Cortical Inhibitory Cell Types Differentially Form Intralaminar and Interlaminar Subnetworks with Excitatory Neurons

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The neocortical circuit is composed of excitatory principal neurons and inhibitory interneurons. Recent advances have established that multiple subnetworks of synaptically coupled excitatory neurons provide distinct pathways for information flow through the cortical circuit. Here we have investigated how inhibitory interneurons are incorporated into these excitatory subnetworks in the rat frontal cortex. In layer 5 (L5), the probability of reciprocal synaptic connections between pyramidal cells and fast-spiking (FS) interneurons was significantly higher than the probability of reciprocal connections between pyramidal cells and non-FS interneurons. Further, the amplitude of synaptic currents in reciprocally connected FS/pyramidal cell pairs was larger than that in pairs connected only in one direction. To examine interlaminar connection specificity, we stimulated layer 2/3 (L2/3) pyramidal cells, using focal glutamate puff stimulation, and recorded evoked EPSCs in L5 cells. Stimulation of L2/3 cells evoked EPSCs in L5 non-FS cells more frequently than in L5 FS cells. Dual recordings from L5 interneurons and neighboring pyramidal cells revealed that connected non-FS/pyramidal cell pairs were more likely to share excitatory inputs from L2/3 cells than were unconnected cell pairs. On the other hand, the connectivity between L5 FS and pyramidal cell pairs did not affect the common input probability from L2/3. Our results suggest that L5 inhibitory interneurons form distinct intralaminar and interlaminar subnetworks with pyramidal cells, depending on inhibitory cell types.

Introduction

The neocortex is composed of functionally distinct areas, each of which shows a unique anatomical pattern of horizontal layers that contain vertical columnar assemblies of neurons (Felleman and Van Essen, 1991; Mountcastle, 1997). As expected from the columnar organization, neocortical cells are well connected vertically in a direction-selective manner (Weiler et al., 2008; Lefort et al., 2009). Inputs from the thalamus to neurons in superficial cortical layers are relayed to neurons in deeper layers that then provide output to the various subcortical areas (Bureau et al., 2006; Lübke and Feldmeyer, 2007). This excitatory feedforward pathway is now assumed to consist of functionally segregated channels that include both intralaminar and interlaminar connections. Multiple recordings from layer 5 (L5) pyramidal cells showed that synaptically connected pairs of pyramidal cells are more likely to share common inputs than are unconnected pairs of L5 cells (Song et al., 2005). The divergent and convergent probabilities of interlaminar excitatory connections depend on the connectivity of recipient and projecting cell pairs, respectively (Shepherd and Svoboda, 2005; Yoshimura et al., 2005; Kampa et

al., 2006; Otsuka and Kawaguchi, 2008). Moreover, interlaminar subnetworks from layer 2/3 (L2/3) to L5 pyramidal cells depend on L5 pyramidal subtypes that correlate with specific subcortical target areas (Otsuka and Kawaguchi, 2008). Similarly, the local connections made between L5 pyramidal cells depend on their subcortical projection areas (Morishima and Kawaguchi, 2006; Brown and Hestrin, 2009). Thus, both intralaminar and interlaminar excitatory synaptic pathways of pyramidal cells are clustered into subnetworks.

The neocortex is composed of numerous types of excitatory principal neurons and inhibitory interneurons (Kawaguchi and Kubota, 1997; Markram et al., 2004). Pyramidal cell excitability is regulated at various surface domains by GABAergic inputs from specific inhibitory cell types (Somogyi et al., 1998; Gupta et al., 2000; Kubota et al., 2007). Dysfunction of cortical inhibition induces abnormal electrical activities and causes various pathologies such as epilepsy, anxiety, and depression (Sanacora et al., 2000; Holmes and Ben-Ari, 2001; Powell et al., 2003). Despite their importance in regulating excitatory networks, little is known about how inhibitory interneurons are incorporated into excitatory intralaminar and interlaminar subnetworks. In L2/3 of the visual cortex, pairs of pyramidal cells and fast-spiking (FS) inhibitory interneurons preferentially form reciprocal connections between them. These connected cell pairs receive common inputs from excitatory neurons in layer 4 (L4) with a higher probability than do other unconnected cell pairs (Yoshimura and Callaway, 2005).

In this study, we investigated how L5 inhibitory interneuron cell types are connected with pyramidal cells in intralaminar and

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interlaminar networks. We examined connections between L5 inhibitory interneurons and neighboring pyramidal cells, and also their excitatory inputs from L2/3 pyramidal cells. Our results suggest that L5 FS and pyramidal cells make reciprocally connected modules. Contrary to observations in the L4 to L2/3 pathway of the visual cortex, we found that these modules do not preferentially receive common inputs from L2/3. On the other hand, we reveal that connected pairs of L5 non-FS/pyramidal cell pairs are preferentially targeted for common input from L2/3 cells.

Materials and Methods

Whole-cell recordings in slice. All experiments were conducted in compliance with the guidelines for animal experimentation of the Okazaki National Research Institutes. To easily identify inhibitory interneurons in slice preparations, VGAT-Venus transgenic rats expressing fluorescent protein Venus in GABAergic neurons were used (Uematsu et al., 2008). VGAT-Venus transgenic rats were generated by Drs. Y. Yanagawa, M. Hirabayashi, and Y. Kawaguchi at National Institute for Physiological Sciences, using pCS2-Venus provided by Dr. A. Miyawaki. VGAT-Venus rats are distributed from The National BioResource Project for the Rat in Japan (<http://www.anim.med.kyoto-u.ac.jp:80/nbr/default.aspx>). We also obtained brain slices from wild-type Wistar rats. Slice preparations including frontal cortex were obtained from animals aged postnatal 19–23 d, as described previously (Otsuka and Kawaguchi, 2008). Slices (300 μ m thick) were incubated in an oxygenated artificial CSF (ACSF) composed of the following (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 1 MgCl_2 , 2 CaCl_2 , 26 NaHCO_3 , and 10 glucose (310 ± 5 mOsm/L, pH 7.4; bubbled with 95% O_2 –5% CO_2) containing 0.2 mM ascorbic acid and 4 mM lactic acid at room temperature. Whole-cell recordings were performed using an EPC-9 double amplifier (HEKA Elektronik). Temperature of the bath solution in the recording chamber was adjusted to 30°C. The recording pipettes were filled with a solution containing the following (in mM): 130 potassium methylsulfate, 0.5 EGTA, 2 MgCl_2 , 2 Na_2ATP , 0.2 GTP, 20 HEPES, 0.1 leupeptin, and 0.75% biocytin (pH 7.2, 290 ± 5 mOsm/L). In some experiments, Cs^+ was included in the pipette solution for pyramidal cell recordings to reduce membrane leak conductance and increase membrane input resistance and, as a consequence, electrotonic distance. Cl^- concentration in the pipette for pyramidal cell recording was also increased for depolarization of GABAergic reversal potential to improve detection sensitivity for IPSCs as inward currents near resting potentials. The pipettes for pyramidal cell recordings were filled with a solution containing the following (in mM): 100 potassium gluconate, 60 CsCl, 0.5 EGTA, 2 MgCl_2 , 2 Na_2ATP , 0.2 GTP, 20 HEPES, and 0.1 leupeptin (pH 7.2, 285 ± 5 mOsm/L) (Cs^+ /high- Cl^- electrodes), predicting GABAergic current reversal potential of -20 mV. To evoke a spike in a L2/3 pyramidal cell, we focally applied glutamate to a L2/3 cell, as described previously (Otsuka and Kawaguchi, 2008). Sodium glutamate (1 mM) were dissolved in ACSF and filled into the same pipettes as those for whole-cell recordings. The pipette filled with glutamate was positioned within 10 μ m distance from the soma of targeted neuron, and air pressure (5–10 psi, 50 ms duration) was added to eject glutamate. To analyze the unitary EPSCs and IPSCs kinetics, we measured the rise time as defined as the time from 10 to 90% of the amplitude from the baseline to the peak. Decay time was defined as interval between peak current amplitude and 30% of amplitude. Data are represented as mean $\pm$ SD, and statistical difference between samples was tested using ANOVA or Mann–Whitney U test for comparison of two groups. Significance was accepted when $p < 0.05$.

Histology. Tissue slices containing biocytin-labeled cells were fixed with a solution containing 4% paraformaldehyde, 1.25% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate-buffered solution (PB). After incubating tissues in PB containing 1% H_2O_2 , slices were resectioned at a thickness of 50 μ m and then incubated with avidin–biotin–horseradish peroxidase complex (1%; Vector Laboratories) in 0.05 M Tris-buffered saline (TBS) with 0.5% Triton X-100 overnight at 4°C. After washing in

TBS, sections were reacted with a mixture of 3,3'-diaminobenzidine tetrahydrochloride (0.02%), nickel ammonium sulfate (0.3%), and H_2O_2 (0.001%) in TBS. Then, sections were postfixed in 1% OsO_4 in PB containing 7% glucose, rinsed in PB, dehydrated in graded ethanol series, mounted on glass slides, and coverslipped with Epon for observation with a light microscope. Neurolucida system (MicroBrightField) was used for a reconstruction of stained cell.

Results

Inhibitory interneurons in layer 5

The neocortex contains numerous types of inhibitory interneurons (Kawaguchi and Kubota, 1997; Markram et al., 2004). To investigate connectional specificity between pyramidal cells and interneurons, we first classified L5 inhibitory interneurons based on their intrinsic membrane properties. In responses to current pulse injections, we obtained three cell types in L5 interneurons. FS cells were identified by high-frequency firing and the lack of adaptation of interspike interval during depolarization (Fig. 1A, left trace). In contrast, regular-spiking (RS) interneurons generated adapting spike trains when injected depolarizing current pulses (Fig. 1A, right trace). Low-threshold spike (LTS) type cells had adapting spike patterns, similar to RS interneurons (Fig. 1A, middle trace), but generated rebound burst spikes following hyperpolarizing current pulse injections (Fig. 1A, middle lower trace, asterisk).

FS cells had dense axonal arbors innervating locally near the soma or at relatively long distance from the soma (Fig. 1B, left and middle cells). Many studies have shown that these neurons are parvalbumin-positive cells (Kawaguchi and Kubota, 1993). Morphologies of RS and LTS type interneurons revealed them to have sparsely spiny dendrites and ascending axonal arbors to layer 1 ($n = 25/28$ morphologically identified non-FS cells) (Fig. 1B, right cell), indicating that these neurons are likely somatostatin-expressing Martinotti cells (Kawaguchi, 1995; Kawaguchi and Kondo, 2002; Wang et al., 2004). Therefore, we classified RS and LTS type interneurons into one broad group identified as “non-FS cells.” Previous studies using VGAT-Venus transgenic rats have shown that parvalbumin- and somatostatin-positive cells comprise $\sim 80\%$ of the total inhibitory interneuron population in layer 5 of the frontal cortex (Uematsu et al., 2008).

Synaptic connections between pyramidal cells and interneurons in layer 5

To investigate intralaminar synaptic networks of pyramidal cells and inhibitory interneurons in layer 5, we obtained dual whole-cell recordings from inhibitory interneurons and neighboring pyramidal cells. Unitary synaptic currents from interneuron to pyramidal cell were detected as outward currents by voltage clamping postsynaptic pyramidal cell at 0 mV (Fig. 2A). Synaptic connections were tested in 73 L5 cell pairs consisting of a pyramidal cell and an FS interneuron. For 70% of these cell pairs ($n = 51/73$ cell pairs), there were no detectable synaptic connections between pyramidal cells and FS cells in either inhibitory or excitatory directions. However, many remaining cell pairs showed reciprocal synaptic connections ($n = 15/73$ cell pairs) (Fig. 2B). We also obtained unidirectionally connected cell pairs connected in both excitatory ($n = 2/73$ pairs) and inhibitory direction ($n = 5/73$ pairs). We have previously classified L5 pyramidal cells into three subtypes, based on their firing properties (Otsuka and Kawaguchi, 2008). All three pyramidal subtypes formed reciprocal connections with FS interneurons (supplemental Table 1, available at www.jneurosci.org as supplemental material).

Figure 1. Firing patterns and morphologies of L5 inhibitory interneurons. **A**, Voltage responses to positive and negative current pulse injections in FS and non-FS interneurons (pulse duration is 1 s, amplitudes are ± 200 pA). The asterisk indicates rebound burst spikes generated by negative current pulse injection. **B**, Morphological reconstructions of FS (left and middle cell) and non-FS interneurons (right cell). Black and gray indicate dendrites and axons, respectively.

For L5 cell pairs of a pyramidal cell and a non-FS interneuron, we tested for synaptic connections in 104 pairs and found that 13.5% of pairs had excitatory connections from the pyramidal cell to the non-FS cell ($n = 14/104$ cell pair) (Fig. 2B). However, inhibitory connections from non-FS to pyramidal cells were rarely found (2.9%, $n = 3/104$ cell pair). No reciprocally connected cell pairs were observed. To better detect inhibitory synaptic connections from interneurons to pyramidal cells, non-FS interneurons were discharged at 50–100 Hz for 50 ms by current pulse injection. This protocol typically generated 4–5 spikes in non-FS cells. However, even with this protocol, few inhibitory connections from non-FS to pyramidal cells were detected in examined cell pairs.

It is known that Martinotti cells form synapses on the dendrites of pyramidal cells (Hendry et al., 1984; de Lima and Morrison, 1989; Kawaguchi and Kubota, 1997). Because of the voltage clamp error, somatic recordings from pyramidal cells might not reliably detect inhibitory inputs from non-FS cells onto electrically distant dendrites. To overcome this problem, we reexamined synaptic connections between pyramidal and non-FS cells, using Cs^+ /high- Cl^- electrodes (see Materials and Methods). Inhibitory synaptic inputs were recorded as inward currents by holding membrane potentials of pyramidal cells to -80 mV. Under these conditions, we found 9.7% of non-FS/pyramidal cell pairs had inhibitory connections from non-FS to pyramidal cells ($n = 7/72$). Excitatory one-way connections were found in 8.3% of non-FS/pyramidal cell pairs ($n = 6/72$). Despite more frequent detection of inhibitory connections in recording by Cs^+ /high- Cl^- electrodes, reciprocal connections were observed in only one cell pair (1.4%). FS/pyramidal cell pairs recorded with Cs^+ /high- Cl^- electrodes showed similar connection probabilities to those found with low- Cl^- pipette solutions. Reciprocal connections in

FS/pyramidal cell pairs were found 15.4% of the time ($n = 8/52$), while one-way excitation was observed 1.9% of the time ($n = 1/52$), and one-way inhibition 7.7% of the time ($n = 4/52$). These results suggest that, in layer 5, pyramidal cells reliably form reciprocal connections with FS cells, but not with non-FS cells.

The kinetics of unitary synaptic currents between pyramidal and FS cell connections were faster than those between pyramidal and non-FS cell connections. The rise and decay times of unitary EPSCs from pyramidal to interneurons were 0.83 ± 0.23 and 4.21 ± 1.88 ms in FS cells ($n = 17$), and 2.35 ± 0.56 and 14.5 ± 4.55 ms in non-FS cells ($n = 14$), respectively ($p < 0.01$). The rise and decay times for unitary IPSCs onto pyramidal cells from FS cells were also faster than those from non-FS cells, investigated by normal K^+ electrodes (1.91 ± 0.47 and 16.7 ± 4.36 ms in FS cells and 3.26 ± 0.42 and 20.96 ± 2.33 ms in non-FS cells, $n = 20, 3$, respectively, $p < 0.01$ and 0.05 for rise and decay time).

We also measured the kinetics of IPSCs from FS and non-FS cells onto pyramidal cells obtained with Cs^+ /high- Cl^- electrodes. In contrast with observations in L5 of the visual cortex (Xiang et al., 2002),

both rise and decay times of IPSCs in non-FS/pyramidal cell pairs were significantly slower than those in FS/pyramidal cell pairs (1.53 ± 0.21 and 12.73 ± 2.81 ms in FS cells and 2.16 ± 0.55 and 16.99 ± 2.86 ms in non-FS cells, $n = 12, 8$, respectively, $p < 0.01$). Kinetics of IPSCs in non-FS/pyramidal cell pairs would be due to distal synaptic location onto pyramidal cells rather than the difference in GABA_A receptor-subunit composition (Maccaferri et al., 2000; Xiang et al., 2002).

We next compared the synaptic strength of reciprocal and one-way connections in FS/pyramidal cell pairs. In inhibitory connections from FS to pyramidal cells, the IPSC amplitude in reciprocally connected cell pairs (mean amplitude = 97.1 ± 57 pA, $n = 15$) was larger than that in cell pairs connected one way (44.1 ± 21.7 pA, $n = 5$) ($p < 0.05$) (Fig. 3). In excitatory connections from pyramidal to FS cells, synaptic strength was also dependent on the connectivity between cells. EPSC amplitudes recorded in reciprocally connected cell pairs (47.9 ± 32 pA, $n = 15$) was larger than the amplitudes observed in cell pairs connected only in the excitatory direction (16.8 ± 1.4 pA, $n = 3$) ($p < 0.05$) (including excitatory connections to FS cells obtained with Cs^+ /high- Cl^- electrodes for pyramidal cells). The EPSC and IPSC amplitudes of reciprocally connected pyramidal/FS cell pairs were not correlated (correlation coefficient = 0.14). Thus, L5 FS interneurons and neighboring pyramidal cells were preferentially connected in reciprocal manner. Similar high reciprocity in FS/pyramidal cell connections has been reported in L2/3 of the visual cortex (Yoshimura and Callaway, 2005).

Synaptic connections from L2/3 pyramidal cells to L5 interneurons

To investigate interlaminar excitatory inputs to L5 inhibitory interneurons, we used focal glutamate puff stimulation of L2/3

pyramidal cells to induce EPSCs in L5 inhibitory interneurons (Fig. 4A) (Otsuka and Kawaguchi, 2008). We have previously confirmed that this technique reliably generates monosynaptic L2/3 to L5 EPSCs at constant latencies in L5 cells (Otsuka and Kawaguchi, 2008). We searched for EPSCs occurring at a constant latency in L5 interneurons following glutamate stimulation to individual L2/3 pyramidal cells (Fig. 4A, left traces).

We then compared the connection probabilities of L2/3 pyramidal cells to L5 FS interneurons and L5 non-FS interneurons. Connection probabilities in individual interneurons were obtained by dividing the number of L2/3 cells evoking EPSCs in the interneuron by the total number of stimulated L2/3 cells. The mean number of stimulated L2/3 cells tested with each L5 interneuron was 39.7 ± 13.7 for FS cells and 35.7 ± 13.3 for non-FS cells (range = 20–60). The mean connection probability from L2/3 pyramidal cells to L5 FS interneurons was 0.087 ± 0.024 ($n = 42$). On the contrary, L5 non-FS interneurons received more frequent synaptic inputs from L2/3 pyramidal cells, with the mean connection probability being 0.15 ± 0.054 ($n = 45$, $p < 0.01$).

Next, we examined depth distribution of presynaptic L2/3 pyramidal cells connected to postsynaptic L5 FS and non-FS interneurons. The somatic position of presynaptic L2/3 pyramidal cells and postsynaptic L5 interneurons were measured vertically from the border between layer 1 and layer 2. Previously, we have shown that one of L5 pyramidal subtypes topographically received synaptic inputs from L2/3 pyramidal cells, depending on the somatic position (Otsuka and Kawaguchi, 2008). However, both L5 FS and non-FS interneurons received synaptic inputs from L2/3 pyramidal cells, independent of their laminar locations (Fig. 4B). To further analyze laminar depth distribution of presynaptic L2/3 pyramidal cells, we obtained cumulative depth plots of presynaptic L2/3 cells connected to L5 FS, non-FS, and three pyramidal cell subtypes (obtained in our previous study; Otsuka and Kawaguchi, 2008) (Fig. 4C). The cumulative depth distribution of presynaptic L2/3 cells showed a uniform distribution in pyramidal cells, but is skewed in favor of deeper neurons in both FS and non-FS interneurons. These results suggest that L2/3 pyramidal cells located deeper within L2/3 are more likely to innervate L5 FS and non-FS interneurons than are superficial L2/3 neurons.

Divergent selectivity from L2/3 to L5 pyramidal cells and interneurons

To investigate whether the intralaminar connectivity between excitatory and inhibitory cells affects the occurrence of interlaminar excitatory inputs to these cell pairs, we obtained dual whole-cell recordings from L5 inhibitory interneurons and neighboring pyramidal cells, while applied glutamate to L2/3 pyramidal cells to induce the spike (Fig. 5A). When both L5 cells receive synaptic inputs from the same L2/3 pyramidal cell, synchronous EPSCs should be observed at constant latencies (Otsuka and Kawaguchi, 2008). Indeed, L2/3 pyramidal cell stimulation induced synchronous EPSCs at constant latencies in both cells of some pyramidal/interneuron pairs (Fig. 5B, left traces). Glutamate stimulation of other L2/3 cells induced EPSCs in either of the two L5 cells (Fig.

Figure 2. Intralaminar connections between pyramidal cells and inhibitory interneurons in layer 5. **A**, Detection of EPSCs and IPSCs in a cell pair of pyramidal cell and interneuron. Left, Unitary EPSCs (lower trace) recorded in an interneuron held at -80 mV and induced by a single action potential in a presynaptic pyramidal cell. Right, Unitary IPSCs (upper trace) in a pyramidal cell held at 0 mV evoked by a single action potential in an interneuron. PC, Pyramidal cell; IN, interneuron. **B**, Percentage of cell pairs (consisting of a pyramidal cell and either an FS or non-FS cell) connected one way from pyramidal cell to interneuron, connected one way from interneuron to pyramidal cell, or reciprocally connected.

Figure 3. Synaptic strength depends on the connectivity between pyramidal and FS cells. Distribution of IPSCs (left) and EPSCs (right) amplitudes from FS to pyramidal cell and pyramidal to FS cell obtained in cell pairs connected one way or reciprocally. Bars in the graph indicate means.

5B, middle and right traces). We calculated the common input probability for individual L5 cell pairs by dividing the number of glutamate-stimulated L2/3 cells that evoked simultaneous EPSCs in both L5 cells by total number of glutamate-stimulated L2/3 cells (supplemental Table 1, available at www.jneurosci.org as supplemental material).

Previously, we have shown that the connection probabilities from L2/3 to L5 pyramidal cells depend on the subtype of the postsynaptic L5 pyramidal cell (Otsuka and Kawaguchi, 2008). Here we found that L2/3 excitatory afferents to L5 show differential selectivity in forming connections with L5 FS and non-FS interneurons. Therefore, to compare the common input probabilities between L5 cell pairs of pyramidal cells and interneurons, we normalized common input probabilities for different postsynaptic cell types relative to the probabilities expected for nonselective innervation to two L5 cells. To calculate the expected probabilities in nonselective cases, we used individual connection probabilities to L5 interneuron subtypes obtained above, and those for L5 pyramidal subtypes described in our previous work (Fig. 5C) (Otsuka and Kawaguchi, 2008). L5 pyramidal firing subtypes were determined from the responses to current pulse injections immediately after membrane rupture for whole-cell recordings. The hypothetical common input probability in nonselective cases was calculated by $p_1 \times p_2$, where p_1 and p_2 are the probabilities that each L5 cell subtype receives synaptic inputs from L2/3 pyramidal cells, as obtained experimentally

Figure 4. Synaptic connection properties from L2/3 pyramidal cells to L5 interneurons. **A**, Detections of EPSCs in L5 interneurons by glutamate puff stimulation to L2/3 pyramidal cells. The triangles and circle show pyramidal cells and interneuron. ** indicates EPSCs evoked at relatively constant timings following glutamate stimulation of the L2/3 pyramidal cell (left traces). An example of a case where glutamate stimulation generated no EPSCs is shown in the traces at right. Arrowheads indicate the timing of puff stimulation onset. **B**, Interlaminar connection maps to L5 FS and non-FS interneurons. Cell positions were measured as vertical distance from the L1 and L2/3 border. Gray, Regression lines. **C**, Cumulative probabilities of the depth location of presynaptic L2/3 pyramidal cells. The data for the L5 pyramidal subtypes were obtained from our previous study.

(Fig. 5C). We observed synchronous inputs in all L5 cell pair combinations of interneuron and pyramidal subtypes (supplemental Table 1, available at www.jneurosci.org as supplemental material).

In L5 cell pairs of pyramidal cells and FS interneurons, there were no differences between normalized common input probabilities in unconnected cell pairs (1.34 ± 0.44 , $n = 10$) and those in connected cell pairs (1.26 ± 0.22 in one-way connection, $n = 4$; 1.31 ± 0.38 in reciprocal connection, $n = 10$) (Fig. 5D). On the other hand, in L5 cell pairs of pyramidal cells and non-FS interneurons, the normalized common input probability was higher in connected pairs (2.59 ± 0.75 , $n = 10$) than for unconnected pairs (1.38 ± 0.56 , $n = 12$; $p < 0.01$). Actual probabilities for common inputs in L5 FS/pyramidal cell pairs were 0.018 ± 0.004 for unconnected pairs, 0.02 ± 0.001 for pairs with one way connection, and 0.019 ± 0.005 for pairs with reciprocal connection. On the other hand, actual common input probabilities in L5 non-FS/pyramidal cell pairs were 0.062 ± 0.018 for connected pairs and 0.029 ± 0.018 for unconnected pairs ($p < 0.01$). These results suggest that L2/3 pyramidal cells selectively coinervate L5 non-FS interneurons and neighboring pyramidal cells when these postsynaptic cells have connections between them.

Discussion

In this study, we investigated the specificity of connections between L5 inhibitory interneurons and pyramidal cells in L5 and L2/3. In terms of intralaminar connections, we found that L5 FS cells and neighboring pyramidal cells preferentially formed reciprocal connections, and that the amplitudes of excitatory and inhibitory synaptic currents were larger in reciprocally connected cell pairs than in pairs connected in only one direction. These results suggest that L5 consists of modules of strongly connected FS and pyramidal cells. In contrast, interlaminar connections from L2/3 pyramidal cells preferred to innervate pairs of synaptically coupled L5 non-FS/pyramidal cells, but

showed no preference for synaptically coupled FS/pyramidal cells. These results suggest that the L2/3 to L5 excitatory pathway includes modules of connected non-FS and pyramidal cells. Thus, our results demonstrate that L5 inhibitory interneurons form distinct subnetworks with pyramidal cells and participate in intralaminar and interlaminar subnetworks in a cell type-dependent manner (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Inhibitory interneurons innervate specific surface domains of pyramidal cells (Somogyi et al., 1998). Each cortical inhibitory cell type shows distinct dendritic and axonal geometries with vertical and horizontal spread (Karube et al., 2004; Kawaguchi et al., 2006), suggesting functional differences in the laminar and columnar selectivity of connections. In intralaminar connections, we found that L5 FS interneurons preferentially form reciprocal connections with neighboring pyramidal cells. Consistent with these data, high reciprocal connection probabilities in cell pairs of pyramidal and FS cells have been reported in layer 2/3 of visual and somatosensory cortex (Holmgren et al.,

2003; Yoshimura and Callaway, 2005). Moreover, synaptic strength between cells depended on the connectivity between them, similar to the observations in the visual cortex (Yoshimura and Callaway, 2005). Together, these results suggest that FS interneurons form highly selective inhibitory connections with local pyramidal cells located in the same layer. However, we cannot rule out the possibility that the increase of synaptic strength in reciprocally connected FS/pyramidal cell pairs reflect well preserved axonal and dendritic arbors from cutting off by slicing and a greater degree of the intact connectivity in reciprocally connected cell pairs. We compared the connection probability from L2/3 pyramidal cells to L5 cells among unconnected, one-way connected, and reciprocally connected cell pairs. If axonal and dendritic arbors are cutoff by slicing, connection probabilities from L2/3 to L5 cells would depend on the connectivity between L5 cells. However, connection probabilities from L2/3 pyramidal cells to individual L5 cells were independent of the connectivity between L5 cells (supplemental Table 1, available at www.jneurosci.org as supplemental material). In addition, no correlation was found between the EPSC and IPSC amplitudes in reciprocally connected FS/pyramidal cell pairs, suggesting that cutting artifact is unlikely to explain the increase of synaptic strength depending on the connectivity between cells.

FS interneurons target the perisomatic domain of pyramidal cells and strongly regulate their action potential output (Somogyi et al., 1998). Therefore, reciprocal connections between pyramidal cells and FS interneurons act as a recurrent feedback inhibition that can regulate the timings of pyramidal cell firing. Indeed, *in vivo* recordings have shown that spike timings of FS interneurons are correlated with local field potential during slow and fast oscillations, including gamma waves (Klausberger et al., 2003; Puig et al., 2008).

Morphological characteristics suggest that most of the non-FS interneurons recorded in this study are Martinotti cells. We found

that L5 non-FS interneurons are rarely reciprocally connected with neighboring L5 pyramidal cell. This suggests that L5 non-FS interneurons receiving excitation from L2/3 and innervating L5 pyramidal cells act as a feedforward lateral inhibition. This notion is consistent with the observations that non-FS Martinotti cells mediate disynaptic inhibition between L5 pyramidal cells (Kapfer et al., 2007; Silberberg and Markram, 2007; Murayama et al., 2009). Martinotti cells target apical dendrites of pyramidal cells and strongly regulate dendritic integration of L5 pyramidal cells (Silberberg and Markram, 2007; Murayama et al., 2009). L2/3 pyramidal cells mainly innervate apical dendrites of L5 pyramidal cells (Letzkus et al., 2006; Sjöström and Häusser, 2006), suggesting that Martinotti cells regulate the integration of synaptic inputs from L2/3 pyramidal cells.

In contrast with projection patterns of L2/3 to L5 pyramidal cells, we have shown that L5 inhibitory interneurons frequently received synaptic inputs from deep L2/3 pyramidal cells, suggesting that deep L2/3 pyramidal cells regulate L5 inhibitory interneuron activities. Recent theoretical study has shown that local inhibition in receiver neurons can act as a gating for the signal flow from sender neurons (Vogels and Abbott, 2009). Information flow from L2/3 to L5 pyramidal cells, which then provide output to the various subcortical areas, may be gated by deep L2/3 pyramidal cells through control of L5 interneuron activities.

Although L2/3 pyramidal cells innervating L5 FS and non-FS cells similarly distributed in the layer, the connection probability was higher in non-FS than FS cells. FS basket and non-FS Martinotti cells are different in vertical extension of dendrites (Kawaguchi et al., 2006). This dendritic geometry difference may be related to varied connection probabilities receiving synaptic inputs from descending axonal arbors of L2/3 pyramidal cells between L5 inhibitory cell types. Moreover, we found that L5 non-FS interneurons and neighboring pyramidal cells frequently received common inputs from L2/3 pyramidal cells when they had connections between them. The dependence of interlaminar common input probability on the local connectivity between recipient cells has previously observed for L4 to L2/3 connections in the visual cortex. However, this preferential divergence occurred in inputs to FS/pyramidal cell pairs, but not to non-FS/pyramidal cell pairs (Yoshimura and Callaway, 2005). In the neocortex, information originating from thalamic neurons first enters the middle layers, and is sequentially relayed to the superficial layers, followed by the deeper layers (Gilbert, 1983; Bureau et al., 2006; Lübke and Feldmeyer, 2007). Thus, both interlaminar connections from L4 to L2/3 and L2/3 to L5 are feedforward excitatory pathways. The discrepancy of the two results suggests that interlaminar connection specificity between pyramidal cells and inhibitory interneurons may differ among cortical areas or layers. Another possibility is that connection

Figure 5. Divergent connection selectivity from L2/3 pyramidal cells to L5 pyramidal cells and interneurons. **A**, Dual whole-cell recordings from pyramidal cell and interneuron in the layer 5, while focal glutamate stimulations were applied to L2/3 pyramidal cells. The triangles and circle indicate pyramidal cells and interneurons, respectively. **B**, EPSCs inductions in the cell pair of pyramidal cell and interneuron. Left, Common inputs induced by L2/3 cell stimulation (indicated by double asterisks). Middle and right, EPSCs observed only in one cell (single asterisks). Arrowheads indicate the timing of stimulation onset. **C**, Upper box, Observed connection probabilities from L2/3 to L5 cells in each cell types. Lower box, Calculation of common input probabilities assuming nonselective cases. Data for connection probabilities from L2/3 to L5 pyramidal subtypes were obtained from previous our study. **D**, The common input probabilities were compared between L5 cell pairs of pyramidal cell and interneuron. Data are means $\pm$ SD. $**p < 0.01$.

specificity may depend on non-FS cell diversity. The composition of inhibitory cell types in L2/3 is more complicated than that in L5 (Kawaguchi, 1995; Uematsu et al., 2008). Each inhibitory cell type receives distinct excitatory and inhibitory laminar input patterns (Dantzker and Callaway, 2000; Xu and Callaway, 2009). Non-FS cells recorded in previous studies may therefore reflect different inhibitory cell types than those recorded in the present study.

We have previously shown that L2/3 pyramidal cells make divergent connection more frequently to the L5 pyramidal cell pairs having similar membrane properties, and that this common input probability was even higher in synaptically connected pairs. These suggest that the L2/3 to L5 excitatory synaptic pathway is composed of functionally segregated channels corresponding to extracortical projection systems (Otsuka and Kawaguchi, 2008). In the present study, we found that connected L5 cell pairs of non-FS interneurons and neighboring pyramidal cells also form interlaminar subnetworks with L2/3 pyramidal cells. We rarely found the bidirectional connections between non-FS interneurons and pyramidal cells. One possibility is that our somatic recordings failed to detect inhibitory inputs to distal dendrites of pyramidal cells. Indeed, the recordings with Cs^+ /high- Cl^- pipettes increased detection probability of the inhibitory connections in non-FS/pyrami-

dal cell pairs. However, even in this recording condition, we seldom found reciprocal connections between pyramidal and non-FS cells. In somatosensory cortex, Martinotti cells and neighboring pyramidal cells make highly convergent and divergent connections among them (Kapfer et al., 2007; Silberberg and Markram, 2007). Together, these results suggest that interlaminar excitatory subnetworks from L2/3 to L5 connected non-FS Martinotti/pyramidal cells do not specifically target particular subtypes of L5 pyramidal cell. However, we cannot rule out the possibility that there is a high rate of shared inputs from L2/3 pyramidal cells to cell pairs depending on a L5 pyramidal subtype for which our samples are very small. In particular, for FA type pyramidal cells there were no more than 3 cell pairs sampled for any of the combinations of connected, unconnected, FS, or non-FS cells.

Non-FS/pyramidal cell pairs connected in either direction were preferentially cotargeted by excitatory afferents from individual L2/3 pyramidal cells. Since multiple synaptic inputs, as occurs during spike trains in pyramidal cells, are necessary to drive non-FS cells (Kapfer et al., 2007; Silberberg and Markram, 2007; Murayama et al., 2009), coincident input from L2/3 and L5 pyramidal cells of the same interlaminar module may be sufficient to drive action potential generation in L5 Martinotti cells that suppress excitation onto the apical dendritic branches of L5 pyramidal cells. Because L5 Martinotti cells do not form intralaminar subnetworks with L5 pyramidal cells, we propose that Martinotti cells provide a form of lateral inhibition by inhibiting neighboring L2/3–L5 interlaminar modules. Dysfunction of this lateral inhibition by Martinotti cells would allow nonselective activation of multiple interlaminar modules, which might contribute to neuropsychiatric disease states and the epilepsy.

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