Distinctive Classes of GABAergic Interneurons Provide Layer-Specific Phasic Inhibition in the Anterior Piriform Cortex

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The primary olfactory (or piriform) cortex is a trilaminar paleocortex that is seen increasingly as an attractive model system for the study of cortical sensory processing. Recent findings highlight the importance of γ-amino butyric acid (GABA)-releasing interneurons for the function of the piriform cortex (PC), yet little is known about the different types of interneurons in the PC. Here, we provide the first detailed functional characterization of the major classes of GABAergic interneurons in the anterior piriform cortex (aPC) and show how these classes differentially engage in phasic synaptic inhibition. By measuring the electrical properties of interneurons and combining this with information about their morphology, laminar location, and expression of molecular markers, we have identified 5 major classes in the aPC of the mouse. Each layer contains at least one class of interneuron that is tuned to fire either earlier or later in a train of stimuli resembling the input received by the PC in vivo during olfaction. This suggests that the different subtypes of interneuron are specialized for providing synaptic inhibition at different phases of the sniff cycle. Thus, our results suggest mechanisms by which classes of interneurons play specific roles in the processing performed by the PC in order to recognize odors.

Keywords: action potential, GABA, GAD67-GFP, olfaction, synaptic transmission

Surprisingly, much less is known about the variety of interneurons found in the PC. It is known that the PC contains a number of classes of interneurons, identified on the basis of morphology and immunohistochemistry (Suzuki and Bekkers, 2007, for review). Fragmented data on the electrophysiology of PC interneurons have also been reported (Satou et al. 1983; Marek and Aghajanian 1996; Kapur et al. 1997; Protopapas and Bower 2000; Luna and Schoppa 2008). However, many questions remain about exactly how many different types of GABAergic interneurons populate the PC and how their properties enable the operation of this sensory circuit. These questions have become more urgent in view of recent studies reporting the importance of synaptic inhibition for coding strategies used by the PC (Luna and Schoppa 2008; Poo and Isaacscon 2009; Stettler and Axel 2009).

In this paper, we present the first rigorous classification of GABAergic interneurons in the anterior piriform cortex (aPC) of the mouse, based on the measurement of cell morphology, molecular markers, and electrical properties of single cells. We identify 5 main classes of GABAergic interneurons and show how these different types of interneurons are able to play unique functional roles in olfactory processing.

Materials and Methods

Slice Preparation

All experiments used acute brain slices (300 μm thick) prepared from GAD67-GFP (Δneo) transgenic mice (14–25 days old; Tamamaki et al. 2003), which we will refer to here as GAD67-GFP mice. These animals, made on a C57BL6/J background, express green fluorescent protein (GFP) in neurons expressing GAD67, 1 of 2 genes encoding isoforms of the GABA-synthesizing enzyme, glutamic acid decarboxylase. GAD67-GFP mice have normal behavior and neuroanatomy (Tamamaki et al. 2003). We also confirmed that our findings were consistent across the full range of ages of animals used here (14–25 days).

For most experiments, coronal slices were prepared from the aPC, defined as that part of the PC anterior to the caudal limit of the lateral olfactory tract (LOT; Neville and Haberly 2004). In a few experiments, parasagittal slices were studied, without effect on the results. Standard slicing procedures were used. Briefly, animals were deeply anesthetized with 2% isoflurane in oxygen and then rapidly decapitated according to procedures approved by the Animal Experimentation Ethics Committee of The Australian National University. Slices were prepared on a Vibroslice (Campden Instruments) under ice-cold cutting solution, comprising (mM) 125 NaCl, 3 KCl, 0.5 CaCl2, 6 MgCl2, 25 NaHCO3, 1.25 NaH2PO4, and 10 glucose (osmolarity 305 mOs/kg), bubbled with 5% CO2/95% O2 (Carbogen). The slices were incubated for 1 h at 35 °C in a holding chamber containing carbogen-bubbled artificial cerebrospinal fluid (ACSF; composition below) and then were maintained at room temperature until required.

Electrophysiology

Whole-cell patch clamp recordings were made from visually identified GFP+ neurons using standard methods (Suzuki and Bekkers 2006). Briefly, slices
were superfused with ACSF containing (mM) 125 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄, and 25 glucose (310 mOsm/kg), bubbled with 5% CO₂/95% O₂ (Carbogen) and maintained at 33–35 °C. Picrotoxin (100 μM) was added to block inhibitory postsynaptic responses. Patch electrodes had resistances of 6–10 MΩ (for current clamp recordings) or 5–7 MΩ (voltage clamp) when filled with internal solution containing (mM) 135 KMeSO₄, 7 NaCl, 0.1 ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetra acetic acid, 2 Na₂ATP, 2 MgCl₂, 0.3 GTP, 100 μM spermine, 10 μM (2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.2, supplemented with 0.2-0.4% biocytin (295–300 mOsm/kg). For consistency with previous work (Suzuki and Bekkers 2006), voltages have not been corrected for junction potentials, measured to be ~7 mV for these solutions. After selecting a GFP neuron, the electrode was targeted to the soma using infrared videomicroscopy with an Olympus BX51WI microscope.

Data were acquired using a Multiclamp 700A amplifier (Molecular Devices). For current clamp recordings, the cell was allowed to remain at its resting membrane potential. Bridge balance and capacitance neutralization were carefully adjusted and checked for stability. To minimize possible changes due to washout, measurement of the passive and firing properties of each cell was begun 1–2 min after attaining the whole-cell configuration; these measurements were typically completed within 8–10 min. For voltage clamp recordings, the soma was clamped at ~70 mV. Voltage or current traces were filtered at 10 kHz and digitized at 20 or 50 kHz by an ITC-18 interface (Instrutech/HEKA) under the control of Axograph (Axograph Scientific).

Extracellular synaptic stimulation was done using a custom-built isolated stimulator that delivered a 100-μs-long constant current pulse with an adjustable amplitude. The concentric bipolar stimulating electrode was constructed from a patch electrode (tip diameter ~5 μm) filled with 1 M NaCl and coated with silver paint (Bekkers and Clements 1999). Layer-specific stimulation of the aPC was achieved using well-established neuroanatomical landmarks to identify laminar (Neville and Haberly 2004). In the aPC close to the LOT, the border between layers Ia and Ib is very sharp and located approximately halfway across the thickness of layer I. Hence, to be safe, we placed our stimulator either on the upper margin of layer I (to stimulate Ia) or in layer I close to its border with layer II (to stimulate Ib). The stimulating electrode was always placed 50–50 μm lateral to an imaginary line drawn perpendicular to the laminar borders and passing through the soma of the cell from which recordings were made. The tip of the stimulator was inserted just below the surface of the slice. In order to avoid polysynaptic excitation in the presence of picrotoxin, all recordings were made using relatively weak stimulus strengths (typically <30 μA) in order to elicit excitatory postsynaptic currents (EPSCs) that were typically <500 pA in amplitude. Measurement of synaptic latencies confirmed that these EPSCs were monosynaptic (Results). Use of extracellular stimulation to assess synaptic connectivity can be uncertain; however, we took care to keep the stimulation strength constant when mapping input strengths onto a single cell and to keep the stimulation strength within a narrow range when mapping inputs onto cells of the same type in different slices (Results).

At the conclusion of the electrical measurements, the electrode was carefully withdrawn while maintaining the seal. The slice was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h and then stored in PBS at 4 °C until processing. Only one cell was filled per slice.

**Immunohistochemistry**

Standard methods (Suzuki and Bekkers 2010) were used to label each slice with antibodies drawn from a palette of 7: the calcium-binding proteins calbindin (CB), calretinin (CR), and parvalbumin (PV) and the neuropeptides cholecystokinin (CCK), neuropeptide Y (NPY), somatostatin (SOM), and vasoactive intestinal peptide (VIP). Briefly, fixed slices were treated with 50 mM Tris-buffered saline (TBS, pH 7.4) plus 1% bovine serum albumin (BSA) and 0.3% Triton X-100 for 1 h. They were then incubated with the primary antibodies in TBS plus 1% BSA and 0.3% Triton X-100 for 18–24 h. The type, dilution, and source of primary antibodies are given in Suzuki and Bekkers (2010). Following washes in TBS (3 washes at 10 min intervals), the slices were incubated with secondary antibodies in TBS plus 1% BSA and 0.3% Triton X-100 for 2 h. The slices were again washed in TBS and then incubated for a further 18 h in a low concentration of streptavidin-Alexa 488 or streptavidin-Alexa 594 (1:1000; Invitrogen), processed with an ABC kit (Vector Laboratories) and diaminobenzidine (DAB), and then mounted on glass slides in order to recover the full morphology.

Fluorescence images of the immunohistochemistry and morphology were acquired on a Zeiss LSM Pascal confocal microscope using a Plan-Apochromat ×20/0.75 numerical aperture (NA) objective or a Plan-Neofluar ×40/1.3 NA oil immersion objective. Confocal sections were taken at 5 μm intervals through the entire dendritic and axonal arbors of the recorded cell and a projection of the z-stack of images was calculated. Full neuronal reconstructions were done by manually tracing DAB-processed cells on a NeuroLucida system (MBF Bioscience). Axons were identified from their smooth, thin, and sometimes beaded appearance, whereas dendrites were thicker, tapering, and sometimes spiny.

The densities of interneuron classes were calculated as previously described (Suzuki and Bekkers 2010). Briefly, the perimeters of all GFP⁺ somata were traced in confocal images of the tissue slice and then the mean intensities of GFP fluorescence and immunolabel fluorescence were measured within each somatic perimeter. GFP fluorescence was normalized for each slice by dividing each mean intensity by the largest somatic GFP fluorescence measured in that slice; this corrected for variation in confocal microscope settings between slices. The laminar location of each soma was also noted, as was the total volume of each layer across the thickness of the tissue slice. This data allowed us to calculate both the absolute density (cells/mm³) and the relative number (% of GFP⁺ cells) of each immunolabeled class of GFP⁺ neuron.

**Analysis of Electrophysiology Data**

All electrophysiology analysis (except cluster analysis, below) was done using either Igor Pro (Wavemetrics) or Axograph. Input resistance was calculated from the voltage responses to hyperpolarizing current steps (duration 500 ms), measuring the response at the end of the step. Membrane time constant was obtained by fitting a single exponential to the relaxation of membrane potential (Vₜ₀) following a ~120 pA current step. sag was calculated as $V_{m2}/V_{m3}$, where $V_{m2}$ was measured at the end of a ~120 pA current step (500 ms) and $V_{m3}$ was measured at the maximal hyperpolarization near the beginning of the step. Single action potential (AP) properties (Table 1) were measured on the first AP that occurred at least 10 ms after the beginning of a current step just above rheobase. Latency to first AP was defined as the time from the beginning of this current step to the peak of the first AP. Rheobase was defined as the amplitude of the 500-ms-long current step that first elicited at least one AP (current steps incremented in 10–40 pA intervals, depending on input resistance). AP voltage threshold was defined as the $V_{th}$ at which $dV/dt$ first exceeded 50 V/s. AP peak was the voltage reached at the peak of the AP, whereas AP height was the difference between the AP voltage threshold and the peak. AP risetime was the time from AP voltage threshold to the AP peak. AP half-width was defined as the width of the AP halfway between the AP voltage

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**Table 1**

<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>aHP</td>
<td>afterhyperpolarization</td>
<td>aPC</td>
<td>anterior periforn cortex</td>
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<tr>
<td>aHP</td>
<td>action potential</td>
<td>assn</td>
<td>associational</td>
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<tr>
<td>BT</td>
<td>bilified</td>
<td>CB</td>
<td>calbindin</td>
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<tr>
<td>CCK</td>
<td>cholecystokinin</td>
<td>CI</td>
<td>calretinin</td>
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<tr>
<td>DP</td>
<td>deep pyramidal</td>
<td>FMP</td>
<td>fast-spiking multipolar</td>
</tr>
<tr>
<td>IZ</td>
<td>horizontal</td>
<td>NG</td>
<td>neuroglamia</td>
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<tr>
<td>IZ</td>
<td>lateral olfactory tract</td>
<td></td>
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</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
<td></td>
<td></td>
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<tr>
<td>PC</td>
<td>periforn cortex</td>
<td></td>
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<tr>
<td>PV</td>
<td>parvalbumin</td>
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<tr>
<td>RMp</td>
<td>regular-spiking multipolar</td>
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<tr>
<td>SOM</td>
<td>somatostatin</td>
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<tr>
<td>SP</td>
<td>superficial pyramidal</td>
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<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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threshold and the peak. Maximum AP upslope and downslope were defined as the maximum dV/
dt during the rising or falling phase, respectively, of the first AP at rheobase. The height, risetime, and half-
width of the afterhyperpolarization (AHP) were calculated for the AHP following the first AP at rheobase, always with respect to the AP voltage threshold. Variability in AP firing during a current step was quantified as a coefficient of variation (CV), defined as the standard deviation of the intervals between successive APs divided by the mean of those intervals. The CV was normally calculated for the first episode (elicited by a 500-
ms-long current step incremented in 10–40 pA intervals) that contained at least 12 APs, ignoring the first AP. For some cells (n = 10), this number of APs could not be elicited because of accommodation; for these cells, the CV was calculated from episodes containing the maximum number of APs (5–10). AP accommodation was calculated as the ratio “interval between last 2 APs/interval between first 2 APs” for the response of each cell to a 500-ms-long current step of 3 different sizes: twice the rheobase current (2IR), 1.5IR + 40 pA, and 1IR + 80 pA. These 3 ratios were then averaged together to give the cited value. Another measure of accommodation—the tendency for APs to cluster at the beginning of the step—was quantified as the AP clustering ratio (Chiang and Strowbridge 2007), defined as the number of APs occurring in the first 100 ms of the step divided by the number of APs during the entire 500-ms step, summed across all step sizes. This measure was less susceptible to stochastic variability than the conventional accommodation ratio defined above and also distinguished cells that fired transiently only at the start of the current step. Peristimulus time histograms (PSTHs) of AP firing (e.g., Fig. 1D) were calculated by counting the total number of APs in each 50-
ms-long time bin elicited by the rheobase current step for each cell, summing the bins counts for all cells, then dividing by the number of cells (n = 15–65 for different classes). The weighted decay time constant of the EPSC was calculated as (a1t1 + a2t2)/(a1 + a2), where a and t are the amplitude and decay time constant, respectively, obtained by fitting a sum of 2 exponentials to the decay phase of the EPSC. The paired-pulse ratio (PPR) of synaptic currents was calculated as the amplitude of the second current divided by that of the first. Synaptic latency was measured from the peak of the stimulus artifact to the foot of the EPSC. Errors are given as the standard error of the mean, with n the number of cells in the data set. Note that the size of n does not necessarily reflect the true density of each cell class in the aPC because of the way in which we targeted our recordings. Statistical comparisons used analysis of variance (ANOVA) or the 2-tailed unpaired t-test with significance as indicated.

Cluster Analysis
This analysis was done for 151 interneurons for which all the following 22 parameters could be measured (Supplemental Fig. S1): resting potential, input resistance, membrane time constant, sag, latency to first AP, AP rheobase, AP voltage threshold, AP peak, AP risetime, AP half-width, maximum AP upslope and downslope, CV of AP firing variability, AP accommodation ratio, AP clustering ratio, AHP peak, AHP height, AHP risetime, AHP half-width, somatic GIP intensity, and layer in which the soma was located. Note that we did not use morphology, synaptic properties, or immunolabeling in this analysis because those parameters were not consistently recovered for every neuron. However, the 151 neurons in the cluster analysis data set were not otherwise selected in any way. Each parameter was normalized across all cells to the range 0–1. The data were then subjected to an unsupervised tree clustering algorithm based on Euclidean distances with Ward’s linkage criterion (Ward 1963; Helmaester et al. 2009). The analysis was done using the statistics toolbox in MatLab (Mathworks).

Results
Before we could explore inhibitory function in the PC, we first needed to undertake a rigorous classification of the main types of GABAergic interneurons in this brain region. We focused on the aPC because it is thought to receive stronger direct inputs from the olfactory bulb (Litaudon et al. 2003) and is commonly studied in vivo (Rennaker et al. 2007; Poo and Isaacson 2009). Our experiments were aided by 2 pieces of information. First, in this study, we used GAD67-GFP mice in which GABA-
synthesizing neurons are labeled with GFP (Tamamaki et al. 2003). We have confirmed that >90% of functional GABAergic neurons in the aPC are correctly labeled with GFP, and the distribution of GABAergic interneurons in the aPC of these animals is identical to that in wild-type mice (Suzuki and Bekkers 2010). Use of GAD67-GFP mice greatly facilitated the identification of GABAergic interneurons in live slices. Second, we have previously used whole-slice immunohistochemistry to identify the broad categories of GABAergic interneurons in the aPC based on their expression of different combinations of

Table 2
Electrical properties of different classes of GABAergic interneurons in the aPC

<table>
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<tr>
<th>Note: NG Ia, NG IIa, NG IIa, NG III, NG cell located in layers Ia, IIa, and III, respectively; Vrest, resting membrane potential; Rm, input resistance; τm, membrane time constant; EPSC τf, weighted decay time constant for EPSCs; PPR, mean difference in the peak of the soma to the peak of the EPSC; −20°C (15); 1.3 ± 0.1 (29); 3.2 ± 0.8 (5); 12.0 ± 1.3 (8); 3.4 ± 0.6 (8); 12.3 ± 0.1 (14)<em>; 1.79 ± 0.17 (4)</em>.</th>
<th>NG Ia</th>
<th>NG IIa</th>
<th>NG III</th>
<th>HZ</th>
<th>BT</th>
<th>tMP</th>
<th>nMP</th>
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<tbody>
<tr>
<td>τm (ms)</td>
<td>3.7 ± 0.3 (12)</td>
<td>4.6 ± 0.3 (14)</td>
<td>5.5 ± 0.3 (29)</td>
<td>3.9 ± 0.3 (28)</td>
<td>7.7 ± 0.5 (15)</td>
<td>4.3 ± 0.2 (43)</td>
<td>14.0 ± 0.7 (25)</td>
</tr>
<tr>
<td>AP threshold (mV)</td>
<td>−34.9 ± 1.1 (12)</td>
<td>−33.5 ± 1.1 (14)</td>
<td>−33.8 ± 0.5 (29)</td>
<td>−37.8 ± 0.7 (26)</td>
<td>−36.4 ± 0.7 (15)</td>
<td>−35.8 ± 0.5 (24)</td>
<td>−41.6 ± 0.6 (25)</td>
</tr>
<tr>
<td>AP Peak (mV)</td>
<td>23.1 ± 2.2 (12)</td>
<td>22.1 ± 1.4 (14)</td>
<td>30.7 ± 0.8 (29)</td>
<td>32.0 ± 1.4 (25)</td>
<td>32.1 ± 2.2 (15)</td>
<td>27.7 ± 0.8 (54)</td>
<td>32.8 ± 1.4 (25)</td>
</tr>
<tr>
<td>AP half-width (ms)</td>
<td>0.53 ± 0.02 (12)</td>
<td>0.63 ± 0.02 (14)</td>
<td>0.57 ± 0.02 (29)</td>
<td>0.42 ± 0.01 (26)</td>
<td>0.49 ± 0.02 (15)</td>
<td>0.32 ± 0.01 (54)</td>
<td>0.44 ± 0.02 (25)</td>
</tr>
<tr>
<td>Variability in interspike interval (CV)</td>
<td>0.27 ± 0.05 (12)</td>
<td>0.20 ± 0.03 (14)</td>
<td>0.08 ± 0.01 (29)</td>
<td>0.37 ± 0.05 (27)</td>
<td>0.75 ± 0.06 (13)</td>
<td>0.59 ± 0.07 (54)</td>
<td>0.24 ± 0.05 (24)</td>
</tr>
<tr>
<td>AP accommodation ratio</td>
<td>2.1 ± 0.5 (12)</td>
<td>1.3 ± 0.2 (14)</td>
<td>1.5 ± 0.1 (29)</td>
<td>2.9 ± 0.6 (26)</td>
<td>2.3 ± 0.3 (15)</td>
<td>1.3 ± 0.1 (54)</td>
<td>1.9 ± 0.2 (24)</td>
</tr>
<tr>
<td>AP clustering ratio</td>
<td>0.26 ± 0.01 (12)</td>
<td>0.24 ± 0.02 (14)</td>
<td>0.27 ± 0.01 (29)</td>
<td>0.36 ± 0.03 (26)</td>
<td>0.92 ± 0.02 (15)</td>
<td>0.23 ± 0.01 (54)</td>
<td>0.30 ± 0.02 (25)</td>
</tr>
<tr>
<td>AHP half-width (ms)</td>
<td>3.2 ± 0.8 (5); 12.0 ± 1.3 (8); 3.4 ± 0.6 (8); 12.3 ± 0.1 (14)<em>; 1.79 ± 0.17 (4)</em></td>
<td>0.22 ± 0.02 (7)</td>
<td>0.81 ± 0.08 (6)</td>
<td>2.28 ± 0.20 (7)</td>
<td>0.82 ± 0.09 (7)</td>
<td>2.58 ± 0.30 (4)</td>
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molecular markers (Suzuki and Bekkers 2010). This work provided us with a road map to guide the single-cell experiments described here.

Here we report that the aPC contains 5 common types of interneuron (and a number of less common types). The following sections describe each of those types in turn, with selected electrophysiological parameters summarized in Table 2 and shown fully in Supplementary Figure S1. We have chosen morphologically descriptive names for the 5 common cell types in an attempt to be as neutral as possible in our terminology (Ascoli et al. 2008). However, our names for these cell types do draw upon earlier work in the PC, neocortex, and hippocampus (McBain and Fisahn 2001; Mott and Dingleline 2003; Freund and Katona 2007; Suzuki and Bekkers 2007; Klausberger and Somogyi 2008).

**Neurogliaform Cell**

Neurogliaform (NG) cells were very prominent in the GAD67-GFP mouse because of their small soma size and very bright somatic GFP fluorescence (Fig. 1, inset). We have previously shown that GFP fluorescence intensity correlates strongly with intensity of GABA immunolabeling (Suzuki and Bekkers 2010); hence, NG cells contain a high cytoplasmic concentration of GABA. This is also the case in wild-type rodents, so it is not an artifact of the GAD67-GFP mouse (data not shown). We have reported that the majority (>98%) of brightly fluorescent GFP+ cells in the aPC are negative for 3 calcium-binding proteins (CB, CR, and PV) and 4 neuropeptides (CCK, NPY, SOM, and VIP; Suzuki and Bekkers 2010). Confirming this result, immunohistochemistry of small, bright NG cells following patch recording showed that every one of them was immunonegative for CB (n = 9 cells), PV (n = 9), CR (n = 3), CCK (n = 12), SOM (n = 9), and VIP (n = 12).

NG cells were found in all layers of the aPC. Their density was estimated by counting all cells with normalized somatic GFP intensity >0.6 (Suzuki and Bekkers 2010). NG cell density was somewhat higher in layers Ia and II and lower in layers Ib and III (Ia: 4.6 ± 0.4 × 10^5 cells/mm^3, n = 781 GFP+ cells counted; Ib: 2.1 ± 0.4 × 10^5 cells/mm^3, n = 142; II: 3.7 ± 0.5 × 10^5 cells/mm^3, n = 1048; III: 1.6 ± 0.3 × 10^5 cells/mm^3, n = 1338). However, expressed as a percentage of the total number of GFP+ cells per layer, NG cells comprised a much larger proportion of GFP+ cells in superficial layers than in deeper layers (Ia: 33.9 ± 5.0%, n = 781; Ib: 35.6 ± 3.2%, n = 142; II: 16.4 ± 1.2%, n = 1048; III: 5.6 ± 0.9%, n = 1338). This reflects the increasing density of interneurons of other types in the deeper layers (see below).

NG cells had the typical neurogliaform morphology seen in other brain regions (Olah et al. 2009) and as described previously in the opossum PC (Haberly 1983), with short aspiny dendrites and profusely ramifying axons that were often restricted to the same layer as the soma (e.g., Fig. 1A, showing a layer Ia NG cell). Dendritic morphology was similar for NG cells in all layers, but the axonal arbor was more variable (Supplementary Fig. S2).

In response to a 500-ms-long current step close to rheobase, NG cells preferentially fired APs toward the end of the current step (Fig. 1C), as revealed by a histogram of AP latencies measured from the beginning of the step (PSTH; Fig. 1D, n = 65 cells). This delay to firing is typical of NG cells in other brain areas (Chu et al. 2003; Price et al. 2005; Simon et al. 2005).

With stronger current steps, the AP firing became more uniformly distributed across the step (Fig. 1E). However, there was a marked difference in the variability of this firing between layers, the NG cells in layer I tending to fire more erratically than those in layers Ib and III (Fig. 2A, bottom; compare left and right panels). This variability was quantified by the CV of AP firing, which was largest for layer I and smallest for layer III (Fig. 2B,C and Table 1; CV: 0.27 ± 0.05, n = 12, for layer Ia NG cells; 0.08 ± 0.01, n = 28, for layer III NG cells; P < 0.001). Variability of firing showed a strong inverse correlation with the half-width of the AHP following each AP, measured from APs evoked by a weak current step in the same cell (Fig. 2A, top, arrows): the most erratic firing (large CV) was observed in layer I NG cells with the smallest AHP half-width (Fig. 2B and Table 1; AHP half-width: 25.2 ± 2.2 ms, n = 9, for layer Ia NG cells; 71.5 ± 5.6 ms, n = 22, for layer III NG cells; P < 0.001).

Some of the passive electrical properties of NG cells also depended on the laminar location. For example, layer III cells had a significantly more depolarized resting potential (Vrest) and larger input resistance (Rin) than those in more superficial layers (Fig. 2C and Table 1; Vrest = -78.1 ± 0.9 mV, n = 9, for layer Ia NG cells; -71.8 ± 0.4 mV, n = 29, for layer III NG cells; P < 0.001; Rin = 80.3 ± 4.7 MΩ, n = 12, for layer Ia NG cells; 120.8 ± 6.2 MΩ, n = 29, for layer III NG cells; P < 0.001). These values

![Figure 1](image-url)
may be compared with those we have previously reported for layer II principal neurons in the same slice preparation (semilunar [SL] cells: $V_{rest} = -68.8 \pm 0.9$ mV, $R_{in} = 240 \pm 10$ MΩ; superficial pyramidal [SP] cells: $V_{rest} = -74.7 \pm 0.6$ mV, $R_{in} = 118 \pm 8$ MΩ; Suzuki and Bekkers 2006).

Taking advantage of the laminar distribution of excitatory afferents in the PC (Fig. 1B; Neville and Haberly 2004; Suzuki and Bekkers 2006), we characterized excitatory synaptic inputs onto NG cells. A whole-cell voltage clamp recording was made from an NG cell in a particular layer and then an extracellular stimulating electrode was placed successively in upper layer Ia (to excite afferent inputs from the LOT) and layers Ib, II, and III (to excite associational inputs). The extracellular solution contained picrotoxin (100 μM) to block GABAergic synaptic responses. A weak stimulus current (10–30 μA for layer Ia NG cells, 9–20 μA for layers Ib–III NG cells) was used in order to avoid polysynaptic excitation. We also confirmed that the mean synaptic latency of the EPSCs was <1.4 mS for all layers and inputs, consistent with monosynaptic excitation (layer Ia NG cells: 1.25 ± 0.06 ms, $n = 4$; layers Ib–III NG cells: 1.35 ± 0.05 ms, $n = 9$). The stimulus setting was kept fixed for all stimulus locations when recording from the same cell.

**Figure 2.** Intrinsic electrical properties of NG cells vary systematically across layers of the aPC. (A) Typical APs elicited in a layer Ia NG cell (left column) or a layer III NG cell (right column) by a just-suprathreshold (top row) or strongly suprathreshold (bottom row) current step. Layer Ia NG cells have APs that are followed by a much briefer AHP (top row, arrowed) and often exhibit greater firing variability (bottom row) than is the case for layer III cells. (B) Summary of data from experiments like in (A), plotted as variability in interspike interval (CV) versus AHP half-width ($n = 48$ NG cells). Different symbols designate cells with somata located in different layers. An inverse correlation between interspike variability and AHP half-width is apparent. (C) Summary of mean intrinsic electrical properties of NG cells that vary across layers. (D) Summary of data from experiments like in (A) and Table 1; PPR at 50 ms interstimulus interval: 2.65 ± 0.25, $n = 4$, for layer Ia input onto layer Ia NG cell cf. 0.99 ± 0.02, $n = 5$, for layer III input onto layer III NG cell; $P < 0.001$). Hence, layer Ia NG cells resemble layer Ib SP cells (Fig. 1B) in that they receive facilitating excitatory input from the olfactory bulb (Suzuki and Bekkers 2006).

**Horizontal Cell**

Horizontal (HZ) cells have previously been described morphologically in the PC of the opossum, where they have horizontally oriented dendrites with profuse spines (Haberly 1983). In the mouse, we found HZ cells exclusively in layer Ia less than about 200 μm from the LOT. In contrast to NG cells, HZ cells had larger and more elongated somata and were weakly GFP fluorescent (Fig. 4A, inset). However, like NG cells, HZ cells were immunonegative for all 7 of the molecular markers mentioned in the previous section ($n = 5$–8 cells for each marker), confirming our finding for putative HZ cells

**Figure 3.** NG cells located in a particular layer receive excitatory synaptic inputs that are largely restricted to that layer, and afferent inputs onto NG cells show paired-pulse facilitation but associational inputs do not. (A) Mean amplitudes of EPSCs recorded in NG cells located in layer Ia (left, $n = 4$ cells) or layer Ib/IIa (right, $n = 4$), following stimulation with an extracellular stimulator placed in layer Ia, Ib, or II/III. For each cell, the stimulus current was set at a value in the range 10–30 μA (layer Ia cells) or 9–20 μA (layer Ib/IIa cells) and maintained at this value as the stimulus was moved to different stimulus locations. Maximal response was seen when the stimulator was placed in the same layer as the cell soma. Insets show typical EPSCs recorded in each cell type following paired-pulse stimulation (50 ms interstimulus interval). Stimulus artifacts have been blanked. (B) Averaged plot of the PPR versus the interval between stimuli, for EPSCs recorded in layer Ia NG cells (left) or layer Ib/IIa NG cells (right, $n = 4$); result for layer III NG cells resembled that for Ib/IIa NG cells; not illustrated). In each case, the stimulating electrode was placed in the same layer as the soma. Paired-pulse facilitation is seen only for the afferent input onto layer Ia cells (fitted decay time constant, 188 ms).

As expected from the small dendritic span of NG cells (Fig. 1A), EPSCs were largest when the stimulator was in the same layer as the soma of the recorded cell, and EPSC amplitude fell off sharply when the stimulus was moved to other layers (Fig. 3A). Paired-pulse stimulation of excitatory afferents revealed that layer Ia (afferent) inputs onto layer Ia NG cells always showed strong paired-pulse facilitation, whereas layer Ib, II, or III (associational) inputs onto NG cells in those layers showed little or no facilitation (Fig. 3A, insets, Fig. 3B and Table 1; PPR at 50 ms interstimulus interval: 2.65 ± 0.25, $n = 4$, for layer Ia input onto layer Ia NG cell cf. 0.99 ± 0.02, $n = 5$, for layer III input onto layer III NG cell; $P < 0.001$). Hence, layer Ia NG cells resemble layer Ib SP cells (Fig. 1B) in that they receive facilitating excitatory input from the olfactory bulb (Suzuki and Bekkers 2006).
stimuli applied to layer Ia (artifacts have been blanked. were observed when stimulating in layer Ia. Inset shows typical EPSCs. Stimulus shown in (A) and III (Fig. 4A). PSTH for APs elicited as in panel (A) and (B), showing accommodation. (C) Mean EPSC amplitudes measured in HZ cells (n = 6) following stimulation in the indicated layer. For each cell, the stimulus current was set at a fixed value in the range 10–15 μA. Maximal responses were observed when stimulating in layer Ia. Inset shows typical EPSCs. Stimulus artifacts have been blanked. (F) Averaged plot of the PPR versus the interval between stimuli applied to layer Ia (n = 6 HZ cells). Paired-pulse depression is seen (fitted decay time constant, 27 ms).

Figure 4. HZ cells have somata that are located in layer Ia close to the LOT and have long, spiny dendrites that are usually restricted to layer I. They fire accommodating APs, receive depressing excitatory inputs, are weakly GFP fluorescent, and are immunonegative for a palette of 7 molecular markers. (A) Tracing of a typical HZ cell showing the dendrites (blue) and axon (red). The axon, like the dendrites, is largely confined to layer I. Inset (lower right) shows GFP fluorescence of this neuron (arrowed). It is weakly GFP positive compared with a nearby NG cell (arrowhead). (B) APs elicited by a just-suprathreshold depolarizing current step (500 ms, 280 pA) in the cell shown in (A) as well as its response to a hyperpolarizing step (–120 pA). (C) PSTH for APs elicited as in panel (B), showing accommodation. (D) APs elicited by a strongly depolarizing current step (500 ms, 640 pA) in the cell shown in (A) and (B), showing accommodation. (E) Mean EPSC amplitudes measured in HZ cells (n = 6) following stimulation in the indicated layer. For each cell, the stimulus current was set at a fixed value in the range 10–15 μA. Maximal responses were observed when stimulating in layer Ia. Inset shows typical EPSCs. Stimulus artifacts have been blanked. (F) Averaged plot of the PPR versus the interval between stimuli applied to layer Ia (n = 6 HZ cells). Paired-pulse depression is seen (fitted decay time constant, 27 ms).

using whole-slice immunohistochemistry (Suzuki and Bekkers 2010). We estimated the percentage of GFP+ cells that were HZ cells by counting the immunonegative somata close to the LOT that had normalized GFP intensity <0.5 (Suzuki and Bekkers 2010), yielding 66 ± 5% (n = 546 GFP+ cells counted). Thus, HZ cells and NG cells together comprise most of the GABAergic interneurons in layer Ia near the LOT.

The morphology of HZ cells was somewhat variable, but they typically had long dendrites that extended horizontally in layer Ia but also frequently into layer Ib and sometimes into layers II and III (Fig. 4A). Their axon was usually restricted to layer I. The dendrites of HZ cells were profusely studded with spines (Supplementary Fig. S3), in agreement with previous reports in the opossum (Haberly 1983).

During a current step at rheobase, HZ cells fired APs that could occur at any time during the step but tended to occur near the beginning, in contrast with the delayed firing seen in NG cells (Fig. 4B,C; latency to first AP: 94.2 ± 20.3 ms, n = 28, for HZ cells cf. 359.9 ± 12.4 ms, n = 64, for NG cells; P < 0.001). With stronger current injections, APs fired throughout the step but showed pronounced accommodation (Fig. 4D and Table 1; accommodation ratio: 2.9 ± 0.6, n = 26, for HZ cells cf. 1.6 ± 0.1, n = 73, for NG cells; P < 0.001). A common feature of HZ cells was a biphasic AHP with a distinctive inflection (Supplementary Fig. S4A). This inflection was clearly seen in 77% of HZ cells in our data set but was never observed in NG cells.

Consistent with their dendritic morphology, HZ cells received strong excitatory synaptic input from LOT/layer Ia stimulation, weaker associational input from layer Ib stimulation, and little input from deeper layers (Fig. 4E; for each cell, the stimulator current was fixed at a value in the range 10–15 μA). The mean synaptic latency was brief (1.09 ± 0.06 ms, n = 6 cells), confirming that our stimulation excited monosynaptic inputs. In marked contrast to layer Ia NG cells, paired-pulse stimulation of LOT/la inputs produced depressing EPSCs in HZ cells (Fig. 4F, inset. Fig. 4F and Table 1; PPR at 50 ms: 0.81 ± 0.08, n = 6, for HZ cells cf. 2.65 ± 0.25, n = 4, for layer Ia NG cells; P < 0.001). Hence, in this respect, HZ cells differ from layer Ib SP cells and are more similar to layer Ia SL cells (Fig. 1B), which receive nonfacilitating LOT inputs (Suzuki and Bekkers 2006). Associational inputs onto HZ cells were also depressing (not illustrated).

**Bitufted Cell**

Bitufted (BT) cells clearly correspond to the VIP+ cells that we have previously described in mice (Suzuki and Bekkers 2010; Fig. 5A) and that others have reported in the rat PC (Ekstrand et al. 2001). BT cells were identified in live slices from the GAD67-GFP mouse by their small, bipolar somata found mainly in layer II and GFP intensity that was considerably weaker than that of the other small cells in the aPC, the NG cells (normalized intensity: 0.343 ± 0.006, n = 290, cf. 0.777 ± 0.003, n = 1854, for NG cells; P < 0.001; Suzuki and Bekkers 2010). Immunohistochemistry following patch recording showed that 100% of BT cells (n = 8 of 8) were VIP+ (Fig. 5A, inset), whereas none were CCK+ (n = 6). Whole-slice immunohistochemistry experiments (Suzuki and Bekkers 2010) showed that VIP+ cells, as a percentage of all GFP+ cells, were most common in layer II (Ia: 0.9 ± 0.6%, n = 643 GFP+ cells counted; Ib: 6.4 ± 3.0%, n = 93; Iia: 25.9 ± 3.8%, n = 239; Ib: 15.3 ± 2.8%, n = 716; III: 4.7 ± 1.1%, n = 1253).

BT cells had a consistent bitufted morphology, with long, sparsely spiny dendrites that traversed layers Ib, II, and III of the aPC and often extended into layer Ia (Fig. 5A). The axon ramified strongly in layers Ib and III, where it would be well placed to form basket terminations on the somata of glutamatergic principal neurons (SP cells and deep pyramidal cells, Fig. 1B). This is consistent with our observation of VIP+ puncta around GFP+ somata in layers II and III in whole-slice immunohistochemistry experiments (Suzuki and Bekkers 2010; see also Ekstrand et al. 2001).

BT cells had the highest input resistance of all GABAergic cells in the aPC, compatible with their small somata and relatively small dendritic trees (Rm: 164.4 ± 10.9 MΩ, n = 15;
BT cells received their largest excitatory synaptic inputs from layer Ia/LOT stimulation and weaker input from layer Ia/LOT stimulation. The expression of the channel blocker, Cd^{2+} (100 μM), converted the transient burst firing to a more regular firing pattern (n = 3; Supplementary Fig. S5), suggesting that a calcium-activated potassium conductance may be responsible for the powerful accommodation that is normally present in BT cells.

Fast-Spiking Multipolar Cell

Fast-spiking multipolar (fMP) cells were the most commonly encountered interneuron type in the deeper layers. They encompassed the CB- and/or PV-expressing cells we previously identified using molecular markers (Suzuki and Bekkers 2010). Following patch recording, immunohistochemistry revealed that 75% (n = 15 of 20) of fMP cells were CB^{+}, 43% (9 of 21) were PV^{+}, and 33% (6 of 18) expressed both markers. None of the fMP cells expressed SOM (n = 15). Whole-slice immunohistochemistry experiments (Suzuki and Bekkers 2010) showed that cells positive for CB and/or PV comprised the highest percentage of GFP^{+} cells in deeper layers of the aPC (Ia: 5.8 ± 0.5%, n = 174 GFP^{+} cells counted; Ib: 0%, n = 21; IIa: 0%, n = 41; IIb: 20.4 ± 9.0%, n = 121; III: 45.0 ± 1.4%, n = 297).

Dye fills revealed fMP cells to have variable multipolar morphologies, with sparsely spiny dendrites that could extend to layer Ia (Fig. 6A). Their axons typically ramified strongly in layer II, where the soma of principal neurons are concentrated (Fig. 6A). This suggests that they form somatic basket-type synapses, consistent with the dense CB^{+} and PV^{+} perisomatic puncta on GFP^{+} neurons found in our whole-slice immunohistochemistry experiments (Suzuki and Bekkers 2010). Morphologically similar CB^{+} and PV^{+} basket cells have previously been described in the rat PC (Ekstrand et al. 2001).

The most prominent electrical feature of fMP cells was their fast-spiking phenotype, similar to that of fast-spiking (FS) cells described in other brain regions (McBain and Fisahn 2001; Freund and Katona 2007). Their APs were briefer than those of any other class of interneuron (Table 1; AP half-width: 0.32 – 0.01 ms, n = 54, cf. mean half-widths in the range 0.42–0.63 ms for other classes; n = 12–40 cells per group; P < 0.001). With strongly depolarizing current steps, fMP cells fired at high frequency (Fig. 6D and Supplementary Fig. S7; see next section) and exhibited weak accommodation (Table 1; AP accommodation ratio: 1.3 ± 0.1, n = 54).

We did not observe any systematic differences among CB^{+}/PV^{+}, CB^{+}/PV^{+}, or CB^{-}/PV^{+} cells for any of the electrical properties listed in Table 1 (ANOVA, n = 4–6 cells per group; P > 0.13). However, we did note some differences in intrinsic electrical properties between fMP cells located in layers II and III (Table 1). For example, layer II fMP cells had a significantly higher input resistance (96.2 ± 9.1 MΩ, n = 14, cf. 58.6 ± 4.7 MΩ, n = 43, for layer III fMP cells; P < 0.001) resulting in
a smaller rheobase current for layer II cells (271 ± 23 pA, n = 14, cf. 609 ± 34 pA, n = 43, for layer III fMP cells; P < 0.001).

As expected from their dendritic span, fMP cells received strong excitatory input from associational fibers in layers Ib, II, and III but little afferent input from the LOT in layer Ia (Fig. 6E; for each cell, the stimulus current was fixed at a value in the range 5–15 μA; mean synaptic latency was 1.39 ± 0.07 ms, n = 13, consistent with monosynaptic stimulation). Paired-pulse stimulation of associational fibers generated depressing EPSCs in layer III fMP cells (Fig. 6E, inset, Fig. 6F) but facilitating EPSCs in layer II fMP cells (Table 1 and Supplementary Fig. S7A; PPR at 50 ms: 0.82 ± 0.09, n = 7, for layer III fMP cells cf. 1.78 ± 0.37, n = 4, for layer II fMP cells; P < 0.009). These results, together with the differences in intrinsic properties for layers II and III fMP cells mentioned above, suggest that IMp cells form a more heterogeneous population than our other categories.

**Regular-Spiking Multipolar Cell**

Regular-spiking multipolar (rMP) cells appear to correspond to the weakly GFP-fluorescent SOM+ neurons that we have previously shown are concentrated in layer III of the GAD67-GFP mouse (Suzuki and Bekkers 2010). Following patch recording, immunohistochemistry showed that 80% of rMP cells (n = 12 of 15) were SOM+. None of the rMP cells expressed PV (n = 8), but 39% (n = 5 of 13) of SOM+ cells also expressed CB, consistent with our findings from whole-slice immunohistochemistry (Suzuki and Bekkers 2010). Whole-slice immunohistochemistry experiments confirmed that SOM+ cells, expressed as a percentage of GFP+ cells, were most common in layer III of the apC (Ia: 0.5 ± 0.3%, n = 488 GFP+ cells counted; Ib: 0%, n = 72; Ia: 2.2 ± 1.1%, n = 208; Ib: 4.8 ± 1.5%, n = 602; III: 25.7 ± 2.4%, n = 1277).

Like IMp cells, rMP cells had variable multipolar dendritic morphologies, but, in contrast to IMp cells, their dendrites were profusely spiny and their axonal projections were more diffuse and not concentrated in layer II (Fig. 7A). Together with our previous report of diffusely distributed SOM+ puncta in all layers (Suzuki and Bekkers 2010), this suggests that the axons of rMP cells preferentially target dendrites.

Electrical recordings from rMP cells revealed striking differences from all other types of apC interneurons. Their resting membrane potential was unusually depolarized (−62.0 ± 9.0 mV, n = 25, cf. −70 to −80 mV for all other types; P < 0.01; Table 1) and their membrane time constant was very prolonged (14.0 ± 7.0 ms, n = 25, cf. 3.7–7.7 ms for other types; P < 0.01; Table 1). Hyperpolarizing current steps uniquely elicited a pronounced sag due to the expression of the hyperpolarization-activated cation current (Ih) in these cells (Fig. 7B and Table 1; sag: 0.70 ± 0.03, n = 25, cf. 0.93–0.98 for all other types; P < 0.001). Application of the selective Ih antagonist ZD 7288 (20 μM) blocked sag in rMP cells (sag: 0.83 ± 0.08 before ZD, 0.99 ± 0.01 after ZD; n = 3 cells; Supplementary Fig. S6).

Depolarizing current steps elicited a weakly accommodating train of APs at a lower frequency than in IMp cells (Fig. 7B and Supplementary Fig. S7A and Table 1). The slope of a plot of mean AP frequency versus amplitude of injected current (f-I plot) was significantly smaller for rMP cells compared with IMp cells (Supplementary Fig. S7B; rMP: 231 ± 11 Hz/nA, n = 25, cf. IMp: 523 ± 35 Hz/nA, n = 57; P < 0.001). The rMP cells also had a more hyperpolarized AP threshold than all other apC interneurons (Table 1; −41.6 ± 0.6 mV, n = 25, cf. −34

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**Figure 6.** fMP cells are found in layers II and III, have widely branching aspiny dendrites that can extend into all layers, and an axon that projects profusely to layer II. They fire high-frequency weakly accommodating APs, receive depressing (layer III cells) or facilitating (layer II cells) excitatory inputs, are weakly GFP fluorescent, and express CB and/or PV. (A) Tracing of a layer III fMP cell showing the extensive dendrites (blue) and the axon (red) that appears to form dense basket-type terminations around the somata of layer II principal cells (Suzuki and Bekkers 2010). Insets (lower right) show GFP fluorescence and PV and CB immunolabeling of the dendrites (blue) and the axon (red) that appears to form dense basket-type cells) or facilitating (layer II cells) excitatory inputs, are weakly GFP fluorescent, and their membrane time constant was very prolonged (14.0 ± 7.0 ms, n = 25, cf. 3.7–7.7 ms for other types; P < 0.01; Table 1). Hyperpolarizing current steps uniquely elicited a pronounced sag due to the expression of the hyperpolarization-activated cation current (Ih) in these cells (Fig. 7B and Table 1; sag: 0.70 ± 0.03, n = 25, cf. 0.93–0.98 for all other types; P < 0.001). Application of the selective Ih antagonist ZD 7288 (20 μM) blocked sag in rMP cells (sag: 0.83 ± 0.08 before ZD, 0.99 ± 0.01 after ZD; n = 3 cells; Supplementary Fig. S6).

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Paired-pulse facilitation is seen (fitted decay time constant, 115 ms). For each cell, the stimulator current was set at a fixed value in the range 7–20 μA; mean synaptic latency was 1.76 ± 0.10 ms, n = 7, consistent with monosynaptic stimulation). However, in contrast to layer III fMP cells, associational inputs onto layer III rMP cells exhibited strong paired-pulse facilitation (Fig. 7E, inset, Fig. 7F; PPR at 50 ms: 2.58 ± 0.30, n = 4, for rMP cells cf. 0.82 ± 0.09, n = 7, for layer III fMP cells; P < 0.001). Thus, rMP cells resembled BT cells in presenting this atypical facilitating response. Finally, the decay time constant of EPSCs in rMP cells was also slow, like that in BT cells (Table 1; 8.1 ± 0.6 ms, n = 7, cf. 12.0 ± 1.3 ms, n = 8, in BT cells; P = 0.020).

**Cluster Analysis Confirms Our Classification of Interneuron Types**

So far we have used a variety of data (intrinsic electrical properties, synaptic inputs, morphology, immunohistochemistry, GFP intensity) in order to converge on a scheme that identifies 5 main classes of GABAergic interneurons in the aPC. To make our classification more rigorous, we next turned to unsupervised cluster analysis, as has been done for interneurons in other brain regions (Tamas et al. 1997; Helmstaedter et al. 2009; Karagiannis et al. 2009). Cluster analysis provides an automated procedure for identifying related groups of objects in multiparameter space (Ward 1963).

The clustering was done for 151 interneurons in which 20 intrinsic electrical parameters (shown in Supplementary Fig. S1) could be measured, plus GFP intensity and laminar location. Synaptic properties and immunohistochemical markers were not used because these parameters were not consistently measured for each neuron in the data set. Morphological information was also not used. All 151 neurons in the data set were included without any prior selection based on morphology or any other parameter.

Cluster analysis was able to assign the neurons to 6 main groups (using threshold linkage distance = 4; dashed horizontal line, Fig. 8). The neurons allocated to 4 of these groups were accurately identified as HZ, BT, rMP, and rMP types, in each case confirming the class to which each neuron was subjectively assigned using the multiple criteria described in previous sections. Neurons in the remaining 2 groups were correctly segregated into NG cells in more superficial layers (I and IIa) and NG cells in deeper layers (IIb and III). This separation of NG cells reflects the grading of their electrical properties with laminar location (Fig. 2). Thus, unsupervised cluster analysis correctly recapitulates the segregation of aPC interneurons into the 5 main groups we identified above, with the exception of NG cells, which separate into 2 subgroups. We prefer to combine these 2 subgroups of NG cells into one class because they are all late-spiking close to rheobase, brightly GFP+, and have a neuroglialform dendritic morphology (Fig. 1).
We also performed a cluster analysis in which information about laminar location was excluded, in order to test whether interneuron types could still be distinguished according to their intrinsic electrical properties. The analysis was still able to accurately segregate NG, BT, and rMP cells into well-separated classes, although there was more intermixing of superficial and deep NG cells than before (19 out of 21 layer I/IIa NG cells correctly grouped; 25 out of 30 layer IIb/III NG cells correctly grouped). However, HZ and fMP cells were incorrectly combined into a single class. This suggests that the intrinsic electrical properties of HZ and fMP cells are more similar than is the case for other cell types, although they can be reliably distinguished by their very different laminar distributions (HZ cells in layer Ia, fMP cells in layer II/III).

Subtypes of Interneurons Preferentially Fire at Different Phases during an In Vivo–Like Stimulus

Having identified the major classes of GABAergic interneurons in the aPC, we next addressed some of the functional consequences of this diversity for olfactory processing. We approached this question by measuring the response of each cell type to a 40-Hz train stimulus that resembles the excitatory input the aPC receives in vivo during olfaction (Cang and Isaacson 2003; Suzuki and Bekkers 2006). Simultaneous whole-cell recordings were made from 2 major classes of interneuron in each layer and then a stimulating electrode was placed in the same layer in order to elicit a train of 5 excitatory postsynaptic potentials (EPSPs) at 40 Hz in both cells (Fig. 9). The stimulus strength was adjusted to simultaneously elicit 1 or 2 APs per train in each cell.

In layer Ia, the 2 main interneuron types are HZ cells and layer Ia NG cells. Placing a stimulating electrode in this layer activates afferent excitatory inputs from the olfactory bulb (Fig. 1B). Because HZ cells exhibit paired-pulse depression of afferent EPSCs (Fig. 4F), they respond to this stimulus train with depressing EPSPs, causing the probability of firing an AP to be largest early in the train (Fig. 9A, black traces and histogram). In contrast, layer Ia NG cells exhibit paired-pulse facilitation of afferent EPSCs (Fig. 3B left), resulting in facilitating EPSPs that tend to fire APs later in the train (Fig. 9A, gray traces and histogram). Thus, the 2 main classes of layer Ia interneurons have opposite response characteristics following an in vivo–like excitatory stimulus.

A similar dichotomy was observed for the 2 main layer II interneurons (layer II NG cells and BT cells; Fig. 9B) and the 2 main layer III interneurons (fMP and rMP cells; Fig. 9C). By stimulating in these layers, associational (intracortical)
excitatory inputs are activated. In layer II, NG cells fire earlier and BT cells later, whereas in layer III, fMP cells fire earlier and rMP cells later. In each case, the main determinant of the timing of AP firing in the train is whether associational EPSP inputs show paired-pulse facilitation or depression. In summary, each layer of the aPC contains at least one main subtype of interneuron that is tuned to fire either earlier or later in response to a physiologically plausible patterned stimulus.

Discussion

In this paper, we present the first detailed classification of subtypes of GABAergic interneurons in the aPC, based on electrophysiology, laminar location, morphology, and expression of molecular markers. We have identified 5 broad classes of interneurons: NG cells, HZ cells, BT cells, and 2 types of multipolar cells (fast-spiking and regular-spiking). (For summary, see Fig. 10.) In addition, we have pointed out the diversity within some of these classes, such as layer-specific variants of NG cells and fMP cells. Finally, by applying in vivo-like trains of stimuli, we have shown that each main layer of the aPC contains 2 functionally distinctive types of interneuron: one that fires earlier in the train and one that fires later, which would give rise to early or late synaptic inhibition of layer II principal neurons. This finding suggests a mechanism by which phasic inhibition might orchestrate the prominent layer-specific oscillations in electrical activity that occur in the PC.

Figure 10. Cartoon summarizing the main findings of this paper. Gray cell at center is a layer II SP neuron, a major recipient of synaptic inhibition. Inhibitory neurons on the left receive depressing excitatory synaptic inputs and fire earlier in a train; inhibitory neurons on the right receive facilitating inputs and fire later. Excitatory inputs (gray lines ending in a triangle) arise from either layer Ia afferent fibers (the LOT) synapsing onto layer Ia HZ and NG interneurons or from associational fibers (in all other layers) synapsing onto all other interneurons. Dashed layer Ia input onto the BT cell indicates that this is a weak input; for clarity, weak inputs onto other interneuronal types are not shown. The locations of inhibitory inputs from each class of interneuron onto the SP cell (i.e., somatic or dendritic; black lines ending in a filled circle) are determined from single-cell fills in this paper, as well as immunolabeling experiments in Suzuki and Bekkers (2010). BT, fMP, and rMP cells express the indicated molecular markers.

Interneuron Diversity in the aPC

Considerable diversity of GABAergic interneurons has been reported in the neocortex (>10; Markram et al. 2004) and hippocampus (>20; Klausberger and Somogyi 2008). In contrast, we find relatively few types of interneurons in the aPC (5 major classes). Why is this so? First, we stress that our classification represents a first pass for the aPC, providing a foundation for future work. We have striven for simplicity by assigning interneurons to broad groupings based on a combination of morphological, molecular, and electrical criteria. However, our classification is also supported by a formal cluster analysis, based primarily on electrical parameters, that clearly recapitulates our major groupings (Fig. 8). Second, we have deliberately focused on the most common subtypes of interneurons, guided by our whole-slice immunohistochemistry experiments (Suzuki and Bekkers 2010). For example, we found relatively few CCK+ and NPY+ cells in those earlier experiments, so have not attempted here to search for corresponding physiological classes. These and other cell types most likely comprise additional categories, although their lower density mitigates against their functional importance. Finally, it is possible that the aPC, being paleocortex, is fundamentally simpler than neocortex or archicortex (such as hippocampus). This might be reflected in a smaller variety of basic interneuron types that are required to perform olfactory processing. As discussed in the next section, the interneurons of the aPC show both similarities to and differences from major classes of interneurons found in other cortical regions.

Note that we avoid a classification based primarily on firing patterns, as is sometimes done (Halabisky et al. 2006; Woodruff and Sah 2007; Young and Sun 2009). Instead, we use a combination of intrinsic and synaptic electrical measurements, immunohistochemistry, and morphology. Our approach has important consequences: for example, in our scheme NG cells form a unitary class because they share a neurogliaform morphology, late-spiking phenotype, and very bright GFP fluorescence (i.e., high cytoplasmic GABA concentration), despite exhibiting a variety of steady-state firing properties (“regular-spiking” to “irregular-spiking”; Fig. 2).

A recent report has used the GAD67-GFP mouse to classify GABAergic interneurons in the posterior PC (Young and Sun 2009). The authors used firing properties to identify 5 classes: late-spiking (LS), irregular-spiking (IS), low-threshold spiking (LTS), fast-spiking (FS), and regular-spiking nonpyramidal (RNSP). LS and IS cells probably correspond to our 2 subgroups of NG cells because they have a similar morphology and, apart from their firing patterns, are reported by the authors to be
electrically similar to each other. LTS cells probably correspond to our rMP cells because they exhibit rebound spiking that is presumably due to the expression of \( \kappa \). Very few LTS cells were found by Young and Sun (2009), perhaps because rMP cells in the GAD67-GFP mouse express GFP weakly and are inconspicuous (Suzuki and Bekkers 2010). FS cells probably correspond to our IMP cells based on their narrow spike width. Unfortunately, Young and Sun (2009) recovered only a relatively small number of immunopositive cells from their electrical recordings. However, they did provide evidence that FS cells were PV positive and soma targeting, consistent with our observations on IMP cells. Finally, RSNP cells were defined as those which did not fall into the other 4 classes. They appear to be a very heterogeneous population, and so might encompass our HZ cells as well as other types of cells that were misassigned in the absence of immunohistochemical information. Transiently firing BT cells were not reported by Young and Sun (2009). Again, these cells are inconspicuous in the GAD67-GFP mouse and are easily missed (Suzuki and Bekkers 2010). It remains possible that the anterior and posterior PC may also differ in their expression of interneuronal types.

Comparison with other Cortices
Assuming that functionally important classes of interneurons are preserved during the evolution of the neocortex from a more primitive form like the PC (DeFelipe 2002), it is instructive to identify subtypes of interneurons that are common to the aPC and other brain regions. Strikingly, each of our 5 subtypes has an identifiable correlate in the neocortex or hippocampus, although the details often differ.

Late-spiking NG cells with compact dendritic arbors are found in neocortex (Kawaguchi and Kubota 1997; Olah et al. 2009) and hippocampus (Klausberger and Somogyi 2008; Price et al. 2008). The axonal arbors of NG cells in the neocortex are quite variable, as we found in the aPC (Supplementary Fig. S2), with reports of both compact (Tamás et al. 2003; Karube et al. 2004) and extended (Chu et al. 2003) morphologies. As in the aPC, hippocampal NG cells can exhibit either regular or intermittent firing phenotypes and receive EPSCs that show short-term depression (Price et al. 2005). However, NG cells in the hippocampus usually express NPY (Price et al. 2005) and tend to be located in stratum lacunosum-moleculare, where they innervate the apical dendritic tuft of CA1 pyramidal cells (Klausberger and Somogyi 2008). This contrasts with NG cells in the aPC, which rarely contain NPY and are found in every layer (Suzuki and Bekkers 2010). NG cells in rat neocortex have been reported to be immunopositive for \( \gamma \)-actinin 2 (Uematsu et al. 2008). Our \( \gamma \)-actinin 2 antibody did not work in either GAD67-GFP or nontransgenic C57BL/6/J mice; however, it did label neurons in all layers of the rat aPC that were also very strongly immunopositive for GABA, that is, putative NG cells (Suzuki and Bekkers 2010).

The profusely spiny HZ cells in the aPC share some of the morphological features of Cajal–Retzius cells in layer I of the neocortex, which have horizontal spiny dendritic processes and axonal arbors confined to layer I (Hestrin and Armstrong 1996). However, Cajal–Retzius cells are usually seen only in juvenile animals (less than postnatal day 10 in rats), in contrast to the PC, where HZ cells are found in adult tissue (Haberly 1983).

Interneurons with a bitufted morphology are common in the neocortex, and one subtype of these has been reported to express VIP, similar to our BT cells (Markram et al. 2004). However, VIP neocortical BT cells are reported to be dendrite targeting, unlike our soma-targeting BT cells, although some types of BT cells do target the soma (Kawaguchi and Kubota 1997). Neocortical BT cells are also heterogeneous in their firing patterns (regular and burst spiking; Kawaguchi and Kubota 1996) and short-term plasticity of their excitatory inputs (Markram et al. 2004). This contrasts with the strongly accommodating APs and short-term facilitation of EPSCs consistently seen in BT cells of the aPC.

Fast-spiking basket cells, similar to our soma-targeting IMP cell, are commonly observed in the hippocampus (Klausberger and Somogyi 2008) and neocortex (Kawaguchi and Kubota 1997; Markram et al. 2004). Moreover, intracortical excitatory inputs onto neocortical FS cells are depressing (Reyes et al. 1998; Beierlein et al. 2003), as we found for layer III IMP cells. FS cells in the neocortex and hippocampus often express PV but rarely CB (Markram et al. 2004). In contrast, we found that IMP cells in the aPC could express either or both PV and CB. This suggests that CB-expressing basket cells are an unusual feature of the PC, confirming earlier work using anatomical approaches (Ekstrand et al. 2001).

Finally, our dendrite-targeting regular spiking rMP cell is similar to Martinotti cells in the neocortex (Kawaguchi and Kubota 1997; Ascoli et al. 2008) and oriens-lacunosum molecular (O-LM) interneurons in the hippocampus (Maccalferri and Lacaille 2003). Like the rMP cell, Martinotti and O-LM cells express SOM, exhibit sag, receive facilitating excitation inputs, and synapse onto dendrites (Reyes et al. 1998; Maccalferri and Lacaille 2003; Kapfer et al. 2007; Ascoli et al. 2008). It seems likely that these cells serve similar roles in their respective cortices.

Oscillations
An emerging principle is that GABAergic interneurons, being highly diverse, underpin the functional complexity needed for neural computation (McBain and Fisahn 2001; Klausberger and Somogyi 2008). In this broad sense, then, the diversity of aPC interneurons described here would equip the PC for complex olfactory processing. Do our findings allow us to draw more specific conclusions about the operation of the PC?

Like other brain regions, the PC exhibits prominent oscillations in electrical activity when it is processing information (Wilson 2001; Neville and Haberly 2003, 2004; Kay et al. 2009; Poo and Isaacson 2009). Although the significance of these oscillations remains controversial (Buzsaki and Draguhn 2004; Haider and McCormick 2009), they may be essential for orchestrating neural processing (Laurent 2002). There are 2 main types of fast oscillation in the PC: beta (15–40 Hz) and gamma (40–100 Hz) (Neville and Haberly 2004; Kay et al. 2009). Gamma oscillations appear to originate in the olfactory bulb (Neville and Haberly 2003) but may be sustained by intrinsic inhibitory circuits in the PC (Luna and Schoppa 2008). Beta oscillations are of unknown origin but also seem to depend upon the PC (Neville and Haberly 2003; Ishikawa et al. 2007). It is likely that inhibitory neurons play a critical role in generating both types of oscillation, as they do in the neocortex and hippocampus (Klausberger and Somogyi 2008). How might this occur?
A common feature of oscillatory circuits is the presence of a phase lag between circuit elements (Buzsaki and Draguhn 2004). In this paper, we show that each layer of the aPC contains 2 main types of interneuron that tend to fire with a ~50- to 100-ms phase difference during a brief train of EPSPs (Fig. 9). This phase lag is within a range that is compatible with the beta oscillation (~10–20 Hz). Some of these interneurons (NG, HZ) have dendrites that are confined to one layer and so receive local excitatory input; others (BT, fMP, rMP) have dendrites that span 2 or more layers and may work to synchronize oscillations between layers. Inhibitory outputs from these different cell types are also distinctive, with some projecting axons to structures in specific laminae (e.g., somata in layer II: fMP), others more diffusely (e.g., dendrites in all layers: rMP). This would add a spatial dimension to the temporal delays mentioned above. Finally, additional complexity may stem from differences in the resting potentials of different types of targeted principal cells in the aPC. Layer II SP cells (Fig. 1B) have an unusually hyperpolarized resting potential (about ~75 mV; Suzuki and Bekkers 2006), so inhibitory postsynaptic potentials (IPSPs) in these neurons may be depolarizing from rest. Another class of principal cell (Sl: Fig. 1B) has a more depolarized resting potential (about ~69 mV; Neville and Haberly 2004; Suzuki and Bekkers 2006). Hence, these may receive hyperpolarizing IPSPs, depending, of course, on the chloride gradient.

**Global Inhibition**

A recent report shows that layer II/III principal cells in the aPC receive odor-evoked inhibition that is widespread and broadly tuned, in contrast to odor-evoked excitation (Poo and Isaacson 2009). The authors provide preliminary evidence regarding the mechanism of this "global" inhibition. They report, firstly, that interneurons in layer I receive relatively unspecific synaptic excitation from the olfactory bulb. They do not attempt to classify these layer I interneurons, but the example they show (Fig. 4A1, Poo and Isaacson 2009) has the morphology of an HZ cell. It would be important to establish whether the other main type of layer I interneuron, the NG cell, behaves differently with regard to input tuning. Poo and Isaacson (2009) also report that electrical stimulation of the LOT more readily evokes disynaptic inhibition than mono-synaptic excitation onto layer II/III principal cells, again suggesting that interneurons receive a higher convergence of broadly tuned olfactory inputs. If their result is due to feedforward inhibition (LOT → layer Ia interneuron → principal cell), then our findings indicate that the responsible interneurons are most likely to be layer Ia NG or HZ cells. (BT cells are also possible, but these receive much weaker LOT input; Figs 5E and 10) On the other hand, if their result is instead due to disynaptic feedback inhibition (LOT → principal cell → layer Ib, II, or III interneuron → principal cell), then the interneuron could be one of at least 4 different classes that are located in deeper layers. Two of these classes, the BT and fMP cells, appear to make powerful perisomatic synapses onto many principal cells and would be well placed to provide global inhibition of the kind suggested by Poo and Isaacson (2009).

In summary, we have shown that the aPC contains 5 broad classes of functionally distinctive GABAergic interneurons that share many features with interneuron classes found in the neocortex and hippocampus. The anatomical simplicity of the aPC, coupled with its well-defined inputs, raises the hope that specific roles for piriform interneurons may soon be identified.