Cellular/Molecular

Two Layers of Synaptic Processing by Principal Neurons in Piriform Cortex

Norimitsu Suzuki and John M. Bekkers
Department of Neuroscience, The John Curtin School of Medical Research, The Australian National University, Canberra, Australian Capital Territory 0200, Australia

The primary olfactory (or piriform) cortex is a trilaminar paleocortex that is thought to construct unified “odor images” from the odor components identified by the olfactory bulb. How the piriform cortex (PC) accomplishes this sophisticated synthetic task, despite its relatively simple architecture, is unknown. Here we used in vitro patch-clamp recordings from acute slices of the anterior PC of mice to identify microcircuits involved in excitatory synaptic processing. Cluster analysis confirmed the presence of two prominent classes of glutamatergic principal cells in the main input layer (layer II) of the PC: semilunar (SL) cells and superficial pyramidal (SP) cells. SL cells received stronger afferent excitatory input from the olfactory bulb, on average, than did SP cells. This was due to the larger mean strength of single-fiber afferents onto SL cells. In contrast, SP cells received stronger associational (intracortical) excitatory inputs, most likely due to their more extensive dendritic trees within the associational layers. Tissue-cut experiments and dual recordings from SL and SP cells in disinhibited slices were consistent with the distinctive patterns of connectivity of these two cell classes. Our findings suggest that the anterior PC employs at least two layers of excitatory synaptic processing: one involving strong afferent inputs onto SL cells, and another involving strong intracortical inputs onto SP cells. This architecture may allow the PC to sequentially process olfactory information within segregated subcircuits.

Introduction

Sensory processing is a complex process that involves distinct levels of coding, typically ranging from simple primitives at the level of receptor cells (e.g., saltiness, light intensity) to an elaborated percept at the level of the cortex (e.g., flavor, a visual scene). In seeking to understand sensory processing, study of the olfactory system offers a number of advantages. One advantage is that only a few synaptic layers interpose between odorant detectors in the periphery and high-level representations of odor space in the brain (Laurent, 2002). For example, in mammals the primary olfactory (piriform) cortex is thought to perform a high-level synthetic role, yet it is only two synapses removed from the olfactory receptor neurons in the nose (Neville and Haberly, 2004). For these and other reasons the piriform cortex (PC) is attracting increasing attention as a promising model system for the study of sensory processing and integration (Suzuki and Bekkers, 2006; Barnes et al., 2008; Poo and Isaacson, 2009; Stettler and Axel, 2009; Isaacson, 2010; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010b; Wilson, 2010).

One strategy for studying the PC is to take a bottom-up cellular/physiological approach, by first characterizing the major classes of neurons and the microcircuits in which they are embedded. This approach has so far identified, for example, classes of glutamate-releasing principal neurons (Franks and Isaacson, 2006; Suzuki and Bekkers, 2006), their dendritic properties (Bathellier et al., 2009), circuits and synaptic plasticity (Franks and Isaacson, 2005; Poo and Isaacson, 2007; Johenning et al., 2009), and classes of GABA-releasing interneurons and their circuits (Young and Sun, 2009; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010a,b). We have previously reported that the main input layer of the PC, layer II, contains two functionally distinctive kinds of principal neurons, semilunar (SL) and superficial pyramidal (SP) cells (Suzuki and Bekkers, 2006). Here, we extend those earlier studies by more rigorously segregating SL and SP cells, and also by examining the properties of the microcircuits in which these cells function. Our findings further highlight the likely distinctive roles of SL and SP cells in olfactory processing.

Franks and Isaacson (2006) have reported that layer II/III pyramidal cells in the PC often receive powerful single-fiber afferent inputs from mitral/tufted (M/T) cells in the olfactory bulb, making it possible for coincident input from just a few M/T cells to drive spiking in cortical pyramidal cells. Their finding allowed them to draw implications for olfactory coding in the PC. Here, we show that principal cells in the PC do, indeed, receive powerful single-fiber afferent inputs, but these are selectively targeted to SL cells. We also show that SP cells preferentially receive powerful intracortical (associational) excitatory inputs. Hence, SL and SP cells participate in two distinctive circuits, the former specializing in afferent processing, the latter in intracortical processing.
Materials and Methods

Slice preparation. Experiments used acute brain slices (300 μm thick) prepared from the anterior PC of C57BL/6 mice of either sex (18–25 d old). The anterior PC was defined as that part of the piriform cortex anterior to the caudal limit of the lateral olfactory tract (LOT) (Neville and Haberly, 2004). In most cases coronal sections of the anterior PC were used, but in some cases sagittal slices containing the LOT were prepared (see Figs. 4, 6). Standard methods of slice preparation were used (Suzuki and Bekkers, 2006, 2010b). Briefly, mice were deeply anesthetized with isoflurane (2% in oxygen), then rapidly decapitated using procedures approved by the Animal Experimentation Ethics Committee of the Australian National University. Slices were prepared using a vibrating slicer with isoflurane (2% in oxygen), then rapidly decapitated using procedures approved by the Animal Experimentation Ethics Committee of the Australian National University. Slices were prepared using a vibrating slicer (Campden Instruments) under ice-cold cutting solution, comprising (in mM) 125 NaCl, 3 KCl, 0.5 CaCl\(_2\), 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), and 10 glucose (osmolality 305 mos/kg), bubbled with 5% CO\(_2\)/95% O\(_2\) (carbogen). The slices were incubated for 1 h at 35°C in a holding chamber containing carbogen-bubbled artificial CSF (ACSF; composition below), and were then held at room temperature until required.

Electrophysiology. Standard infrared-enhanced videomicroscopy techniques were used to make visualized whole-cell patch-clamp recordings from glutamatergic principal neurons in layer II of the PC (Suzuki and Bekkers, 2006). In dual recording experiments (see Fig. 3), SL and SP cells were selected such that both somata lay on an imaginary line drawn perpendicular to the borders of layer II and immediately below the LOT. Identification of SL and SP cells was confirmed by measuring their distinctive passive and spiking properties (Suzuki and Bekkers, 2006) and by recovering each cell for morphological reconstruction (details below). GABA-releasing interneurons, which were occasionally encountered and identified by their characteristic passive and firing properties (Suzuki and Bekkers, 2010b), were discarded.

Slices were continuously superfused (2–3 ml/min) with ACSF comprising (in mM) 125 NaCl, 3 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), and 25 glucose (310 mos/kg), bubbled with 5% CO\(_2\)/95% O\(_2\) (carbogen) and maintained at 33–35°C. Unless stated otherwise, the ACSF also contained picrotoxin (100 μM) to block GABA\(_A\) receptor-mediated inhibitory postsynaptic responses. For the synaptic train experiments (see Fig. 8), the ACSF contained n-2-amino-5-phosphonovaleric acid (n-APV, 50 μM; Tocris Bioscience) plus a low concentration (1 μM) of 6,7-dinitroquinoxaline-2,3-dione (DNQX; Tocris Bioscience) to partially block EPSCs, minimizing polysynaptic excitation. For current-clamp recordings, patch electrodes had resistances of 6–10 MΩ when filled with internal solution containing (in mM) 135 KMeSO\(_4\), 7 NaCl, 0.1 EGTA, 2 Na\(_2\)ATP, 2 MgCl\(_2\), 0.3 GTP, 10 HEPEs at pH 7.2, supplemented with 0.2–0.4% biocytin (295–300 mOs/kg). For voltage-clamp recordings, 135 mos Cs methane sulfonate replaced the KMeSO\(_4\), and electrodes had resistances of 4–7 MΩ. Unless stated otherwise, all compounds were present in the main input layer (layer II) of the anterior PC.

Electrical parameters were measured (see Fig. 1). The electrical parameters were as follows: resting potential, input resistance, membrane time constant, sag, latency to first AP, AP rheobase, AP voltage threshold, AP peak, AP height, AP rise time, AP half width, maximum AP upstroke and down slope, coefficient of variation of AP firing variability, AP accommodation ratio, AP clustering ratio, peak of the afterhyperpolarization (AHP). AHP height, AHP rise time, AP half width, threshold membrane potential slope, and burst index [calculated as described by Suzuki and Bekkers (2006, 2010b)]. The morphological parameters were as follows: soma cross-sectional area, number of dendrites emanating from the soma, number of dendritic branch points, and total dendritic length, where each dendritic parameter was measured separately for the apical and (where present) the basal dendrites. Morphological parameters were calculated using Neurolucida software following neuronal tracing. Note that soma location was not used as an input parameter in the cluster analysis; rather, soma location was used as an independent variable to test the outcome of the analysis (see Fig. 1D). Each of the above parameters was normalized across all cells to the range 0–1, and then the data were subjected to an unsupervised tree clustering algorithm based on Euclidean distances with Ward’s linkage criterion (Suzuki and Bekkers, 2010b). This analysis was done using the statistics toolbox in Matlab (MathWorks).

Results

Two broad classes of principal neurons are found in layer II of the anterior PC

We have previously reported that two functionally distinctive types of glutamatergic principal neurons, SL and SP cells, are present in the main input layer (layer II) of the anterior PC (Suzuki and Bekkers, 2006), confirming neuroanatomical studies.
Whole-cell patch-clamp recordings were made from layer II principal neurons \((n = 61)\) selected randomly, but in such a way that their somata were distributed approximately uniformly across the full span of layer II (Fig. 1C). GABAergic neurons, identified by their electrical properties (Suzuki and Bekkers, 2010b), were occasionally encountered and were discarded. The intrinsic electrical properties of each principal neuron were assayed using a series of current steps (see Materials and Methods). After \(~15\) min (sufficient time for biocytin in the electrode to equilibrate within the cell), the patch electrode was carefully withdrawn while maintaining the seal. The tissue was then fixed and processed to reveal the morphology of the recorded cell. The dendritic arbors of all 61 neurons in our dataset were traced using reconstruction software (see Materials and Methods).

Typical dendritic morphologies are shown in Figure 1A. Classic SL cells, lacking basal dendrites, had their somata concentrated in the upper half of layer II (layer IIa) (Fig. 1A, left two cells). Typical SP cells, with profuse apical and basal dendrites, were concentrated in the lower half of layer II (layer IIb) (Fig. 1A, right two cells). A third group of neurons, often with their somata located near the center of layer II, had an intermediate dendritic morphology, with apical dendrites and a small number of basal dendrites (Fig. 1A, middle two cells).

The intrinsic electrical properties of these neurons are shown in Figure 1B (traces are shown directly below the cell in A from which the recordings were made). These recordings indicated that, notwithstanding the range of morphologies, layer II neurons appeared to fall into just two broad categories: those with SL-like electrical properties [e.g., higher input resistance, longer membrane time constant, smaller spike amplitude (Fig. 1B, left three cells)] and those with SP-like properties [e.g., lower input resistance, shorter membrane time constant, larger spike amplitude (Fig. 1B, right three cells; Table 1)] (Suzuki and Bekkers, 2006). This distinction was formalized by performing an unsupervised cluster analysis on 22 intrinsic electrical parameters plus 7 morphological parameters for each of the 61 neurons in our dataset (Fig. 1D; selected mean parameters in Table 1). The dendrogram confirmed a segregation into two broad groupings: 34 “SL-like” cells, found preferentially in layer IIa (Fig. 1D, somatic locations mainly in layer IIa, indicated by predominantly blue colors; see color scale in C), and 27 “SP-like” cells, found only in layer IIb (Fig. 1D, somatic locations in layer IIb, indicated by red colors).

These results suggest that there are approximately equal numbers of functionally distinctive SL- and SP-like neurons, concentrated in the superficial and deep subdivisions, respectively, of layer II. However, this separation was not perfect, indicated by some intermixing of “red” and “blue” somatic locations within the SL-like grouping (Fig. 1D). Accordingly, for the remainder of this study we took care to select classic SL and SP neurons located in layers IIa and IIb, respectively, as confirmed from their dendritic morphology and electrical properties (Suzuki and Bekkers, 2006).

**Figure 1.** Two broad classes of principal neurons can be identified in layer II of the anterior PC, based on their morphologies and intrinsic electrical properties. A, Typical dendritic morphologies of layer II principal neurons from which electrical recordings were made. These examples are drawn from a dataset of \(n = 61\) neurons distributed across the thickness of layer II (C). Cells with more superficial somata (in upper layer IIa) resemble typical SL cells, which lack basal dendrites. Cells with deeper somata (in lower layer IIb) resemble typical SP cells, with profuse apical and basal dendrites. Cells with soma near the middle of layer II have intermediate dendritic morphologies. Dashed lines indicate the approximate locations of the layers of the PC. All inputs from the olfactory bulb via the lateral olfactory tract are restricted to layer Ia; allamina inputs from intracortical principal cells are restricted to all other layers (Ib, II, III). B, Voltage responses (top) of the neurons in A to 500-ms-long current steps (bottom). The hyperpolarizing step was always \(+80\) pA, and the gray traces show the response to a step to just below rheobase. Horizontal dashed lines represent 0 mV. The electrical traces are shown directly below the cell from which the recordings were made. Note that the leftmost three cells have regular-spiking APs, while the rightmost three cells have burst-firing APs. C, Histogram of the somatic locations of the 61 neurons in our dataset, expressed as a fraction of the distance of the soma across the width of layer II (0, superficial border; 1, deep border). The cell somata were distributed across layer II. Loci of the color code shown at the bottom is used in D. D, Dendrogram showing the result of an unsupervised cluster analysis of the neurons in our dataset, calculated using 7 morphological parameters and 22 electrical parameters measured for each neuron. Colored bars at right indicate the fractional depth (across layer II) of the soma of each neuron, using the color code in C. Note the separation into two broad classes, as follows: SL-like cells that tend to be found more superficially (mostly blue colors), and SP-like cells that tend to be found deeper (red colors).

Baclofen selectively blocks associational inputs onto both SL and SP cells

Our aim was to compare excitatory synaptic inputs onto SL and SP cells. Both receive two main types of excitatory synaptic input:
Figure 2. Excitatory synaptic inputs onto both SL and SP cells in the PC can be selectively inhibited with baclofen. A. Plot of mean peak amplitude of EPSCs, normalized to the mean amplitude during the first 5 min of recording, versus time for SL cells (open symbols, n = 5 cells) and SP cells (filled symbols, n = 5) while stimulating the LOT afferent input from the olfactory bulb. Error bars show ± SEM. (±)-Baclofen (100 μM, bath-applied during period shown by the horizontal bar) had little effect on EPSC amplitude in both SL and SP cells. Insets at right show typical EPSCs recorded at the indicated time points (1, baseline, black traces; 2, at end of baclofen application, gray traces). B. Same as A, except for EPSCs recorded while stimulating the assn input in layer Ib (n = 5 for SL cells; n = 6 for SP cells). The Ib input to both SL and SP cells was strongly inhibited by 100 μM baclofen.

Next, we wished to perform a simple experiment comparing the strength of the two kinds of excitatory synaptic input (aff and assn) onto the two classes of layer II principal cells (SL and SP). Simultaneous whole-cell voltage-clamp recordings from an SL cell and a nearby (<150 μm) SP cell were obtained (Fig. 3A), and then an extracellular stimulator was placed either at the LOT/Ia border (to excite aff inputs) or at the layer Ib/Ila border (to excite assn inputs). Baclofen (100 μM) was present in the bath when stimulating the LOT to minimize contamination from polysynaptic excitation, although broadly similar results were also obtained in the absence of baclofen.

Over a wide range of stimulation intensities, LOT stimulation produced larger EPSCs in SL cells than in SP cells (Fig. 3B, left; responses to two stimulation intensities shown superimposed). The opposite result was found for layer Ib stimulation, which elicited much larger EPSCs in SP cells (Fig. 3B, right). These results were summarized by plotting the EPSC amplitude measured in an SL cell versus that measured simultaneously in an SP cell for a range of stimulus strengths, averaging across n = 6 cell pairs (Fig. 3C). With LOT stimulation, the points lay below the diagonal (averaged slope 2.41 ± 0.34), indicating that SL cells receive stronger aff inputs. Conversely, with layer Ib stimulation, the points lay below the diagonal (averaged slope 0.063 ± 0.017), showing that SP cells receive stronger assn inputs.
Minimal stimulation reveals the basis for different synaptic strengths in SL and SP cells

What is the physiological basis for these different input strengths? We addressed this question by using minimal extracellular stimulation to isolate single aff or assn fibers contacting SL and SP cells (Fig. 4). Small increments in a weak stimulation current often elicited a step-like increase in the EPSC (Fig. 4A); this is interpreted as being due to the all-or-none excitation of a single axon, producing a unitary synaptic response (Bekkers and Clements, 1999; Franks and Isaacson, 2006). The amplitude of this unitary response, estimated from the mean amplitude of the plateau in the stimulus-response plot (Fig. 4A), was measured for a number of different cells and accumulated into a frequency histogram for each kind of input onto each cell type (Fig. 4B).

The distribution of amplitudes of unitary aff inputs onto SL cells was \( \sim 3.6 \times \) larger on average and more skewed to the right than was the distribution for unitary aff inputs onto SP cells (Fig. 4B, left) (SL: mean amplitude of unitary EPSCs, 70.7 \( \pm \) 9.5 pA, \( n = 29 \); SP: mean amplitude, 19.4 \( \pm \) 2.3 pA, \( n = 20 \); \( p \approx 0.001 \)). This suggests that the larger aff response in SL cells described earlier (Fig. 3) is due to a more powerful unitary synaptic connection from aff axons onto SL cells. Note that we never observed larger unitary EPSC amplitudes in SP cells with stronger LOT stimulation, as has been reported for unidentified principal cells in layer II/III of rat PC (Franks and Isaacson, 2006). Thus, SP cells in mouse PC appear to receive only relatively weak single-fiber inputs.

In contrast to aff inputs, the distribution of unitary amplitudes for assn inputs was not significantly different in SL and SP cells (Fig. 4B, right) (SL: mean amplitude of unitary EPSCs, 42.6 \( \pm \) 4.9 pA, \( n = 10 \); SP: mean amplitude, 41.3 \( \pm \) 8.4 pA, \( n = 10 \); \( p = 0.89 \)). How can this be reconciled with the earlier finding (Fig. 3) that multifiber stimulation of assn inputs gives a larger response in SP cells? A possible explanation is that SP cells are more densely innervated by assn fibers than SL cells are; that is, stronger extracellular stimulation may simply recruit more SP cell-targeting assn axons. This is consistent with the observation that SP cells have many more dendrites and dendritic spines in the assn layers than SL cells have (Haberly, 1983); that is, SP cells provide a larger target for assn axons and so receive more inputs (see Discussion).

Input- and cell type-specific stimulus thresholds for synaptically evoked action potentials

Having used voltage-clamp recordings to measure differential synaptic input strengths to SL and SP cells, we next turned to
The opposite result was obtained when stimulating assn inputs in deep layer Ib (Fig. 5B): synaptically evoked APs occurred with much weaker stimulus strengths in SP cells (8.9-fold lower stimulus current required to elicit APs in the SP cell in Fig. 4B compared with the SL cell; average 7.0 ± 1.8-fold lower stimulation required in SP cells, n = 6). This behavior reflects the much larger assn synaptic input received by SP cells (Fig. 3).

In summary, these results show that synaptically driven APs mimic the behaviors described earlier for EPSCs: SL cells fire more readily in response to single-shock aff inputs, whereas SP cells fire more readily in response to single-shock assn inputs.

**Tissue cut experiments are consistent with stronger aff input to SL cells**

We next explored another consequence of the stronger aff input to SL cells by using a mechanical method to isolate assn inputs. A cut was made across the LOT and layer Ia, then the stimulating electrode was placed at the LOT/Ia border on one side of the cut, and a recording was made from an SP cell on the other side of the cut (Fig. 6A). With this arrangement, direct aff input from the stimulator to the SP cell is prevented; instead, only polysynaptic feedforward excitation via deeper assn fibers should be visible in the SP cell. This feedforward excitation could be provided by either an SP or an SL cell (Fig. 6A, two cells at left). It is also possible that other principal neurons may interpose between the directly stimulated (SL or SP) cell and the recorded (SP) cell, prolonging the delay between the stimulus and response. However, here we are only interested in the circuit which gives the shortest possible delay. This will allow us to determine whether SL or SP cells are preferentially stimulated by LOT input.

First, we measured the latency between stimulation of the LOT and the appearance of a monosynaptically evoked action potential in an SL or SP cell; i.e., the first stage of the circuit in Figure 6A. The stimulus strength was adjusted to be just above threshold for eliciting an AP in the recorded cell (5–30 μA for SL; 30–70 μA for SP) (Fig. 6B). The mean latency from the stimulus to the AP peak was significantly shorter in SL cells than in SP cells (SL: 3.9 ± 0.1 ms, n = 4; SP: 8.7 ± 0.1 ms, n = 4; p < 0.001) (Fig. 6B). Both latencies became smaller as the LOT stimulus strength was increased, but the latency in SL cells was consistently 2–4 ms briefer than that in SP cells (Suzuki and Bekkers, 2006).

Next, we weakly stimulated the LOT while recording from an SP cell on the other side of the tissue cut (as in Fig. 6A). Initially, GABA<sub>A</sub> receptor antagonists were not present for these experiments. A typical response is shown in Figure 6C. Top. Most commonly, the EPSC in the SP cell had a mean latency of 3.9–5.5 ms (n = 14 cells; e.g., for the cell in Fig. 6C, mean latency is 4.40 ± 0.02 ms, n = 230 trials). This EPSC was inhibited by 100 μM

---

**Figure 5.** The differential strength of LOT and layer Ib synaptic inputs onto SL and SP cells (as shown in Fig. 3, using voltage clamp) is also manifested as differential AP firing thresholds (measured using current clamp). A<sub>1</sub>, Simultaneous whole-cell current-clamp recordings from an SL cell (top row) and an SP cell (bottom row) while stimulating (Stim) the LOT with the indicated currents (15, 40, 80 μA). Inset at top shows schematically the recording configuration. A<sub>2</sub>, Analysis of data from the cells in A<sub>1</sub>, plotting the probability of firing at least one AP versus the current strength applied to the stimulator in the LOT. The SL cell (open symbols) reaches AP threshold at much lower stimulation currents than the SP cell (filled symbols). Similar results were obtained in six cell pairs. The bath solution contained 100 μM baclofen to block contamination by assn inputs. B<sub>1</sub>, Similar experiment while stimulating in layer Ib. Now, the pattern of excitability is reversed: with assn inputs, the SP cell reaches AP threshold at much lower stimulation currents than the SL cell. Similar results were obtained in five cell pairs. Baclofen was not present during these measurements.
Critically, these data show that the majority population of early EPSCs (those appearing at 4.6 ± 0.1 ms after the stimulus) could only have been driven by action potentials in SL cells (which occur at 3.9 ± 0.1 ms after the stimulus) (Fig. 6B2). In contrast, SP cells only fire after these early EPSCs appear. Moreover, the difference between these two latencies (4.6—3.9 = 0.7 ms) is compatible with a propagation delay in the SL cell axon plus a delay at the SL → SP cell synapse. These results strongly suggest that, following LOT stimulation, SL cells provide the main excitatory feedforward input that drives assn pathways, again consistent with the idea that SL cells receive the strongest aff input. However, we cannot exclude the possibility that a minority of EPSCs (the 30% with a longer latency) are driven by SP cells, or perhaps by a chain of SL and SP cells.

**Epileptiform activity is associated with stronger assn input to SP cells**

With the exception of Figures 3 and 5, the experiments described so far used relatively weak stimulation currents (<70 μA) to minimize epileptiform discharges in these disinhibited slices. [Picrotoxin (100 μM) was present in most experiments to prevent contamination of excitatory postsynaptic responses by GABA_A receptor-mediated inhibitory responses.] For the next series of experiments we used stronger stimulation currents (up to 150 μA) to elicit epileptiform polysynaptic activity.

Simultaneous whole-cell voltage-clamp recordings were made from an SL–SP cell pair (Fig. 7A1) or from two SP cells (Fig. 7A2) while stimulating the LOT. The initial EPSC, largely due to monosynaptic aff input (see below), was often followed by an asynchronous burst of EPSCs with a much longer latency (Fig. 7A, arrowed). The latency of this burst was highly variable between stimuli, but was synchronized in both cells (quantification not shown). When recording from SL–SP cell pairs, the asynchronous burst appeared to be consistently smaller in the SL cell (Fig. 7A1; compare arrowed events), whereas the bursts were of similar size in SP–SP cell pairs (Fig. 7A2, arrowed). This observation was quantified by integrating the charge carried by the delayed phase of each current trace (see Materials and Methods), and then plotting the charge measured in one cell against that measured in the other (Fig. 7B). Sweep-to-sweep fluctuations in the amplitudes of this charge gave rise to a cloud of points distributed along a straight line (correlation coefficients: SL + SP: 0.56; SP + SP: 0.93). The different slopes of these lines confirm that the asynchronous response is much smaller in the SL cell (Fig. 7B1; SL + SP pair: slope of line, 0.10; SP + SP pair: slope, 1.71). Averaged data from a number of cells confirmed this finding (Fig. 7B2) (averaged mean slow charge: SL: 4.2 ± 1.0 pC, n = 4 cells; SP, 26.5 ± 4.3 pC, n = 12 cells; significantly different, p = 0.012).

We hypothesized that the delayed asynchronous EPSCs observed in this experiment were generated by recurrent activity via assn fibers. If so, addition of baclofen (100 μM), which selectively blocks assn inputs to both SL cells and SP cells (Fig. 2), should abolish the asynchronous responses. This was found to be the case (Fig. 7C) (SL cells: mean slow charge 4.2 ± 1.0 pC in control, 0.42 ± 0.11 pC in baclofen, n = 4, p < 0.05; SP cells: mean slow charge 25.8 ± 6.5 pC in control, 0.84 ± 0.31 pC in baclofen, n = 8, p < 0.01).

For the SP cell shown in Figure 7C, baclofen also inhibited the early EPSC (Fig. 7C, bottom; compare amplitudes of EPSCs immediately following the stimulus). However, examination on a faster timescale shows that this early EPSC contains two components: an initial baclofen-resistant component, presumably due to

---

**Figure 6.** When direct aff input to SP cells is prevented by a tissue cut, weak stimulation of the LOT reveals a feedforward excitatory assn input that is largely driven by SL cells. A, Schematic diagram showing the recording configuration, illustrated for a parasagittal slice containing the LOT. The LOT and layer Ia (gray) are transected by a tissue cut. The extracellular stimulating electrode (Stim) is placed at the LOT/Ia border on one side of the cut, while recordings are made from an SP cell on the other side of the cut. Weak stimulation excites aff input to either an SP cell or an SL cell, which in turn provides assn input to the recorded SP cell. B1, Two superimposed episodes recorded in an SL cell (top) and an SP cell (bottom) showing synaptically evoked APs at stimulus strengths just above threshold for eliciting APs. The bath solution did not contain picrotoxin. B2, Histograms of the latency from the stimulus to the peak of the synaptically evoked AP (arrowed in B1), combining measurements on n = 4 SL cells and n = 4 SP cells. C, Top, Superimposed episodes recorded from an SP cell following weak LOT stimulation with the electrode configuration as in A; showing EPSCs that result from the feedforward excitation of assn inputs. The synaptic latency is arrowed. The mean synaptic latency in this cell was 4.40 ± 0.02 ms (n = 230 trials). C, Bottom, Episodes from the same cell after application of 100 μM baclofen. Inhibition of the EPSCs confirms the involvement of assn fibers. D, Histogram of mean synaptic latencies for 20 experiments as in C. Two classes of cells are apparent, with “Early” (mean 4.6 ± 0.1 ms, n = 14) and “Late” (8.0 ± 0.2 ms, n = 6) EPSC latencies.
to monosynaptic aff input, and a slightly delayed component, presumably mediated by disynaptic assn excitation, which is selectively blocked by baclofen (Fig. 7C, inset at lower right). In contrast, the early EPSC in the SL cell (Fig. 7C, top) was unaffected by baclofen and must be entirely due to monosynaptic aff input. Similar results were obtained in \( n = 4 \) cell pairs.

In summary, stronger stimulation of the LOT in disinhibited slices evokes a much larger delayed, polysynaptic response in SP cells than in SL cells, and this polysynaptic response is mediated by baclofen-sensitive assn inputs. This result is consistent with the finding (Figs. 3, 5) that SP cells receive a larger assn input than SL cells receive.

**SL and SP cells respond differentially to trains of synaptic stimuli**

So far we have focused on the responses of SL and SP cells to single-shock stimuli. In *vivo* these cells are thought to receive brief bursts of excitation at \( \sim 10–40 \) Hz (Cang and Isaacson, 2003; Suzuki and Bekkers, 2006). Therefore, we next examined the responses of these two cell types to trains of 10 pulses of synaptic stimulation at frequencies in the range of 5–100 Hz.

Whole-cell voltage-clamp recordings were made from either SL cells or SP cells (Fig. 8, top and bottom, respectively) while stimulating either the LOT or layer Ia (Fig. 8, left and right, respectively). EPSC amplitudes during each train were normalized to the first in the train. Aff (LOT-evoked) responses in SP cells showed the most complex changes in EPSC amplitude during trains, with initial facilitation at all frequencies tested, followed by depression at the higher (20–100 Hz) stimulation frequencies (Fig. 8C). The facilitation is consistent with previous reports of paired-pulse facilitation at these inputs (Bower and Haberly, 1986; Hasselmo and Bower, 1990; Suzuki and Bekkers, 2006). In contrast, assn (layer Ia-evoked) inputs onto SP cells and aff and assn inputs onto SL cells all showed similar behavior, with little facilitation at any frequency and pronounced depression at the higher stimulation frequencies (Fig. 8A, B, D).

**Short-term synaptic facilitation in SP cells partially compensates for their weaker aff input**

The results in the previous section emphasize that aff inputs onto SP cells are unusual in that they exhibit striking short-term synaptic dynamics. This behavior is likely to have ramifications for our earlier conclusions about the responsiveness of SP cells to LOT stimulation (Figs. 3, 5). Accordingly, we revisited our earlier experiments studying the strength of aff inputs onto SP cells (Fig. 5), this time using trains of stimuli rather than single shocks.

Simultaneous whole-cell current-clamp recordings were made from an SL cell and a nearby SP cell, as before (Fig. 5). A 50 Hz train of three stimuli at a range of stimulus strengths (5–100 \( \mu A \)) was applied to the LOT/layer Ia border. Baclofen (100 \( \mu M \)) was present to inhibit assn inputs. During the train, the amplitude of the EPSP in SP cells increased due to short-term facilitation (Fig. 9A, bottom gray inset), whereas the amplitude in SL cells did not (Fig. 9A, top gray inset). As a result, SP cells tended to fire APs more readily later in the train. The data were analyzed by plotting the probability of firing at least one AP on any EPSP in the train versus stimulus current (Fig. 9B, black symbols and lines). Also plotted on the same axes is the probability of firing at least one AP on the first EPSP in the train (Fig. 9B, red symbols and lines), to allow easy comparison with the experiment in Figure 5 in which only a single EPSP was elicited.

By allowing APs to occur anywhere in a train of three EPSPs, the difference between the two cell types was now reduced (Fig.
9B, red arrow) as follows: SP cells now required only $1.9 \pm 0.3$-fold larger stimulus current ($n = 10$ pairs) than did SL cells to elicit at least one AP in a train (compared with a $3.7 \pm 0.5$-fold stronger stimulation for a single stimulus; $p < 0.02$; compare Fig. 5A). Thus, the relative sensitivity of SL and SP cells to aff input depends on the dynamics of that input.

**Discussion**

It has long been appreciated that layer II of the PC contains morphologically distinctive types of glutamate-releasing principal neurons (Haberly, 1983; Haberly and Bower, 1984), broadly divisible into semilunar cells and superficial pyramidal cells (Neville and Haberly, 2004). Here, we extend earlier work by showing that SL- and SP-like cells are present in approximately equal numbers in layer II, and that they differ in their contributions to cortical microcircuits as follows: SL cells receive stronger afferent excitation from the olfactory bulb via the lateral olfactory tract, whereas SP cells receive stronger associative excitation from other principal neurons within the cortex. This conclusion holds both for unitary spike inputs and, less markedly, for trains of spike inputs. Thus, our findings suggest that SL and SP cells are specialized to provide two layers of synaptic processing (afferent and associative, respectively) within the piriform cortex. These results stress the importance of maintaining a distinction between these two cell types, rather than merging them into a homogeneous class of pyramidal cells (cf. Franks and Isaacson, 2005, 2006; Poo and Isaacson, 2009; Stettler and Axel, 2009; Stokes and Isaacson, 2010; Wilson, 2010).

**Measurement of synaptic strengths**

Our conclusions depend upon our ability to measure the strength of defined excitatory inputs onto identified neurons. Ideally, such experiments should be done using pair recordings in vivo, to correctly register all connections, including those that might be amputated in vitro. However, for reasons of feasibility, we used extracellular stimulation in acute brain slices. How reliable are our findings using such a system?

Many studies have confirmed that excitatory inputs to the PC are strictly laminated, enabling selective extracellular stimulation of aff and assn fibers (Stevens, 1969; Haberly and Price, 1978; Tang and Hasselmo, 1994). Moreover, we obtained identical results from both coronal and sagittal slices, suggesting that differential amputation of inputs is not a concern. In many of our experiments we made dual recordings from SL and SP cells, allowing us to compare simultaneously the response of each cell to the same stimulus (Figs. 3, 5, 7, 9). The difference in assn input strengths between SL and SP cells was so striking (SP input >15 times SL input, on average) (Fig. 3) that systematic disparities in extracellular stimulation are unlikely to be an explanation. The difference in
aff input strengths was less marked (SL input ~2.4× SP input, on average, for single shocks). However, this finding was directly confirmed by minimal stimulation (Fig. 4), which is a method that is widely used to specifically excite single afferents and so is resistant to the usual concerns about bulk extracellular stimulation (Bekkers and Clements, 1999; Franks and Isaacson, 2006). These minimal stimulation experiments confirmed that aff excitation was, indeed, stronger in SL cells.

Finally, we provide two other pieces of evidence, less dependent on stimulus strength, in support of our conclusions. First, the tissue cut experiments (Fig. 6) drew upon information about synaptic and spike latencies, not stimulus strength, to confirm that SL cells were preferentially excited by aff input. Second, the hyperexcitability experiments (Fig. 7) used extracellular stimulation only to initiate delayed epileptiform activity, the properties of which were consistent with preferential excitation of SP cells by assn input. In summary, then, we have used an interlocking series of approaches to confirm our major findings.

Mechanisms of differential input strengths
What mechanisms could give rise to the differential input strengths we observe? Minimal stimulation of the LOT (Fig. 4 B, left) shows that single aff fibers can generate surprisingly large EPSCs in SL cells (>200 pA), but consistently produce small EPSCs in SP cells (<50 pA). One possible explanation is that single aff axons make multiple synaptic contacts with the dendrites of SL cells, but only one or a few contacts with the dendrites of SP cells. There is some neuroanatomical evidence for this suggestion. The distal dendrites of SL cells (i.e., in layer Ia, where they are recipients of aff input) are studded with unusually large spines (Haberly, 1983). These structures are reminiscent of mossy fiber inputs on CA3 pyramidal neurons in the hippocampus, where clustered presynaptic release sites provide powerful single-fiber input (Hallermann et al., 2003). In a similar way, the anatomy of SL cells might be adapted for receiving strong afferent input from the olfactory bulb.

Minimal stimulation of layer Ib (Fig. 4 B, right) shows that unitary assn inputs onto SL and SP cells are identical in size (~40 pA). Hence, the much larger responses to gross stimulation we observe in SP cells (Figs. 3 B, 5 B) cannot be due to stronger single-fiber inputs. Instead, it is likely that SP cells are simply the target of larger numbers of intracortical assn axons. This would be unsurprising, because SP cells, with their basal dendrites, seem optimized for sampling greater numbers of associational inputs (Fig. 1 A) (Neville and Haberly, 2004). Furthermore, anatomical data suggest that SP cell dendrites are profusely spiny in associationl layer Ib, which is a region where SL cell dendrites are comparatively aspiny (Haberly, 1983; Haberly and Behan, 1983).

Implications for olfactory coding
Franks and Isaacson (2006) have reported that pyramidal cells in layers II/III of the rat PC can receive powerful single-fiber afferent inputs (as well as weaker inputs) from the olfactory bulb, and that this finding has implications for the coding strategies used by the PC. Strong single-fiber input means that only a few coactive M/T cells in the olfactory bulb are required to bring a PC pyramidal cell to spike threshold, suggesting that odor stimulation is likely to produce broad and distributed firing in the PC (Franks and Isaacson, 2006). Indeed, such distributed activity has been reported in the PC using histological, electrophysiological, and imaging approaches (Wilson, 2001; Illig and Haberly, 2003; Rennaker et al., 2007; Poo and Isaacson, 2009; Stettler and Axel, 2009).

Here, we confirm the presence of strong and weak single-fiber afferent inputs to PC principal cells, but, in contrast to Franks and Isaacson (2006), we show that these two kinds of input are segregated onto two types of neuron, viz. SL and SP cells, respectively. Our findings are also consistent with those of Bathellier et al. (2009), who found little evidence of strong unitary inputs in SP cells. Interestingly, our distribution of unitary afferent strengths onto SL cells (Fig. 4 B) closely resembles that obtained by Franks and Isaacson (2006) for their unspecified pyramidal cells (their Fig. 1 D). In support of the idea that their dataset contained SL cells, their Figure 3 shows trains of EPSPs that do not strongly facilitate, consistent with the behavior of SL cells in response to aff inputs (Fig. 9 A).

Our findings are still compatible with the idea that odor stimulation can produce broad and distributed firing in the PC (Franks and Isaacson, 2006), but with the refinement that this pattern of activity may be initiated in the SL cell layer (Ila). Presumably this excitation is then transmitted to and between the SP cells via their abundant assn inputs. These results suggest a simple two-layer model of excitation in the PC following odor stimulation, as follows: input from the olfactory bulb initially produces distributed firing in SL cells (layer Ila), followed rapidly by an additional pattern of distributed firing in SP cells (layer Ib). A further complication, however, is the presence of short-term synaptic facilitation that is restricted to aff inputs to SP cells (Fig. 8).

As a result, the relative strength of inputs to SL and SP cells will alter during bursts of activity, enriching the dynamic features of olfactory coding (Mazor and Laurent, 2005).

The significance of our findings has several caveats. First, the distinction between SL and SP cells might not always be clear-cut; indeed, our cluster analysis (Fig. 1 D) indicates some intermixing of SL- and SP-like cells in layer II, perhaps resulting in a more complex pattern of responses. Second, we have ignored the minority population of principal neurons that are found in layer III (Tseng and Haberly, 1989). Further work will be needed to incorporate these deep cells into models of cortical connectivity. Finally, most of our experiments were done in the presence of a GABAA receptor antagonist to block synaptic inhibition. Although this was appropriate for studying synaptic excitation in isolation, it is obvious that any model of the PC must include synaptic inhibition to achieve a realistic understanding of olfactory processing (Poo and Isaacson, 2009; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010a,b).

In summary, we have shown that the two main classes of glutamatergic neurons in the piriform cortex, SL cells and SP cells, engage differentially in afferent and associational circuits. These findings point to additional subtleties in the mechanisms used by the PC to process olfactory information.

References
Franks KM, Isaacson JS (2005) Synapse-specific downregulation of NMDA


