Segregated Excitatory–Inhibitory Recurrent Subnetworks in Layer 5 of the Rat Frontal Cortex

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Abstract

A prominent feature of neocortical pyramidal cells (PCs) is their numerous projections to diverse brain areas. In layer 5 (L5) of the rat frontal cortex, there are 2 major subtypes of PCs that differ in their long-range axonal projections, corticopontine (CPn) cells and crossed corticostriatal (CCS) cells. The outputs of these L5 PCs can be regulated by feedback inhibition from neighboring cortical GABAergic cells. Two major subtypes of GABAergic cells are parvalbumin (PV)-positive and somatostatin (SOM)-positive cells. PV cells have a fast-spiking (FS) firing pattern, while SOM cells have a low threshold spike (LTS) and regular spiking. In this study, we found that the 2 PC subtypes in L5 selectively make recurrent connections with LTS cells. The connection patterns correlated with the morphological and physiological diversity of LTS cells. LTS cells with high input resistance (Ri) exhibited more compact dendrites and more rebound spikes than LTS cells with low Ri, which had vertically elongated dendrites. LTS subgroups differently inhibited the PC subtypes, although FS cells made nonselective connections with both projection subtypes. These results demonstrate a novel recurrent network of inhibitory and projection-specific excitatory neurons within the neocortex.

Key words: corticopontine cell, corticostriatal cell, frontal cortex, FS cell, LTS cell

Introduction

Cortical circuits consist of various types of excitatory and inhibitory neurons (Jones 1984; Kawaguchi and Kubota 1997; Markram et al. 2004; Gabbott et al. 2005). Maintaining a balance between excitatory and inhibitory synaptic networks is essential for controlling information processing and synaptic plasticity (Markram et al. 1998; Maffei et al. 2004; Hasenstaub et al. 2005; Yoshimura and Callaway 2005). It is therefore important to identify the organizational principles of excitatory and inhibitory synaptic connectivity. Pyramidal cells in layer 5 (L5 PCs) of the cortex are the final output neurons to subcortical and other cortical areas, and have been well classified according to their axonal projection patterns (Jones 1984; Gabbott et al. 2005; Shipp 2007). The rodent frontal cortex contains 2 major PC subtypes in L5: crossed corticostriatal (CCS) cells projecting to both sides of the striatum, and corticopontine (CPn) cells projecting...
to the ipsilateral striatum and pons (Wilson 1987; Reiner et al. 2003). We previously reported that these 2 subtypes differ in their dendritic morphologies, physiological properties, and synaptic connection patterns (Morishima and Kawaguchi 2006; Morishima et al. 2011). There is a hierarchical relationship between these 2 PC subtypes, as CCS cells innervate CPn cells, but the inverse connections are rarely observed. This connection specificity is also reported for the visual (Brown and Hestrin 2009) and motor cortices (Kiritani et al. 2012). These 2 PC subtypes are thought to play different roles in slow wave (Ushimaru and Kawaguchi 2015) and awake states, for example, in movement-planning tasks (Masamizu et al. 2014).

Compared with PCs, cortical inhibitory GABAergic cells are more characterized by the details of their morphologies, firing patterns, and chemical markers. Two major subtypes of GABAergic cells in L5 are the parvalbumin (PV)-positive cells and the somatostatin (SOM)-positive cells. PV cells are a fast-spike (FS) type that generate time-locked inhibition and synchronize activity (Traub et al. 1996; Hasenstaub et al. 2005; Cardin et al. 2009) by innervating the perisomatic area of neighboring PCs (Kawaguchi and Kubota 1997, 1998; Kubota et al. 2015). By contrast, SOM cells have heterogeneous firing patterns, such as low threshold spike (LTS) and regular spike (RS) patterns, as well as a wide variety of morphologies (Kawaguchi and Kubota 1996, 1997; Ma et al. 2006; McGarry et al. 2010; Munoz et al. 2017). Martinoit cells (MCs) are a major subpopulation of SOM cells; these have moderately spiny dendrites and axons that ascend to layer 1, and LTS or RS patterns, and innervate the dendrites of PCs (Kawaguchi and Kubota 1998; Wang et al. 2004). MCs easily induce disynaptic inhibition between tufted L5 PCs (Silberberg and Markram 2007; Hilscher et al. 2017), which suggests that MCs are strongly innervated by subtypes of L5 PCs. The LTS firing pattern typically exhibits rebound spikes following hyperpolarization (Kawaguchi and Kubota 1993, 1996; Goldberg et al. 2004; Hilscher et al. 2017). X98 or Chrm2cre mouse lines may be useful to identify SOM LTS cells (Ma et al. 2006; Hilscher et al. 2017). This specific LTS firing property, in addition to the axonal arborization, may be suitable for generating the slower oscillatory activities (Fanselow et al. 2008; Kopell et al. 2011) that seem to differ from those elicited by another GABAergic cell subtype, the FS cells (Traub et al. 1996; Hasenstaub et al. 2005; Cardin et al. 2009).

LTS cells are mostly located in L5 (Kawaguchi and Kubota 1996; Goldberg et al. 2004; Jiang et al. 2015), which suggests that they play an important role in inhibiting L5 PCs. However, it is not known how LTS cells and PC subtypes in L5 make recurrent networks at the circuit level.

In the present study, we investigated how L5 CPn and CCS cells are connected to LTS and FS cells. Using paired or triple patch-clamp recordings, we found that the synaptic connections of LTS cells with these PC subtypes were more specific than those of FS cells. LTS cells exhibit morphological and physiological diversity in L5 of the rat frontal cortex. CPn cells made connections with diverse LTS cells, but CCS cells only made connections with a part of the LTS cells. Furthermore, both CPn and CCS cells made reciprocal connections with different populations of LTS cells. Consequently, LTS cells formed segregated recurrent inhibitory circuits with each of the 2 PC subtypes. By contrast, CPn and CCS cells made similar recurrent networks with FS cells. These results reveal a novel form of excitatory/inhibitory recurrent microcircuits in L5 of the frontal cortex.

**Materials and Methods**

**Animals**

Male and female Wistar rats (postnatal days [p] 17–21) were anesthetized with a mixture of ketamine (40 mg/kg, intramuscular injection [i.m.]) and xylazine (4 mg/kg, i.m.), followed by an injection of glycerol (0.6 g/kg, intraperitoneal injection [i.p.]) and dexamethasone (1 mg/kg, i.m.). Alexa Fluor 555-conjugated cholera toxin subunit B (CTB555, Invitrogen, Carlsbad, CA, USA) and red RetroBeads (Lumafluor, Naples, FL, USA) were injected into the pons or contralateral striatum with glass pipettes and a Picopump (PV820, World Precision Instruments, Sarasota, FL, USA) (Morishima and Kawaguchi 2006; Morishima et al. 2011).

**Tracer Injection**

Wistar rats (Charles River Laboratories Japan, Inc., Tsukuba, Japan) of either sex and aged from 2 to 6 weeks were used for the experiments. All experiments were conducted according to the guidelines of The Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences, Japan.

**Viral Injection**

Adeno-associated virus (AAV) DJ-Ef1a-DIO ChETA-EYFP (Addgene 26968) (Gunaydin et al. 2010) vectors were prepared using the Helper Free Expression System (Cell Biols, Inc., San Diego, CA, USA). A neuron-specific retrograde gene transfer virus construct (NeuRet-Cre) was prepared as previously described (Kato and Kobayashi 2014), by pseudotyping the HIV-1 vector encoding Cre-recombinase with fusion glycoprotein type E (PuG-E). The NeuRet-Cre virus is deficient in amplification ability. Wistar rats at p14–17 were anesthetized with ketamine (40 mg/kg, i.m.) and xylazine (4 mg/kg, i.m.), followed by isoflurane anesthesia (1% in O2, v/v). Glycerol (0.6 g/kg, i.p.) and dexamethasone (1 mg/kg, i.m.) were administered. Glass pipettes (tip size: 20 μm for the frontal cortex and 40 μm for the contralateral striatum) and Nanoject II injectors (Drummond Scientific, Broomall, PA, USA) were used to inject AAV into the frontal cortex (2–3 mm anterior to the bregma, 1.0–2.5 mm lateral to the bregma, and 0.5–1.0 mm in depth) and NeuRet-Cre into the contralateral striatum.

**Slice Recording**

After 2 or 3 days of tracer injection (rats at p19–23), animals were deeply anesthetized by isoflurane and decapitated. Oblique horizontal slice sections of 300 μm thickness were made from the medial agranular area of the frontal cortex in ice-cold oxygenated buffer solution (containing [in mM] 124 NaCl, 3 KCl, 1 NaH2PO4, 26 NaHCO3, 10 glucose, 5 MgCl2, 1 CaCl2, pH 7.4). For adult rats (>5 weeks old), 3–4 weeks after viral injection, oblique slice sections (300 μm) were made in ice-cold oxygenated buffer solution (containing [in mM] 95 NMDG [N-methyl-d-glucamine], 93 HCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 25 glucose, 10 MgSO4, 0.5 CaCl2, 2.5 sodium ascorbate, 1.5 sodium pyruvate) (Ting et al. 2014). Slices were incubated in oxygenated artificial cerebrospinal buffer solution (containing [in mM] 124 NaCl, 3 KCl, 1 NaH2PO4, 26 NaHCO3, 10 glucose, 1.2 MgCl2, 2.4 CaCl2, 4 lactic acid, pH 7.4) in a submerged chamber at room temperature. Lactic acid was omitted during the recordings. The K+-based pipette solutions...
**Biocytin Staining**

After recording, slices containing biocytin-loaded cells were fixed overnight with 4% paraformaldehyde in phosphate buffer (PB). The slices were washed with PB and resectioned to a thickness of 50 μm. Some of the slices were stained by streptavidin-conjugated Alexa Fluor 594 (1:2000; s11227, Invitrogen) or Alexa Fluor 488 (1:2000; s11223, Invitrogen) in 0.05 M Tris-buffered saline (TBS) containing Triton X-100 (TX) for 40 min to 2 h. The sections were then incubated overnight at 4 °C with 1% ABC Elite (Vector Laboratories, Inc. Burlingame, CA, USA) in 0.05 M TBS containing 0.04% TX. After washing in TBS, the sections were reacted with 0.02% diaminobenzidine tetrahydrochloride and 0.001% H2O2 in 0.05 M Tris buffer. Cells were postfixed in 1% OsO4 in 0.1 M PB containing 7% glucose, and then dehydrated and embedded on glass slides in Epon resin.

**Data Analysis**

Electrophysiological data were analyzed using IGOR Pro (WaveMetrics, Oswego, OR, USA) (Morishima and Kawaguchi 2006; Morishima et al. 2011). Input resistances, time constants, and sag ratios were measured by 50 pA negative current injections for 1 s with a K+-based internal solution. Steady-state voltage was used for Ri measurement. Ri values of LTS cells obtained by linear fitting of voltage changes from −10 to −50 pA pulses at 10 pA interval steps were compared with those from the −50 pA pulse alone. There were no statistically significant differences between the Ri values obtained from the linear fitting of 5 different pulses and those obtained from the −50 pA pulses (P = 0.84, n = 16). The time constant was obtained by exponential fitting of the initial 50 ms trace, while the sag ratio was measured from the difference between the maximum and steady-state voltage. To obtain the release probability, a mathematical model of presynaptic release was applied to successive amplitudes of EPSC in response to a train of 8–15 presynaptic spikes at 40 Hz, followed by a single presynaptic spike 500 ms later (the phenomenological approach) (Tsodyks and Markram 1997; Markram et al. 1998; Wang et al. 2006). Presynaptic action potentials were induced by current injection of 5 ms duration.

The stained cells were reconstructed using NeuroLucida (MBF Bioscience, Williston, VT, USA), and analyzed quantitatively with NeuroExplorer (MBF Bioscience). Approaching sites between the LTS cell axon and PC dendrite were identified by their coexistence in the same voxel (5 μm × 5 μm × 5 μm) using IGOR Pro (WaveMetrics, Oswego, OR, USA).

Data are given as mean ± SD. Statistical comparisons between 2 groups were performed using the nonparametric Mann–Whitney U test or Fisher’s exact test. Multiple comparisons were performed using Dunn’s test. For one-sample t-test, a normality test was performed when the sample size was larger than 7. A value of P less than 0.05 was considered to indicate a statistically significant difference.

**Results**

**PC Subtypes Selectively Connected to LTS Cells, But not to FS Cells**

To investigate the excitatory connections with the 2 major GABAergic cells, we performed dual or triple patch-clamp recordings from the 2 PC subtypes in L5 and neighboring FS or LTS cells (Fig. 1A1, A2). The 2 PC subtypes were identified by retrograde tracer injection into the ipsilateral pontine nuclei and contralateral striatum, respectively (Fig. 1B). FS and LTS cells were then identified by their firing patterns (Fig. 1A1, A2). FS cells expressing parvalbumin account for about half of the GABAergic population in L5 (Uematsu et al. 2008), and were identified as those with no adaptation firing to the depolarizing current injection (Fig. 1A1). LTS cells were identified as those having rebound spikes on slow depolarizing humps resulting from hyperpolarized current injection (Fig. 1A2).

To examine the connections from the 2 PC subtypes to FS or LTS cells, we elicited 2 or 4 repetitive presynaptic spikes in PCs (40 ms interval, 25 Hz; Fig. 1C1, C2), and compared the occurrences of EPSCs in FS or LTS cells, as a measure of connection probability. We found that the connection probability from CPn to FS cells (CPn/FS pairs, 0.32) was not different from that from CCS to FS cells (Fig. 1D; CCS/FS pairs, 0.22, P = 0.26, Fisher’s exact test). By contrast, the connection probability of CPn/LTS pairs was 2-fold higher than that of CCS/LTS pairs (Fig. 1D; 0.28 in CPn/LTS pairs; 0.13 in CCS/LTS pairs; P = 0.032, Fisher’s exact test). The 2 PC subtypes similarly innervated FS cells, whereas CPn cells innervated LTS cells more frequently than did CCS cells. These results indicate that the 2 PC subtypes make recurrent networks with LTS cells in a different manner.

**Physiological Diversity of LTS Cells in L5**

L5 LTS cells have been previously classified as one subgroup of SOM-positive cells (Kawaguchi and Kubota 1996; Goldberg et al. 2004). They have moderately spiny dendrites and axons that extend vertically towards layer 1 (Fig. 2A1). These morphological features are characteristic of MCs. LTS cells induced rebound spikes on slow depolarizing humps after the cessation of hyperpolarized currents; these were blocked by tetrodotoxin (TTX) and NiCl2, respectively (Fig. 2A2). This implies that the rebound sodium spikes were elicited by the depolarizing humps mediated by T-type calcium channels. To examine the physiological property of LTS cells, we measured the area of the depolarizing humps after TTX application. The hump area (range: 72–486 mV ms) correlated with the number of rebound spikes prior to the TTX application (n = 12, Supplementary Data 1). The hump durations varied from 120 μs to 1.8 ms even in the same cell (Fig. 2B) and correlated with the rebound amplitudes of LTS cells (Fig. 2C).
Fig. 1A). The input resistances (Ri) of LTS cells were diverse (353 ± 144 MΩ, n = 125; Fig. 2B, left). The Ri values were comparable to those reported previously (337 ± 11 MΩ, n = 36 in Hilscher et al. 2017). In addition to the hump area, the rebound spikes correlated positively with the Ri (n = 119, P < 0.001; Fig. 2B, right). The sag ratio obtained from the hyperpolarizing voltage response negatively correlated with the Ri (n = 125, P < 0.001; Supplementary Fig. 1B), suggesting larger hyperpolarization-activated (Ih) currents in LTS cells with lower Ri. Thus, LTS cells demonstrate diverse intrinsic physiological characteristics.

The relationship between the intrinsic physiological characteristics and dendritic morphology of L5 LTS cells has not been well studied, and so we therefore quantitatively investigated the dendritic morphology of L5 LTS cells (Fig. 2C). The horizontal length of the dendrites was not significantly correlated with Ri (n = 29, P = 0.22; Fig. 2D), but the vertical dendritic length demonstrated a strong negative correlation with Ri (n = 29, P = 0.002; Fig. 2E). The higher-Ri LTS cells had shorter vertical dendrites and larger rebound spike numbers. The position of the soma measured from the pia correlated weakly with the input resistance: LTS cells with lower Ri tended to be located in deeper L5 (n = 52, P = 0.0119, Supplementary Fig. 1C). The Ri was not different between L5a and L5b. On the other hand, the axonal length in L1 and L2/3 of LTS cells did not correlate with Ri (n = 7, P = 0.57, Supplementary Fig. 1D). Thus, LTS cells were shown to have diverse physiological properties correlating with dendritic length.

PC Subtypes Selectively Innervate Diverse LTS Cells

Next, we examined whether the 2 PC subtypes innervated the specific or diverse LTS cell types mentioned above. We found that CPn cells innervated a whole population of LTS with a range of low to high Ri values (467 ± 164 MΩ, range: 195–831 MΩ), while CCS cells only innervated a part of the population having low Ri (220 ± 78.5 MΩ, range: 115–369 MΩ; P = 0.0006, Mann–Whitney U test; Fig. 3A). These findings indicate that CCS cells specifically innervate a subpopulation of LTS cells.

The findings shown in Figure 3A suggest that CCS cells innervate low-Ri LTS cells, but not high-Ri LTS cells. To confirm this connection specificity with another approach, we performed optogenetic stimulation of the CCS cell population (Fig. 3B). Light-activated opsins ChETA was selectively expressed in CCS cells by injection into the contralateral striatum of a retrogradely transferred virus encoding Cre-recombinase, coupled with an injection of a virus encoding Cre-dependent ChETA into the frontal cortex (Fig. 3B, left). After allowing 3 weeks for expression of ChETA in CCS cells, we prepared brain slices and recorded the synaptic responses induced by photo-activation of ChETA. We confirmed that the ChETA-expressing CCS cells fired action potentials in response to depolarizing current pulses that were comparable to those of noninfected CCS cells (Fig. 3B, lower right), and that they also fired in response to photostimulation (n = 10; Fig. 3B, upper right). We recorded from pairs of neighboring non-PCs, where each pair included a LTS cell, with the other cell being used as a reference (e.g., a FS cell; Fig. 3C, D). EPSCs in the reference cell confirmed the effective optogenetic stimulation of neurons in the surrounding area. LTS cells responding to optogenetic excitation of CCS cells had low Ri (<400 MΩ, n = 4), but nonresponsive LTS cells were more diverse, and included cells with higher input resistances (Fig. 3E). This result confirms that CCS cells preferentially innervate a subpopulation of LTS cells.

Excitatory Synaptic Properties of the PC Subtypes Onto LTS and FS Cells

We compared the excitatory synaptic properties from the different PC subtypes to FS cells and LTS cells. We induced
repetitive action potentials in presynaptic PCs (2–4 spikes, 40 ms intervals [25 Hz]; Fig. 1), and analyzed the EPSCs induced by the first action potentials (E1), and the amplitude ratios of successive EPSCs.

In the connections from PCs to FS cells, E1 was always detected. The E1 amplitudes were similar between CPn/FS and CCS/FS pairs (32.0 ± 25.5 pA in CPn/FS and 37.3 ± 28.8 pA in CCS/FS, P = 0.46, Mann–Whitney U test; Fig. 4A). The EPSC latencies, rise times, and decay time constants were also not significantly different between CPn/FS and CCS/FS pairs (Fig. 4A).

By contrast, in connections from the PCs to LTS cells, E1 was not always detected. Although in some pairs E1 was not detected, the second (E2) or third (E3) response appeared due to strong synaptic facilitation (Supplementary Fig. 2A). E1 was detected in 58% of CPn/LTS connected pairs (n = 11 of 19) and in 89% of CCS/LTS connected pairs (n = 8 of 9; Supplementary Fig. 2B). We compared the EPSC characteristics between CPn/LTS and CCS/LTS pairs, and found that the E1 amplitudes did not differ significantly between them (4.6 ± 3.5 pA in CPn/LTS and 5.4 ± 3.5 pA in CCS/LTS, P = 0.54, Mann–Whitney U test; Fig. 4B). The EPSC latencies and rise times were also not significantly different between them, but the EPSC decay time constant was faster in CPn/LTS pairs than in CCS/LTS pairs (P = 0.0153, Mann–Whitney U test; Fig. 4B).

We previously reported that short-term plasticity (STP) depends on the presynaptic PC subtype (Morishima et al. 2011). This suggests that the excitatory STP to GABAergic cells may also depend on the presynaptic PC subtype. We then compared the paired pulse ratio (PPR) of 2 successive EPSCs elicited by PC stimulation at 10 Hz (pp10), 25 Hz (pp25), and 40 Hz (pp40; Fig. 4C, D).

Figure 2. Physiological and morphological heterogeneity of LTS cells. (A1) Reconstruction of a LTS cell (soma and dendrites in black, axons in red). (A2) Firing patterns of LTS cells. *Low threshold spike (LTS). The LTS was resistant to application of TTX (1 μM), but was reduced by further application of NiCl2 (20 μM). (B) Relationship of the numbers of spikes riding on the humps of LTS cells with Ri. The maximum number of spikes was used. Left, distribution of the Ri. (C) Reconstructions of LTS cells (somata and dendrites). The reconstructed LTS cells are positioned according to their Ri. Dendrite morphology is related to the Ri. (D) The relationship between the horizontal length (shown in the inset) and Ri. (E) Relationship of Ri with vertical dendritic lengths (shown in the inset).

Figure 3. Target-dependent differences in the postsynaptic LTS property. Input resistances of LTS cells innervated by CPn cells or CCS cells. **P < 0.01. (B) Schematic of retrograde virus labeling of CCS cells. NeuN-Cre lentivirus was injected into the contralateral striatum, and AAV was injected into the frontal cortex. Upper, a ChETA-expressing CCS cell elicited 8 repetitive action potentials in response to each laser stimulation at 40 Hz. Lower, firing pattern of the same cell in response to an intracellular current (200 pA). (C) EPSCs induced in LTS and FS cells by photostimulation at 40 ms intervals. Their firing patterns in response to current injection are shown on the right. (C1) ChETA expression in CCS cells. The red fluorescent cells are the FS and LTS cells recorded in C1. (D) Photo-activated EPSC amplitudes of 2 cells (including a LTS cell) recorded in the same slice (connected by lines). (E) Input resistances of LTS cells with and without the photo-activated synaptic response. Ri = 235 ± 60 MΩ for LTS cells with response (n = 4) and 384 ± 202 MΩ for LTS cells with no response (n = 16), P = 0.48.
Excitatory STP is Dependent on Both the Presynaptic PC Subtypes and Postsynaptic LTS Cell Subpopulations

In the previous section, the PPR was calculated only from PC/LTS pairs in which E1 was detected. To observe the STP for repetitive PC inputs in all pairs, we compared 8 consecutive responses induced by presynaptic stimulation at 40 Hz (Fig. 5A). The successive EPSC amplitudes were normalized to the eighth EPSC. The EPSCs in CCS/LTS pairs reached maximum amplitude earlier than in CPn/LTS pairs, and then reduced (Fig. 5B). However, in CPn/LTS pairs the EPSCs demonstrated a more gradual increase in amplitude. The normalized amplitudes for the first four EPSCs were significantly different between the CPn/LTS pairs and CCS/LTS pairs (Fig. 5B). Thus, the 2 PC subtypes drove the different LTS populations with different time courses.

This different temporal activation of LTS cells probably reflects a difference in presynaptic properties. To compare the presynaptic release properties between the PC subtypes, we applied phenomenological analysis of successive EPSCs to calculate U (baseline level of fraction of resources U, analogous to the release probability) (Markram et al. 1998). U was significantly smaller in CPn/LTS pairs (0.05 ± 0.07; n = 13) than in CCS/LTS pairs (0.13 ± 0.12, n = 7; P = 0.0324; Fig. 5C right). Furthermore, U was more variable in CPn/LTS (CV = 1.38) than in CCS/LTS (CV = 0.95), which was probably because the LTS cells targeted by CPn cells were more diverse. Larger U from CPn cells was found in connections to low-Ri LTS cells (Fig. 5C left). These data suggest that CCS cells make excitatory synapses with higher release probabilities on lower-input resistance LTS cells, whereas CPn cells make excitatory synapses with higher and lower release probabilities on diverse LTS cells.

Different Populations of LTS Cells Develop Distinct Reciprocal Connections With CPn and CCS Cells

We next investigated how FS or LTS cells innervate the 2 PC subtypes in L5 (Fig. 6A, D). The inhibitory connection probabilities of FS/CPn pairs were similar to those of FS/CCS pairs (Fig. 6B; 0.48 in FS/CPn pairs and 0.37 in FS/CCS pairs, P = 0.23, Fisher’s exact test). For both pairs, the inhibitory connection probability (reciprocal connection probability) to the PC/FS pairs was higher than the overall inhibitory connection probability (0.83 in CPn/FS pairs, P = 0.0038, 0.8 in CCS/FS, P = 0.0037, Fisher’s exact test Fig. 6B). The IPSC amplitudes from FS cells to each PC subtype with an internal solution containing K⁺ were not different (P = 0.403; Supplementary Fig. 3A). To improve the spatial clamp of the membrane potential, we also used a Cs⁺-based internal solution for postsynaptic PC recordings (K⁺-based internal solution for presynaptic recordings); the inhibitory connection

In connections from CPn to FS cells, the PPR was almost constant at each frequency (0.97 for 10 Hz [P = 0.56; one-sample t-test], 1.0 for 25 Hz [P = 0.97], and 1.0 for 40 Hz [P = 0.99]). By contrast, in connections from CCS to FS cells, the PPR was depressive at each frequency (0.66 for 10 Hz [P < 0.01], 0.61 for 25 Hz [P < 0.01], and 0.42 for 40 Hz [P < 0.01]; Fig. 4C). Thus, the STP from PC subtypes to FS cells is dependent on the presynaptic PC subtype.

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probability and IPSC amplitude from FS cells were not different between the PC subtypes (P = 0.56 and 0.97, respectively; Supplementary Fig. 3B,C).

In FS/CPn pairs, the IPSC amplitude was larger in reciprocal (50 ± 38.6 pA) than one-way connections (21.1 ± 15 pA, P = 0.014; Fig. 6C). In FS/CCS pairs, however, the IPSC amplitude did not differ between reciprocal (64.7 ± 73.2 pA) and one-way connections (45.5 ± 55.2 pA, P = 0.23; Fig. 6C). This result indicates that inhibition strength depends on the reciprocity of FS/CPn pairs, but not on that of FS/CCS pairs.

Furthermore, we analyzed whether individual FS cells innervate both CPn and CCS cells, performing triple recordings from a FS cell and 2 PCs of the same or different subtypes. In dual inhibition cases (divergence from a single FS cell), there were no significant differences in the double innervation probability among the 3 types of postsynaptic cell combinations (Supplementary Fig. 3D).

In contrast to inhibitory connections from FS cells, those from LTS cells were different between the PC subtypes. The inhibitory connection probability of LTS to CPn (LTS/CPn) pairs was higher than that of LTS to CCS (LTS/CCS) pairs (Fig. 6E,F; 0.39 in LTS/CPn pairs and 0.086 in LTS/CCS pairs, P < 0.0001, Fisher’s exact test). This connection specificity was also found in Cs+-based internal solution for postsynaptic PC recordings (P = 0.0065; Supplementary Fig. 4B). The inhibitory connection probability (reciprocal connection probability) to the CPn/LTS pairs was not different from the overall inhibitory connection probability in LTS/CPn pairs (0.58, P = 0.192; Fig. 6E,F), whereas that to the CCS/LTS pairs was higher than the overall inhibitory connection probability in LTS/CCS pairs (0.5, P = 0.0079, Fisher’s exact test; Fig. 6E,F).

The IPSC amplitudes from LTS cells did not significantly differ between the PC subtypes in the K+-based solution (P = 0.87, Mann–Whitney U test; Supplementary Fig. 4A), and also in a Cs+-based internal solution for postsynaptic PC recordings (P = 0.87; Supplementary Fig. 4C).

To know which domains of PC dendrites the LTS cells innervate, the approaching sites were obtained from LTS/CPn or LTS/CCS pairs (Supplementary Fig. 5A). LTS cell axons approached L5 PCs not only on the distal dendrites in L1 and L2/3 but also proximal dendrites in L5 (Supplementary Fig. 5A,B). These results suggest that LTS Martinotti cells innervate basal and apical oblique dendrites in addition to the apical tufts.

Similar to the excitatory connections from PC subtypes to LTS cells, inhibitory connections depended on both the PC subtype and LTS cell heterogeneity. CPn cells were innervated by LTS cells with either low or high Ri (381 ± 131 MΩ, range: 196–742 MΩ), but that CCS cells were innervated by those with a low-Ri (234 ± 145 MΩ, range: 115–519 MΩ; P = 0.011, Mann–Whitney U test; Fig. 6G). The IPSC amplitude was similar in one-way and reciprocal connections in both LTS/CPn and LTS/CCS pairs, suggesting the inhibition strength is not dependent on the reciprocity of LTS/PC pairs (Fig. 6F). However, LTS cells reciprocally connected with CPn cells had higher Ri (469 ± 142 MΩ, range: 196–742 MΩ) than LTS cells reciprocally connected with CCS cells (176 ± 51.5 MΩ, range: 115–240 MΩ; P < 0.01, Dunn’s test; Fig. 6H). This suggests that the high-Ri LTS cells predominantly have reciprocal connections with CPn cells, while the low-Ri LTS cells mainly have reciprocal connections with CCS cells. These findings therefore indicate that subpopulations of LTS cells make reciprocal connections with CCS or CPn cells.

**Discussion**

Excitatory and inhibitory interactions are critical for processing cortical information (Markram et al. 1998; Maffei et al. 2004;
Figure 6. Inhibitory synaptic properties in CPn and CCS cells originating from FS and LTS cells. (A) Morphologies of synaptically connected FS and CCS cells. Presynaptic FS cell, dendrites/soma in black and axons in red; postsynaptic CCS cell, dendrites/soma in gray. Upper inset, successive IPSCs in the CCS cells in response to FS cell spikes at a 40 ms interval. Lower inset, firing pattern in response to a depolarizing current pulse (200 pA, 1 s). (B1) Inhibitory connection probabilities of FS/CPn and FS/CCS pairs in K^+ internal solutions. Gray colored area, reciprocally connected pairs. (B2) Connection patterns between FS cells and the 2 PC subtypes. Four patterns: no connection (NC), unidirectional excitation (Ex), reciprocal connection (Rec), and unidirectional inhibition (In). The inhibitory connection probability with excitatory connection in FS/CPn pairs (thick border; 19/23) was higher than the overall inhibitory connection probability in FS/CPn pairs (B1; 35/73), and that in FS/CCS pairs (thick border; 12/15) was also higher than the overall probability in FS/CPn pairs (B1; 25/69). (C) Comparison of IPSC amplitudes in FS/CPn and FS/CCS pairs between reciprocal and one-way connections recorded with a K^+ based internal solution. Filled and open triangles, CPn cells; filled and open circles, CCS cells. (D) Morphologies of synaptically connected LTS and CPn cells. Presynaptic LTS cell, dendrites/soma in black and axons in red; postsynaptic CPn cells, dendrites/soma in gray. Lower inset, IPSCs from the LTS to CPn cells. (E1) Inhibitory connection probabilities recorded with a K^+ based internal solution. Gray colored area, pairs with a reverse excitatory connection from the PC (reciprocal connections). **P < 0.01. (E2) Connection patterns between LTS cells and the 2 PC subtypes. The inhibitory connection probability with excitatory connection in LTS/CPn pairs (thick border; 11/19) was similar to the overall inhibitory connection probability in LTS/CPn pairs (E1, 27/69). The inhibitory connection probability with excitatory connection in LTS/CCS pairs (4/8) was higher than the overall probability in LTS/CCS pairs (E1, 6/70). In 1 of 5 LTS/CCS pairs with excitatory connection, the inhibitory connection was not tested. (F) Comparison of IPSC amplitudes in LTS/CPn and LTS/CCS pairs between reciprocal and one-way connections recorded with a K^+ based internal solution. Filled and open triangles, CPn cells; filled and open circles, CCS cells. *P < 0.05. (G) Input resistances of LTS cells innervating CPn and CCS cells. **P < 0.01. (H) Input resistances of reciprocally and one-way connected LTS/CPn and LTS/CCS pairs. *P < 0.05.
Hasenstaub et al. 2005; Yoshimura and Callaway 2005). It is therefore important to understand the synaptic connection patterns between different subtypes of excitatory and inhibitory cortical neurons. Neocortical GABAergic cells are well classified by chemical markers such as PV and SOM, and are functionally distinct (Kawaguchi and Kubota 1997; Markram et al. 2004; Kepecs and Fishell 2014). We previously reported that the 2 PC cell subtypes, CPn (corresponding to pyramidal tract types) and CCS (belonging to intratelencephalic types), make distinct excitatory recurrent networks that differ in their target specificity and synaptic transmission properties (Morishima and Kawaguchi 2006; Morishima et al. 2011). More specifically, there exists a hierarchical connection between CCS and CPn cells, because CCS cells connect with CPn cells unilaterally. However, the specificity of recurrent networks, including both PC and GABAergic cell subtypes, remains poorly understood. In this study, we demonstrated novel synaptic circuits between GABAergic cells and the 2 PC subtypes, CPn and CCS cells (Fig. 7). We found that CPn cells make connections with diverse populations of LTS cells, but have more selective reciprocal connections with a cell population with high Ri. The release probability from CPn cells to high-Ri LTS cells was lower than that to low-Ri LTS cells. CCS cells make reciprocal connections with a population of low-Ri LTS cells, with the high release probability from CCS cells (Fig. 7). The excitatory STPs between the 2 PC subtypes and LTS cells were significantly different. By contrast, the synaptic properties between the FS cells and these PC subtypes were similar, except for the frequency-dependent STP onto the FS cells (Fig. 7).

Connections Between FS cells and the 2 PC Subtypes

Individual PV cells randomly make inhibitory synapses with PCs in L5 of the somatosensory cortex (Fino and Yuste 2011; Packer and Yuste 2011). In addition, a single FS cell receives local excitatory inputs from different PC subtypes (Otsuka and Kawaguchi 2013). Here, we report that the excitatory and inhibitory connection probability and reciprocity did not differ between the 2 PC subtypes and FS cells (Fig. 7, left). Thus, FS cells connect with PCs irrespective of the projection subtype, although there are some reports describing connection selectivity. Groups of PV cells that are excited optogenetically by commissural afferents induce inhibition in a L5 PC subtype, the strength of which depends on the cortical area; that is, PV cells more often innervate the pyramidal tract type in the prefrontal cortex, whereas they do the intratelencephalic type in the auditory cortex (Lee et al. 2014; Rock and Apicella 2015). FS cells that receive input from the thalamus preferentially innervate intrinsic bursting cells that are supposed to be the pyramidal tract type in L5b of the auditory cortex (Sun et al. 2013). FS cells receive convergent excitation from the PC subtypes locally, but may differ in inputs from the other cortical areas or thalamus. Thalamic input to PV cells is heterogeneous, depending on the layer location of the PV cells in the frontal cortex (Shigematsu et al. 2016). FS basket cells preferentially innervate the perisomatic area in some PCs, and the dendritic region in other PCs, with the synaptic strength depending on the contact domain of the PCs (Kubota et al. 2015). FS cells receiving specific external inputs may preferentially innervate the perisomatic area of a particular PC subtype, which could generate differences in inhibition strength among PCs.

FS cells preferentially inhibit PCs that excite them (Yoshimura and Callaway 2005; Otsuka and Kawaguchi 2009), and electrically connected FS cells share excitation from common PCs (Otsuka and Kawaguchi 2013). These features may be related to gamma oscillations generated by the reciprocal connections between FS cells and PCs (Hasenstaub et al. 2005; Sohal et al. 2009). During the gamma rhythm within the cortical up-state, both CPn and CCS cells fire in earlier phases of gamma cycles in comparison with FS cells, suggesting that excitation from the PC to FS cell is important for gamma wave generation (Ushimaru and Kawaguchi 2015).
Connections Between LTS Cells and the 2 PC Subtypes

A previous study showed that, in L2/3 of the frontal cortex, SOM cells make highly divergent connections with nearby PCs (Fino and Yuste 2011). This divergent connection pattern can be explained by just the spatial overlap of SOM cell axons and PC dendrites (Hill et al. 2012; Packer et al. 2013). By contrast, we found that a group of LTS cells selectively inhibit CPn cells, but rarely inhibit CCS cells (Fig. 7, right).

Considering difficulty of the voltage clamp of distal dendrites from the soma, the recorded IPSCs were thought to originate mainly from the proximal synaptic contacts (Bar-Yehuda and Kornberg 2008). By anatomical reconstructions, we found that the approaching sites distribute not only on the apical tufts but also on the basal and apical oblique dendrites of PCs (Supplementary Fig. 5). Therefore, the connections of LTS cells onto the 2 PC subtypes in our findings may represent innervation to the proximal parts of dendrites. As the apical tufts are known to be densely innervated by thalamo-cortical fibers as well as remote cortico-cortical inputs, the inhibition mechanisms by LTS cells in the distal parts might be different from those of the proximal parts.

LTS cells with compact dendrites, high Ri, and more rebound spikes reciprocally connect with CPn cells, but not with CCS cells. The response of LTS cells to repetitive firing of CPn cells is more strongly facilitating. This group of LTS cells probably exerts disynaptic inhibition on L5 thick-tufted PCs in the somatosensory cortex (Le Be et al. 2007; Silberberg and Markram 2007; Munoz et al. 2017). However, CCS cells make reciprocal connections, at lower probabilities, with low-Ri LTS cells. The excitatory response of LTS cells to repetitive firing of CCS cells is significantly different from that from the repetitive firing of CPn cells. These findings could explain why disynaptic inhibition is not observed among commissural cells projecting to the contralateral cortex, including CCS cells (Le Be et al. 2007; Hilscher et al. 2017). Thus, the recurrent inhibition pattern is dependent on both the PC subtype and the MC population.

Functional Roles of GABAergic Cell Subtypes in the L5 Excitatory Circuits

The contrasting recurrent networks between LTS cells and the 2 PC subtypes may regulate cortical synchronized activity differently. Compact LTS cells with high Ri and more rebound spikes preferentially connect with CPn cells. CPn cells exhibit stronger burst firing than CCS cells during up-states of the slow oscillation (Ushimaru and Kawaguchi 2015). Burst firing of L5 PCs could be induced intrinsically by a dendritic plateau spike (Williams and Stuart 1999). SOM cells are often connected by electrical synapses, but rarely by inhibitory synapses (Gibson et al. 1999; Jiang et al. 2015). Therefore, the summated excitatory postsynaptic potentials induced in LTS cells by burst firing may spread to other LTS cells through gap junctions, resulting in synchronized firing among LTS cells. Spikes in one cell could also propagate as spikelets to neighboring coupled cells and help synchronize them (Hu and Aghmon 2015). The membrane potentials of SOM cells, which affect the firing pattern, also depend on the behavioral state (Gentet et al. 2012). Thus, the unique recurrent subnetworks formed with the heterogeneous LTS cells contribute to synchronization of CPn cells, depending on the behavioral situation.

The gamma oscillation of about 40 Hz is generated by the reciprocal connections between FS PV cells and PCs (Hasenstaub et al. 2005; Bartos et al. 2007; Sohal et al. 2009). As PV and SOM cells are mutually connected (Urban-Ciecko and Barth 2016), it would be of relevance to reveal the synaptic properties of SOM cells during 40 Hz oscillatory activity. In the visual cortex, L2/3 SOM cells contribute to generation of the context-dependent gamma rhythm (Veit et al. 2017). In the present study, the repetitive excitatory inputs to LTS cells from CPn cells were more augmented and sustainable at 40 Hz than those from CCS cells. This suggests that, in higher-frequency rhythmic states such as gamma oscillations, CPn cells could effectively drive the connected LTS cells more than the CCS cells could. Thus, different populations of LTS cells may be activated in different oscillatory cortical states.

PC and GABAergic cell subtypes exhibit specific responses to various neuromodulators (Kawaguchi 1997; Kawaguchi and Shindou 1998; Dembrow et al. 2010; Avesar and Gulludge 2012; Gee et al. 2012; Seong and Carter 2012). Spiking and membrane fluctuations of electrically coupled LTS cells are synchronized by activation of metabotropic glutamate or muscarnic acetylcholine receptors (Beierlein et al. 2000). The activities of 2 recurrent subnetworks may be controlled differently by various neuromodulators, depending on the cortical oscillatory states.

The 2 parallel output running from L5 of the frontal cortex to the striatum, the CCS and CPn cells are important for generating appropriate behaviors in cooperation with the basal ganglia (Friend and Kravitz 2014; Gunaydin and Kreitzer 2016). CPn cells are often reciprocally connected with facilitating synapses (Morishima et al. 2011). This connection pattern could work as a reverberating circuit, maintaining the information of past states/actions, which may be used for calculation of the temporal difference error in the basal ganglia (Morita et al. 2012). CPn cells also excite a subpopulation of LTS-Martinotti cells with strong synaptic facilitation. The facilitatory connections from CPn cells to both CPn cells and a subpopulation of GABAergic LTS cells could achieve the excitation/inhibition balance in the reverberating circuits.

Supplementary Material

Supplementary data is available at Cerebral Cortex online.

Funding

JSPS KAKENHI Grant nos. 25250005, 15K14324, 15H01456, and 17H06311 to Y.K.; 15KT0013 to M.M.

Notes

We thank Dr. Kenji Morita for help in electrophysiological analysis, and Drs. Ariel Aghmon, Allan Gulludge, and Attila Losonczy for comments on the article. Conflict of Interest: None declared.

References


