of each other, connected CCS cells tended to have similar dendritic morphologies (Fig. 6A). No such correlation was found between the dendritic morphologies of synaptically connected CCS and CPn pairs. The dendritic lengths of tuft branches in layer I were found to be similar in connected CCS pairs, (Fig. 6B; c.c. = 0.68, $p<0.05$; slope against the presynaptic cell = 0.6), as were their basal dendritic structures. The mean internode intervals of basal dendrites were similar in CCS pairs that were synaptically connected (c.c. = 0.46, $p = 0.16$; slope = 0.71). The connected CCS pairs correlated in horizontal distances (Fig. 6C; c.c. = 0.91, $p<0.01$; slope = 1.01) and in vertical distances (c.c. = 0.74, $p<0.01$; slope = 1.12). The area of basal dendritic field was also correlated in connected CCS pairs (c.c. = 0.84, $p<0.01$; slope = 0.82). Because the somata of recorded pairs were typically within 100 μm 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. 7A1-3). Contact points between boutons and postsynaptic dendrites were mapped on dendrograms (Fig. 7A1, 2), and their

distances from somata were compared (Fig. 7B). 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 (Table3), or the dendritic order of contact sites (Table3). However, CCS axons contacted apical branches more frequently in postsynaptic CPn cells than in CCS cells (Fig. 7B,8C). The apical contact ratio (contacts on apical branches / total contacts) was lower in CCS than CPn cells in CPn cells ($p < 0.05$; table3).

To test whether unitary currents were correlated with the morphological distribution of synaptic contacts in the postsynaptic cells, we compared mean EPSC amplitudes with the number and position of synaptic contacts (Fig. 7B). EPSCs were detected even in the case of a single bouton located 220 μm from the soma (4.5 pA; Fig. 7B). 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 and 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 (Fig. 7B, C). Further, 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 and 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. 7D). 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 ($p = 0.01$; Table3). In the pairs for which both presynaptic and postsynaptic cells were reconstructed, mean somatic EPSC amplitudes were similar between CCS and CPn cells ($p = 0.91$; table3). The EPSC amplitude divided by the number of contacts tend to be larger in CCS cells than CPn cells ($p = 0.06$; table3), 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 neurons show specificity in synapse formation onto postsynaptic dendrites. To investigate whether CCS neurons 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. 8C). 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. 8A). 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. 8B). A distance of 2.5 µm was selected because the average spine length varies from 1.8 – 2.6 µm (Stepanyants et al., 2002). Approach points included contact sites.

In non-reciprocally 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 non-synaptically and synaptically connected neurons, respectively, was 7.2 ± 6.7 (n =6) and 11.1 ± 5 (n = 13; p = 0.1) (Fig. 8C). In the CCS–CPn pairs, neurons with no EPSC detected had about 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 about one third of those in neurons with observable EPSCs (3.4 ± 2.6; p =0.05) (Fig. 8C). Similarly, in CCS–CPn pairs, the number of apical approaches in non-responding 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 non-targeted 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. 9B1, Table4; Kolmogorov-Smirnov test, p = 0.59 in basal dendrites] or CCS to CPn cells [Fig. 9B2, Table4; p = 0.56 in basal dendrites]. The vertical spatial distributions of contact sites and approaches were similar in CCS to CPn pairs [Fig. 9A2, Table4; p = 0.34 in basal dendrites]. In CCS to CCS pairs, however, contact sites were more skewed toward the white matter side than approaches [Fig. 9A1, Table4; $p < 0.05$ in basal dendrites], 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. 8D). CCS to CCS pairs significantly favored synapse generation onto basal dendrites, with the contact ratio of the basal dendrites being 0.28 ± 0.15 (n = 13), compared to a contact ratio of only 0.09 ± 0.28 ($p<0.01$) in apical branches. Conversely, CCS to CPn pairs showed greater balance in synapse formation onto basal and apical dendrites, with the contact ratio being 0.31 ± 0.14 (n = 7) in the basal dendrites and 0.18 ± 0.2 (p = 0.14) in the apical branches. These data suggest that the location of synapse formation between presynaptic CCS neurons and nearby pyramidal neurons is target-specific, and

that synapses onto basal dendrites are favored when establishing contacts onto other CCS neurons, while synapse formation onto CPn neurons occurs on both basal and apical branches.

Discussion

Synaptic connection selectivity between pyramidal cell subtypes

Pyramidal cells are functionally connected with each other by local collaterals (Thomson and Deuchars, 1994; Markram et al., 1997; Mercer et al., 2005), and pyramidal cells in different layers are selectively connected (Thomson and Bannister, 2003; Feldmeyer et al., 2005). Pyramidal cells in the same layer appear to be connected in nonrandom ways (Markram et al., 1997; Song et al., 2005). While subtypes of pyramidal neurons show differential patterns in their axonal projections to subcortical structures (Jones, 1984), it remains to be investigated how selective and precise are the recurrent connections among pyramidal cell subtypes within the neocortex. In this study, for the first time, we investigated the synaptic connection patterns of corticostriatal subtypes identified by both their axonal projection pattern and their dendritic structure. Our data demonstrate that there is significant specificity in the connectivity of cortical pyramidal neurons.

Layer V pyramidal neurons with distinct tuft structures were differentially connected in a direction-selective way (Fig. 10): CCS cells formed synapses onto CPn cells, but there was almost a complete absence of connectivity from CPn neurons to CCS cells, even though the axons of CPn neurons frequently approached CCS dendrites at distances close enough to facilitate synapse formation. In addition to direction selectivity, CCS cells preferentially innervate the 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, farther 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 ratio of the EPSC amplitudes to contact number was found in 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, while 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 neuron identified in rats projects to the brainstem (Donoghue and Kitai, 1981; Cowan and Wilson, 1994; Lévesque et al., 1996a). While 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, most brainstem-projecting layer V neurons also frequently innervate the ipsilateral striatum (Lévesque et al., 1996a; Lévesque and Parent, 1998). In this paper we have 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 between somatic depth and the degree of dendritic arborization in layer I.

Since CCS and CPn cells are differentiated in their dendritic structures, synaptic connectivity, and their 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 crlls. Further 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 intralaminar 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).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). These two types are considered to affect basal ganglia outputs in opposite ways (Albin et al., 1989; Alexander and Crutcher, 1990). Direct pathway cells are considered to promote desired movements, and

GPe-exclusive cells 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 discrete motor output to 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 due to their enlarged dendritic trees in layer I relative to CCS cells. In addition, CPn cells are excited by CCS neurons 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 intrastriatal 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 neurons may correspond to the striatal cell differentiation and compartmentalization.

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References

Albin RL, Young AB, Penney JB (1989) The functional anatomy of basal ganglia disorders. Trends Neurosci 12: 366-375.

Alexander GE, Crutcher MD (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci 13: 266-271

Anderson J, Lampl I, Reichova I, Carandini M, Ferster D (2000) Stimulus dependence of two-state fluctuations of membrane potential in cat visual cortex. Nat Neurosci 3: 617-621.

Bauswein E, Fromm C, Preuss A (1989) Corticostriatal cells in comparison with pyramidal tract neurons: contrasting properties in the behaving monkey. Brain Res 493: 198-203.

Cebrián C, Parent A, Prensa L (2005) Patterns of axonal branching of neurons of the substantia nigra pars reticulata and pars lateralis in the rat. J Comp Neurol 492: 349-369.

Cowan RL, Wilson CJ (1994) Spontaneous firing patterns and axonal projections of single corticostriatal neurons in the rat medial agranular cortex. J Neurophysiol 71: 17-32.

DeLong MR (1990) Primate models of movement disorders of basal ganglia origin. Trends Neurosci 13: 281-285.

Deschénes M, Bourassa J, Doan VD, Parent A (1996) Asingle-cell study of the axonal projections arising from the posterior intralaminar thalamic nuclei in the rat. Eur J Neurosci 8: 329-343.

Donoghue JP, Kitai ST (1981) A collateral pathway to the neostriatum from corticofugal neurons of the rat sensory-motor cortex: an intracellular HRP study. J Comp Neurol 201: 1-13.

Feldmeyer D, Egger V, Lübke J, Sakmann B (1999) Reliable synaptic connections between pairs of excitatory layer 4 neurones within a single 'barrel' of developing rat somatosensory cortex. J Physiol 521: 169-190.

Feldmeyer D, Roth A, Sakmann B (2005) Monosynaptic connections between pairs of spiny stellate cells in layer 4 and pyramidal cells in layer 5A indicate that lemniscal and paralemniscal afferent pathways converge in the infragranular somatosensory cortex. J Neurosci 25: 3423-3431.

Gabbott PLA, Warner TA, Jays PR, Salway P, Busby SJ (2005) Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. J Comp Neurol 492: 145-177.

Gao W-J, Krimer LS, Goldman-Rakic PS (2001) Presynaptic regulation of recurrent excitation by D1 receptors in prefrontal circuits. Proc Natl Acad Sci U S A. 98: 295-300.

Gao WJ, Zheng ZH (2004) Target-specific differences in somatodendritic morphology of layer V pyramidal neurons in rat motor cortex. J Comp Neurol 476: 174-185.

Gerfen CR (1984) The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems. Nature 311: 461-464.

Gerfen CR (1989) The neostriatal mosaic: striatal patch-matrix organization is related to cortical lamination. Science 246: 385-388.

Gerfen CR, Young WS (1988) Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an in situ hybridization histochemistry and fluorescent retrograde tracing study. Brain Res 460: 161-167.

Graybiel AM, Aosaki T, Flaherty AW, Kimura M (1994) The basal ganglia and adaptive motor control. Science 265: 1826-1831

Hallman LE, Schofield BR, Lin CS (1988) Dendritic morphology and axon collaterals of corticotectal, corticopontine, and callosal neurons in layer V of primary visual cortex of the hooded rat. J Comp Neurol 272: 149-160.

Hikosaka O, Nakamura K, Sakai K, Nakahara H (2002) Central mechanisms of motor skill learning. Curr Opin Neurobiol 12: 217-222.

Holmgren C, Harkany T, Svennenfors B, Zilberter Y (2003) Pyramidal cell communication within local networks in layer 2/3 of rat neocortex. J Physiol 551: 139-153.

Hübener M, Schwarz C, Bolz J (1990) Morphological types of projection neurons in layer 5 of cat visual cortex. J Comp Neurol 301: 655-674.

Jones EG, Coulter JD, Burton H, Porter R (1977) Cells of origin and terminal distribution of corticostriatal fibers arising in the sensory-motor cortex of monkeys. J Comp Neurol 173: 53-80.

Jones EG (1984) Laminar distribution of output cells. In: Cerebral cortex, vol 1, cellular components of the cerebral cortex (Peters A, Jones EG, eds), pp 521-553. New York: Plenum.

Jones EG (2001) The thalamic matrix and thalamocortical synchrony. Trends Neurosci 24: 595-601.

Kalisman N, Silberberg G, Markram H (2005) The neocortical microcircuit as a tabula rasa. Proc Natl Acad Sci U S A 102: 880-885.

Karube F, Kubota Y, Kawaguchi Y (2004) Axon branching and synaptic bouton phenotypes in GABAergic nonpyramidal cell subtypes. J Neurosci 24: 2853-2865.

Kasper EM, Larkman AU, Lübke J, Blakemore C (1994) Pyramidal neurons in layer 5 of the rat visual cortex. I. Correlation among cell morphology, intrinsic electrophysiological properties, and axon targets. J Comp Neurol 339: 459-474.

Kawaguchi Y (1992) Receptor subtypes involved in callosally-induced postsynaptic potentials in

rat frontal agranular cortex in vitro. Exp Brain Res 88: 33-40.

Kawaguchi Y (1993) Groupings of nonpyramidal and pyramidal cells with specific physiological and morphological characteristics in rat frontal cortex. J Neurophysiol 69: 416-431.

Kawaguchi Y, Wilson CJ, Emson PC (1990) Projection subtypes of rat neostriatal matrix cells revealed by intracellular injection of biocytin. J Neurosci 10: 3421-3438.

Kuroda M, Yokofujita J, Murakami K (1998) An ultrastructural study of the neural circuit between the prefrontal cortex and the mediodorsal nucleus of the thalamus. Prog Neurobiol 54: 417-458.

Lei W, Jiao Y, Del Mar N, Reiner A (2004) Evidence for differential cortical input to direct pathway versus indirect pathway striatal projection neurons in rats. J Neurosci 24: 8289-8299.

Lévesque M, Charara A, Gagnon S, Parent A, Deschenes M (1996a) Corticostriatal projections from layer V cells in rat are collaterals of long-range corticofugal axons. Brain Res 709: 311-315.

Lévesque M, Gagnon S, Parent A, Deschenes (1996b) Axonal arborizations of corticostriatal and corticothalamic fibers arising from the second somatosensory area in the rat. Cereb Cortex 6: 759-770.

Lévesque M, Parent A (1998) Axonal arborization of corticostriatal and corticothalamic fibers arising from prelimbic cortex in the rat. Cereb Cortex 8: 602-613.

Lévesque M, Parent A (2005) The striatofugal fiber system in primates: a reevaluation of its organization based on single-axon tracing studies. Proc Natl Acad Sci U S A 102(33): 11888-11893.

Marini G, Pianca L, Tredici G (1996) Thalamocortical projection from the parafascicular nucleus to

layer V pyramidal cells in frontal and cingulate areas of the rat. Neurosci Lett 203: 81-84.

Markram H (1997) A network of tufted layer 5 pyramidal neurons. Cereb Cortex 7: 523-533.

Markram H, Lübke J, Frotscher M, Roth A, Sakmann B (1997) Physiology and anatomy of synaptic connections between thick tufted pyramidal neurones in the developing rat neocortex. J Physiol 500: 409-440.

Mercer A, West DC, Morris OT, Kirchhecker S, Kerkhoff JE, Thomson AM (2005) Excitatory connections made by presynaptic cortico-cortical pyramidal cells in layer 6 of the neocortex. Cereb Cortex 15: 1485-1496.

Metherate R, Ashe JH (1993) Ionic flux contributions to neocortical slow waves and nucleus basalis-mediated activation: whole-cell recordings in vivo. J Neurosci 13: 5312-5323.

Parent A, Charara A, Pinault D (1995) Single striatofugal axons arborizing in both pallidal segments and in the substantia nigra in primates. Brain Res 698: 280-284.

Reiner A, Jiao Y, Del Mar N, Laverghetta AV, Lei WL (2003) Differential morphology of pyramidal tract-type and intratelencephalically projecting-type corticostriatal neurons and their intrastriatal terminals in rats. J Comp Neurol 457: 420-440.

Song S, Sjöström PJ, Reigl M, Nelson S, Chklovskii DB (2005) Highly nonrandom features of synaptic connectivity in local cortical circuits. PLoS Biol 3: 507-519.

Stepanyants A, Hof PR, Chklovskii DB (2002) Geometry and structural plasticity of synaptic connectivity. Neuron 34: 275-288.

Steriade M, Nunez A, Amzica F (1993) Intracellular analysis of relations between the slow (< 1 Hz) neocortical oscillation and other sleep rhythms of the electroencephalogram. J Neurosci 13: 3266-3283.

Stern EA, Jaeger D, Wilson CJ (1998) Membrane potential synchrony of simultaneously recorded striatal spiny neurons in vivo. Nature 394: 475-478.

Stern EA, Kincaid AE, Wilson CJ (1997) Spontaneous subthreshold membrane potential fluctuations and action potential variability of rat corticostriatal and striatal neurons in vivo. J. Neurophysiol. 77, 1697-1715.

Thomson AM, Bannister AP (1998) Postsynaptic pyramidal target selection by descending layer III pyramidal axons: dual intracellular recordings and biocytin filling in slices of rat neocortex. Neuroscience 84: 669-683.

Thomson AM, Bannister AP (2003) Interlaminar connections in the neocortex. Cereb Cortex 13: 5-14.

Thomson AM, Deuchars J (1994) Temporal and spatial properties of local circuits in neocortex. Trends Neurosci 17: 119-126.

Thomson AM, Deuchars J (1997) Synaptic interactions in neocortical local circuits: dual intracellular recordings in vitro. Cereb. Cortex 7, 510-522.

Thomson AM, Morris OT (2002) Selectivity in the inter-laminar connections made by neocortical neurones. J Neurocytol 31: 239-246.

Vercelli AE, Garbossa D, Curtetti R, Innocenti GM (2004) Somatodendritic minicolumns of output neurons in the rat visual cortex. Eur J Neurosci 20: 495-502.

Wang XJ (2001) Synaptic reverberation underlying mnemonic persistent activity. Trends Neurosci 24, 455-.

Wilson CJ (1987) Morphology and synaptic connections of crossed corticostriatal neurons in the rat. J Comp Neurol 263: 567-580.

Wilson CJ (2004) Basal ganglia. In The Syantic Organization of the Brain, fifth edition. (ed Shepherd GM) pp 361-413. Oxford University Press, New York.

Wilson CJ, Groves PM (1981) Spontaneous firing patterns of identified spiny neurons in the rat neostriatum. Brain Res 220: 67-80.

Wilson CJ, Kawaguchi Y (1996) The origins of two-state spontaneous membrane potential fluctuations of neostriatal spiny neurons. J Neurosci 16: 2397-2410.

Zheng T, Wilson CJ (2002) Corticostriatal combinatorics: the implications of corticostriatal axonal arborizations. J Neurophysiol 87:1007-1017.