Multi-functional feedforward inhibitory circuits for cortical odor processing

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1 **Abstract**

2 Feedforward inhibitory circuits are key contributors to the complex interplay between
3 excitation and inhibition in the brain. Little is known about the function of feedforward
4 inhibition in the primary olfactory (piriform) cortex. Using *in vivo* two-photon targeted
5 patch clamping and calcium imaging in mice, we find that odors evoke strong excitation
6 in two classes of interneurons – neurogliaform (NG) cells and horizontal (HZ) cells –
7 that provide feedforward inhibition in layer 1 of the piriform cortex. NG cells fire much
8 earlier than HZ cells following odor onset, a difference that can be attributed to the
9 faster odor-driven excitatory synaptic drive that NG cells receive from the olfactory
10 bulb. As a consequence, NG cells strongly but transiently inhibit odor-evoked
11 excitation in layer 2 principal cells, whereas HZ cells provide more diffuse and
12 prolonged feedforward inhibition. Our findings reveal unexpected complexity in the
13 operation of inhibition in the piriform cortex.

14

15 Introduction

16 Synaptic inhibition in the cortex is an intricate process with many interacting
17 parts. Cortical interneurons are classified into dozens of subtypes with distinctive
18 morphological, transcriptomic and electrophysiological properties (Kawaguchi and
19 Kubota, 1997; Tremblay *et al.*, 2016; Zeng and Sanes, 2017; Gouwens *et al.*, 2020).
20 These diverse interneurons are embedded in at least three types of cortical inhibitory
21 circuits – feedforward inhibition, feedback inhibition and disinhibition (Kullmann and
22 Lamsa, 2007; Isaacson and Scanziani, 2011; Denève and Machens, 2016; Tremblay *et al.*
23 *et al.*, 2016; Lourenco *et al.*, 2020). Within these three categories there exist numerous
24 variants that implement a variety of neural computations. For example, feedforward
25 and feedback inhibition exert different effects depending on whether the effector
26 interneurons innervate the perisomatic or distal dendritic regions of their targets (Bruno
27 and Sakmann, 2006; Isaacson and Scanziani, 2011; Pardi *et al.*, 2020), and disinhibition
28 can depend on the subtypes of interneurons involved and the origin of their inputs (Pi
29 *et al.*, 2013; Fu *et al.*, 2014; Tremblay *et al.*, 2016). In this paper we focus on
30 feedforward inhibition in the primary olfactory (piriform) cortex, and report that a
31 surprising complexity of inhibitory processing is also a feature of this type of inhibition
32 in the paleocortex.

33 Feedforward inhibition is conventionally understood as an input-tracking
34 mechanism that does not depend on local circuit activity (Tremblay *et al.*, 2016). In
35 the primary neocortex, feedforward inhibition is often mediated by parvalbumin-
36 expressing fast-spiking basket cells localized in the deeper layers that receive direct
37 afferent input from first-order thalamic nuclei (Beierlein *et al.*, 2003; Gabernet *et al.*,
38 2005; Cruikshank *et al.*, 2007). In this role feedforward inhibition improves sensory
39 discrimination by favoring coincidence detection, but in addition it can provide gain

modulation through input normalization (Bruno and Sakmann, 2006; Carandini and Heeger, 2011). More recently it has been reported that feedforward inhibition is also prominent in layer 1 of the neocortex, where it can engage afferent input from other cortical regions and from higher-order thalamic nuclei (Pardi *et al.*, 2020; Anastasiades *et al.*, 2021). However, the properties and functions of layer 1 neocortical interneurons have received relatively little attention and remain enigmatic (Schuman *et al.*, 2019; Fan *et al.*, 2020; Lourenco *et al.*, 2020).

The piriform cortex (PCx) is unusual in that it receives all of its primary afferent input from the olfactory bulb into the superficial part of layer 1 (layer 1a; Neville and Haberly, 2004; Bekkers and Suzuki, 2013). Layer 1a mostly contains the apical dendrites of glutamatergic principal cells located in layers 2 and 3, as well as the somas, axons and dendrites of two classes of GABAergic interneurons, namely, neurogliaform (NG) cells and horizontal (HZ) cells (Suzuki and Bekkers, 2010a). NG and HZ cells in layer 1a are thus ideally situated to provide feedforward inhibition onto the distal dendrites of layer 2/3 principal cells. Classic work (Biedenbach and Stevens, 1969; Haberly, 1973), as well as more recent studies (*e.g.* Luna and Schoppa, 2008; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2012; Sheridan *et al.*, 2014; Stokes *et al.*, 2014; Large *et al.*, 2016a; Large *et al.*, 2016b), have used electrical stimulation to explore some of the basic properties of feedforward inhibition in the PCx. However, less is known about how particular types of interneurons respond to odor stimulation. The existence and importance of odor-evoked inhibition in the PCx has been established (Poo and Isaacson, 2009; Zhan and Luo, 2010; Franks *et al.*, 2011; Poo and Isaacson, 2011; Sturgill and Isaacson, 2015; Bolding and Franks, 2017; Tantirigama *et al.*, 2017; Bolding and Franks, 2018) but the identities and properties of neurons responsible for the different kinds of inhibition are, for the most part, uncertain.

Here we approach this question by using 2-photon targeted whole-cell patch clamping and functional Ca^{2+} imaging to record from visually identified NG and HZ cells in layer 1a of the PCx *in vivo*. We find that the odor-evoked feedforward inhibition provided by these two types of interneurons is strikingly different: NG cells express a powerful and transient inhibition that begins rapidly after odor onset, whereas HZ cells provide a more diffuse and delayed form of feedforward inhibition. Thus, a multi-functional feedforward inhibitory circuit exists in the superficial layer of the PCx, where it is well-placed to generate dynamically complex patterns of inhibition in the distal dendrites of principal cells.

Results

Interneurons that provide feedforward inhibition in the PCx can be targeted ‘in vivo’

In this study we took advantage of the simple architecture of the PCx to examine in isolation only those neural circuits that generate feedforward synaptic inhibition. Afferent input from the olfactory bulb via the lateral olfactory tract (LOT) to the PCx is strictly confined to layer 1a (L1a) of the PCx (Figure 1A; Neville and Haberly, 2004). Hence, only those interneurons with dendrites concentrated in L1a, where they are able to intercept axons from the LOT, are able to generate feedforward inhibition (red cells, Figure 1A). Two such interneuron types have been identified in the PCx, HZ cells and L1a NG cells (Suzuki and Bekkers, 2010a). Conversely, feedback inhibition is largely mediated by interneurons with dendrites concentrated in deeper associational layers, two prominent types being fast-spiking (FS) and regular-spiking (RS) interneurons (blue cells, Figure 1A). In this paper we focus on the feedforward inhibitory neurons, HZ cells and L1a NG cells (here called ‘NG cells’ for short), located in the anterior PCx.

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Figure 1. Layer 1a neurogliaform (NG) and horizontal (HZ) cells are reliably distinguished using two-photon-targeted patch clamping *in vivo*. NG and HZ cells respond differently to odors.

Pair recordings in slices show that all types of interneurons in the anterior PCx synaptically inhibit principal cells (Figure 1A; Suzuki and Bekkers, 2010a; Suzuki and Bekkers, 2012). Inhibition is commonly observed at the soma of principal cells in response to odors *in vivo* (e.g. Poo and Isaacson, 2009; Zhan and Luo, 2010; Franks *et al.*, 2011; Tantirigama *et al.*, 2017; Bolding and Franks, 2018), although it is not immediately clear whether this inhibition is dominated by feedforward or feedback inhibitory circuits. We decided to explore feedforward inhibition in this study because the relevant interneurons (NG and HZ cells) are close to the cortical surface and more readily accessible to 2-photon targeted patch clamping *in vivo*.

In GAD67-GFP (Δ neo) mice, NG and HZ cells can readily be distinguished under the 2-photon microscope: HZ cells are only found close to the LOT whereas NG cells are distributed throughout L1a; HZ cell somata have an elongated shape whereas NG somata are spherical; and HZ cells express much lower levels of GFP than NG cells (Figure 1B, top; Suzuki and Bekkers, 2010a; Suzuki and Bekkers, 2010b). After whole-cell recording, their identities could be confirmed by their dendritic morphology (NG: short, thin, highly branched dendrites; HZ: longer, less branched, often spiny; Figure 1B, bottom) and distinctive intrinsic electrical properties (Figure 1C, D), similar to *in vitro* (Suzuki and Bekkers, 2010a). Thus, we were confident that we could record from identified NG and HZ cells *in vivo*.

NG and HZ cells respond strongly to odors

We applied up to 15 odorants from a variety of chemical functional groups to urethane-anesthetized mice and measured the voltage responses of identified NG and HZ cells in the anterior PCx (Figure 1E, F). Three features were apparent in these responses: first, both cell types responded to many different odors (*i.e.* they were

broadly tuned); second, regular oscillations in the membrane potential (V_m) often became larger in the presence of odor; and third, HZ cells appeared to respond more slowly to odors than did NG cells. Each of these features is examined in the following sections.

NG and HZ cells are broadly excited by odors

Our palette of odorants was drawn from a variety of chemical functional groups intended to span a large part of ‘odor space’ (although this term is difficult to define; Pashkovski *et al.*, 2020; Ravia *et al.*, 2020). We measured odor responsiveness by median-filtering each trace to remove action potentials (APs) then testing whether the z-scored V_m amplitude exceeded a positive threshold during the period of odor application (Experimental Procedures; [Figure 1 – Figure Supplement 1](#)). These data were analyzed in two different ways: (i) by calculating the fraction of odors that each cell responded to, then averaging across cells (‘cell-averaged index’); and (ii) by calculating the fraction of cells each odor activated, then averaging across odors (‘odor-averaged index’). NG and HZ cells both had large cell-averaged indices that were not significantly different from each other (NG: 0.78 ± 0.04 , $n = 28$ cells; HZ: 0.66 ± 0.12 , $n = 9$; $p = 0.34$, Welch’s 2-sample unpaired t-test). The odor-averaged index was also large for both cell types but was significantly smaller for HZ cells (NG: 0.75 ± 0.03 , $n = 15$ odors; HZ: 0.60 ± 0.06 , $n = 15$; $p = 0.025$, Welch’s 2-sample unpaired t-test). Similar results were found by using an alternative pair of measures, the lifetime and population sparseness, which are analogous to the cell- and odor-averaged indices, respectively (Willmore and Tolhurst, 2001; Poo and Isaacson, 2009).

Respiration-locked oscillations in V_m are prominent in NG and HZ cells

Subthreshold oscillations in V_m were frequently seen in both NG and HZ cells (e.g. [Figures 2A and 2B](#), top, show the same cells as in [Figures 1E and 1F](#) responding to ethyl-n-butylate; gray traces are respiration, with upward spikes indicating onset of exhalation). Expanding the traces in windows before (*b*), during (*d*) and after (*a*) odor application revealed that oscillations in V_m were synchronized to respiration and, at least

Figure 2. Oscillations in membrane potential (V_m) are synchronized to respiration in NG and HZ cells, and the oscillation amplitude is enhanced in NG cells during odors.

in the NG cell, the amplitude of V_m oscillations appeared to increase during the odor ([Figures 2A and 2B](#), bottom). These observations were quantified by excising the segments of V_m that lay between successive positive peaks of the respiration trace, linearly warping them to have the same time axis, then averaging together all such segments within each of the three windows (*b*, *d*, *a*) for each odor. The peak-to-peak amplitude of the average V_m and the time of the positive peak of average V_m , expressed as a phase of the respiration cycle, were plotted for each odor application ([Figure 2C](#);

each triplet of connected points is from each odor; only the data from the NG cell in [Figure 2A](#) are shown). For this particular NG cell, the peak-to-peak V_m amplitude increased significantly during the odor ($b: 7.7 \pm 0.8$ mV; $d: 11.1 \pm 1.2$ mV; $n = 12$, $p = 0.002$, 1-way ANOVA with Tukey contrasts), whereas the phase of the peak was unchanged ($b: 0.49 \pm 0.02$; $d: 0.45 \pm 0.03$, both expressed as a fraction of the respiration cycle; $p = 0.47$).

A similar analysis was done for all NG and HZ cells in our dataset and the summary is shown in [Figure 2D](#). The mean peak-to-peak V_m amplitude increased significantly during odors in NG cells but not in HZ cells ([Figure 2D](#), left; $n = 232$ trials in 22 NG cells, $p < 0.0002$; $n = 99$ trials in 10 HZ cells, $p = 0.78$; 1-way ANOVA with Tukey). In contrast, the phase of the peak V_m was unchanged by odors in both NG and HZ cells ([Figure 2D](#), right). The same result was obtained if we used a different measure of phase-locking between V_m and respiration, the cross-covariance ([Figure 2 – Figure Supplement 1](#)). Thus, while NG and HZ cells both show strong respiration-coupled oscillations in their sub-threshold V_m , odors affected these cells differently: in NG cells the oscillation amplitude increased, whereas in HZ cells V_m tended to depolarize without a change in the oscillation.

We wondered if the oscillations in V_m we found in NG (and HZ) cells in the PCx were also seen in NG cells in other cortical areas and, if so, whether they were synchronized to respiration. We made targeted whole-cell recordings from NG cells in the upper layers of primary somatosensory cortex *in vivo* and found that, although V_m oscillations were strongly present, their temporal structure was different from those in PCx and they were not synchronized to respiration ([Figure 2 – Figure Supplement 2](#)).

NG and HZ cells tend to fire early and late, respectively, following odor onset

Next, we turned to the apparent difference in the kinetics of the odor response, with NG cells appearing to be excited more quickly following odor application (Figures 1E and 1F). We confirmed this impression by constructing AP raster plots and peristimulus time histograms (Figure 3A): spiking in NG cells reached a peak at 0.43 ± 0.14 s after odor onset ($n = 7$ cells), whereas in HZ cells the peak occurred at 1.92 ± 0.24 s ($n = 5$ cells; significantly different, $p = 0.0013$, Welch's 2-sample unpaired t-test). Control experiments confirmed that none of these responses were limited by the rate of delivery of odorants by our olfactometer (Figure 3 – Figure Supplement 1).

Figure 3. NG cells tend to fire earlier than HZ cells in response to odors.

To test whether this difference in dynamics could be observed using a less invasive approach, we turned to 2-photon Ca^{2+} imaging with the red-shifted indicator Cal-590. NG and HZ cells could be distinguished as before from their soma location, soma shape and GFP fluorescence (Figure 3B, top), and odor-evoked spiking could be resolved from changes in $\Delta F/F_0$ (Figure 3B, bottom). Consistent with whole-cell patch clamping (Figure 3A), Ca^{2+} imaging showed that NG cells fired quickly after odor onset and HZ cells fired with a delay (Figure 3B; traces are averages of $n = 6$ NG cells and $n = 5$ HZ cells; individual traces were too noisy to reliably determine the average time to peak).

We also took advantage of the Ca^{2+} imaging approach to examine the effect of anesthetics on this neural circuit. (Because the surgery to expose the PCx is so invasive, our experiments could not use awake animals.) All of the above experiments used urethane (0.7 g/kg). We repeated the imaging experiment in Figure 3B using fentanyl plus medetomidine, which has been used to induce a more awake-like state of anesthesia (Constantinople and Bruno, 2011; Altwegg-Boussac *et al.*, 2014). The result was the same (Figure 3 – Figure Supplement 2): in response to odors, NG cells fired early, HZ cells late. These findings, together with a report that urethane at the relatively low concentration we used here has little effect on ligand-gated synaptic receptors (Hara and Harris, 2002), suggest that the different odor-response dynamics of NG and HZ cells are not related to anesthesia.

Different kinetics of odor-evoked EPSPs and EPSCs in NG and HZ cells

Having found that NG and HZ cells differ in their AP responses to odors, we next asked whether similar differences could be observed in the underlying EPSPs and EPSCs. Odor-evoked EPSPs were median-filtered to remove APs and notch-filtered at

2-4 Hz to remove respiration-coupled oscillations, then all EPSPs measured in the same cell for different odors were averaged together (responses to 4-15 odors per cell; [Figure 4 – Figure Supplement 1](#)). Because these averages were still relatively noisy ([Figure 4 – Figure Supplement 1](#)), a smooth curve (Methods) was fitted to each averaged EPSP and the key parameters were measured from this curve. The analysis showed that odor-evoked EPSPs reached their peak about 2-fold faster in NG cells than in HZ cells ([Figure 4A](#); NG: 0.53 ± 0.05 s, $n = 16$ cells; HZ: 1.26 ± 0.26 s, $n = 6$ cells; $p = 0.038$, unpaired t-test). Thus, the slower-rising EPSPs in HZ cells ([Figure 4A](#)) can explain the longer delay to firing of these cells ([Figure 3](#)).

To confirm this result, we used a different method that focused on the rising phase of individual, unaveraged EPSPs. Traces were again median-filtered to remove APs, then the z-scored mean V_m for each respiration cycle was plotted *versus* time and a straight line was fitted over the period of odor application ([Figure 4B](#); typical NG cell on left, HZ cell on right). The slope of this line tended to be negative for NG cells and positive for HZ cells (red and blue line, respectively; [Figure 4B](#), bottom). These data were quantified for all odors and cells by plotting the peak z-scored V_m amplitude during the odor period *versus* the fitted slope ([Figure 4C](#), top; NG cells in red, $n = 310$ trials; HZ cells in blue, $n = 188$ trials). Trials that did not give an odor response (*i.e.* with peak z-scored V_m values ≤ 2.5) are grayed out ([Figure 4C](#), top). Histograms of all the remaining trials confirm that the EPSP slopes for NG and HZ cells were significantly different ([Figure 4C](#), bottom; NG slope, -0.27 ± 0.02 , $n = 232$ trials above the z-score threshold; HZ slope, 0.21 ± 0.04 , $n = 84$ trials above threshold; $p < 0.001$, Kolmogorov-Smirnov [KS] test). Thus, during odors, EPSPs in NG cells tend to decline from an early peak while EPSPs in HZ cells tend to rise to a later peak. These behaviors are consistent with the odor-evoked AP firing observed in NG and HZ cells ([Figure 3](#)).

Figure 4. Odors cause V_m to depolarize more slowly in HZ cells than in NG cells.

Differences in excitatory synaptic input can explain the odor response differences between NG and HZ cells

What cellular mechanisms might explain the different odor response dynamics of NG and HZ cells? Perhaps the simplest explanation is that NG cells receive depressing EPSPs from the olfactory bulb via the LOT, whereas HZ cells receive facilitating EPSPs (Figure 5A). To test this idea, we first recorded from NG and HZ cells in slices and applied patterned electrical stimuli to the LOT. For brief trains of stimuli (6 pulses at 40 Hz, modeled on *in vivo* patterns; Suzuki and Bekkers, 2006), the response was the

Figure 5. Differences in EPSC kinetics partly account for the different odor responsiveness of NG and HZ cells

exact opposite of what was required: NG cells facilitated and HZ cells depressed (Figure 5B, top). We next tried a more realistic model of odor-evoked afferent excitation, *i.e.* a train of 40 Hz stimuli repeating at 3 Hz (the approximate respiration frequency in mice; Poo and Isaacson, 2009). This stimulus produced a modestly depressing envelope of EPSPs in both NG and HZ cells (Figure 5B, bottom). Thus, the short-term dynamics of the LOT afferents could explain the declining response to odors in NG cells, but not the facilitating response in HZ cells.

To further explore this question, we returned to *in vivo* recordings but now used whole-cell voltage clamp to isolate odor-evoked EPSCs at a holding potential of -70 mV (Figure 5C). Similar to the result for EPSPs (previous section), the time between odor onset and EPSC peak was about 2-fold faster in NG cells than in HZ cells (NG: 0.72 ± 0.09 s, $n = 30$ cell-odor pairs; HZ: 1.24 ± 0.12 s, $n = 21$ cell-odor pairs; $p = 0.0014$, unpaired t-test; Figure 5C inset, bottom). Thus, given that the membrane time constant is fast (~ 4 ms) and not significantly different between NG and HZ cells (Suzuki and Bekkers, 2010a), the slower EPSC risetime in HZ cells is consistent with the slower-rising EPSP (Figure 4A) and the delayed onset of odor-evoked spiking in HZ cells (Figure 3).

In summary, despite the *in vitro* finding that HZ cells do not receive facilitating EPSPs from the LOT (Figure 5B), both current clamp (Figure 4A) and voltage clamp (Figure 5C) experiments *in vivo* show that HZ cells do receive slower-rising synaptic inputs than NG cells. Thus, the dynamics of excitatory synaptic inputs at least partially determine the delayed odor responses of HZ cells, although the origin of these slower dynamics remains unclear (see Discussion).

Synaptic inhibition also contributes to the odor responses of NG and HZ cells

Although we have so far focused on excitatory synaptic inputs, a likely contribution of inhibitory inputs cannot be excluded. NG and HZ cells may engage in mutual inhibition, thereby modifying the odor-evoked excitation both receive from the LOT. We addressed this possibility by making *in vivo* whole-cell voltage clamp recordings to look for odor-evoked IPSCs in isolation at a holding potential of +10 mV (Poo and Isaacson, 2009). Such IPSCs were, indeed, present in both HZ and NG cells (Figure 6A). Their properties were not significantly different between the two cell types (mean amplitude: 50.3 ± 4.3 pA vs 49.9 ± 11.0 pA; time to peak: 0.58 ± 0.12 s vs 0.35 ± 0.05 s; data for NG and HZ cells, respectively; all $p > 0.1$, $n = 14$ and 5 cell-odor pairs, respectively). Current clamp experiments confirmed that odor-evoked IPSPs

Figure 6. NG and HZ cells receive odor-evoked feedforward inhibition, predominantly from other NG cells.

could also be observed in HZ cells when they were depolarized by somatic current injection (Figure 6 – Figure Supplement 1).

What might be the origin of these inhibitory inputs? We addressed this question by making pair recordings in slices. These experiments revealed that NG→HZ cell connections were frequent and strong (Figure 6B, C; 14/43 = 32.6% connectivity; mean connection conductance 0.87 ± 0.41 nS, range 0.10 – 5.86 nS, $n = 14$ pairs) whereas the reverse connection was never seen (0/13 = 0%; significantly different, $p = 0.02$, Chi-square 2 x 2 contingency test). Synaptic connections were also comparatively rare between NG cells and between HZ cells (11.8% and 5.3% respectively; Figure 6C). Thus, odor-evoked synaptic inhibition of both NG and HZ cells is mostly likely provided by NG cells after they are excited by input from the LOT.

Feedforward inhibition alters the synaptic responsiveness of SP cells

Lastly, we explored the functional consequences of HZ and NG cell inhibition for one of their major targets, layer 2 superficial pyramidal (SP) cells. Because it is difficult to disambiguate these two types of feedforward inhibition *in vivo*, we conducted the experiments in slices.

We began by eliciting *in vivo*-like IPSPs in SP cells. Extracellular stimuli were applied to layer 1a in patterns obtained from the *in vivo* odor-evoked firing patterns of NG and HZ cells (Figure 7A, red traces), and averaged postsynaptic IPSPs were recorded in SP cells while pharmacologically blocking ionotropic glutamate receptors (Figure 7A, black traces; average of $n = 44$ or 51 single traces for 13 or 3 different NG or HZ cell stimulus patterns, respectively, while recording from 6 different SP cells).

Two further manipulations were done to make the recordings more *in vivo*-like. First, NG stimulus recordings were made distant (>400 μ m) from the LOT to avoid

stimulating HZ cell axons, which are clustered around the LOT (Suzuki and Bekkers, 2010a). On the other hand, HZ stimulus recordings were made near the LOT, where a mixture of HZ and NG cell axons were likely excited. Second, the stimulus patterns were linearly warped so that the respiration trace recorded for each stimulus pattern matched a reference respiration trace (shown in gray, Figure 7A, bottom). This was

Figure 7. NG and HZ cells produce different patterns of synaptic inhibition in postsynaptic SP cells.

done to preserve any respiration-synchronized structure in the stimulus patterns when averaging across stimuli. Such synchronization is apparent in the respiration-locked oscillations in the averaged IPSPs (Figure 7A, black traces). These results show that NG cells generate a large, rapid IPSP in SP cells, whereas HZ cells generate a smaller and more diffuse IPSP that persists beyond the end of odor application.

In a final series of experiments we examined the effect of these two types of synaptic inhibition on spiking patterns in postsynaptic SP cells. The method described in the previous paragraph was employed, except that a dynamic clamp was used to replay into the SP cell an odor-evoked excitatory postsynaptic conductance (EPSP) that had previously been recorded from an SP cell *in vivo*. Again, the EPSP and all stimulus patterns were linearly warped to match their respiration traces. When injecting the EPSP alone, an *in vivo*-like train of action potentials was evoked in the SP cell (Figure 7B, black traces). When patterned extracellular stimuli were applied at the same time, eliciting IPSPs, fewer action potentials were evoked in the SP cell by the EPSP (Figure 7B, blue traces; spikes that dropped out are indicated by asterisks above the black traces). These effects were quantified by calculating normalized PSTHs for action potentials in the SP cell (Figure 7B, bottom; averages from $n = 7$ [with NG stimulation] or $n = 4$ [with HZ stimulation] SP cells). The results confirm that NG cells strongly suppress early odor-evoked firing in SP cells ($p < 0.001$, KS test), whereas HZ cells tend to have a weaker, delayed effect (here, not significant; $p = 0.81$, KS test).

Discussion

In this paper we used whole-cell patch clamping and 2-photon Ca^{2+} imaging *in vivo* to characterize the odor responses of two types of GABAergic interneurons that provide feedforward inhibition in the input layer (layer 1a) of the anterior piriform

cortex. We find that both NG cells and HZ cells are broadly excited by different odors, but the time to reach peak excitation is much slower in HZ cells than in NG cells. This difference can be explained by a 2-fold slower time to peak of the odor-evoked compound EPSC in HZ cells compared to NG cells. Synaptic inhibition of HZ cells by NG cells may also help to suppress early firing of HZ cells. In addition, these two cell types differ in their oscillatory response to odors, with NG cells showing larger-amplitude respiration-coupled EPSPs during odor sampling. Lastly, we show that NG and HZ cells have distinctive effects on the excitability of downstream pyramidal neurons: NG cells generate powerful inhibition immediately after odor onset, whereas HZ cells exert more diffuse and prolonged inhibition. Our findings reveal a remarkable richness in inhibitory control at the very first stage of cortical odor processing, and add to a growing understanding of the role of afferent-driven feedforward inhibition in cortical processing more broadly (Khubieh *et al.*, 2016; Fan *et al.*, 2020; Anastasiades *et al.*, 2021).

Use of anesthesia

Two-photon targeted patch clamping and Ca²⁺ imaging as implemented in this study require direct access to the surface of the PCx (Margrie *et al.*, 2003; Tantirigama *et al.*, 2017). Although similar experiments can be done during wakefulness and semi-paralysis of mice (Pashkovski *et al.*, 2020), our animal ethics protocols required us to conduct all surgery and experiments under general anesthesia. We used urethane at the minimum concentration empirically determined to be effective in providing stable anesthesia with complete abolition of reflexes (0.7 g/kg *s.c.*). Urethane has been widely used in previous *in vivo* studies of the PCx (*e.g.* Barnes *et al.*, 2008; Poo and Isaacson, 2009; Poo and Isaacson, 2011; Wesson *et al.*, 2011; Chapuis and Wilson, 2012; Sturgill

and Isaacson, 2015), and we have confirmed that urethane at this concentration has no effect on electrical activity in the PCx compared with mice anesthetised with fentanyl/medetomidine (which induces a more awake-like brain state; [Figure 3 – Figure Supplement 2](#); Constantinople and Bruno, 2011; Tantirigama *et al.*, 2017). We avoided using ketamine/xylazine anesthesia which has been reported to alter PCx activity through its blockade of NMDA receptors (Fontanini and Bower, 2005; Tantirigama *et al.*, 2017; Bolding *et al.*, 2020). Despite these considerations, however, it remains possible that our findings are affected by urethane and further study is warranted.

Comparison with previous findings

Most previous work has used electrical stimulation in slices to study feedforward inhibition in the PCx (Luna and Schoppa, 2008; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010a; Suzuki and Bekkers, 2012; Sheridan *et al.*, 2014; Stokes *et al.*, 2014; Large *et al.*, 2016a; Large *et al.*, 2016b). Only two reports, to our knowledge, have explicitly examined this feedforward circuit using odor stimulation *in vivo*. Poo and Isaacson (2009) made blind whole-cell recordings from neurons in layer 1, while Bolding and Franks (2018) made unit recordings from optogenetically identified GABAergic neurons in layer 1. However, neither study distinguished interneuron subtypes.

We found broad tuning of odor-evoked EPSPs in NG and HZ cells, consistent with previous findings (Poo and Isaacson, 2009; odor-averaged index, 0.50 ± 0.04 *cf* 0.75 and 0.60 for NG and HZ cells, respectively, found here). Interestingly, broad odor-tuning has also been reported for some (Zhan and Luo, 2010; Poo and Isaacson, 2011; Bolding and Franks, 2017) but not all (Sturgill and Isaacson, 2015) classes of feedback inhibitory neurons located in deeper layers of the PCx. It is likely that this diversity in

stimulus tuning reflects the number and variety of functional inputs from upstream neurons. Indeed, it has been shown that Layer 1 interneurons receive a higher convergence of afferent input from the olfactory bulb than do principal neurons (Poo and Isaacson, 2009; Miyamichi *et al.*, 2011). Future work could repeat these experiments while distinguishing NG and HZ cells. Differences might arise because HZ cells, unlike NG cells, are clustered around the LOT where they may encounter a higher density of afferents (Suzuki and Bekkers, 2010a).

We often observed oscillations in subthreshold V_m that were phase-locked to respiration, consistent with previous reports that such oscillations are ubiquitous in the olfactory system (Fontanini and Bower, 2006; Kay *et al.*, 2009; Poo and Isaacson, 2009; Wilson, 2010; Oswald and Urban, 2012; Kay, 2014; Jiang *et al.*, 2017). Our novel finding was that respiratory oscillations in V_m often increased in amplitude during odor sampling, but only in NG cells. These odor-dependent oscillations were functionally relevant because they generated respiratory-patterned IPSPs in target neurons (Figure 7A) and provided rhythmic inhibition of similarly patterned EPSPs (Figure 7B; see also Poo and Isaacson, 2009). This difference between NG and HZ cells suggests that NG cells provide fast, phase-critical feedforward inhibition whereas HZ cells provide a slower, tonic form of inhibition. Interestingly, Bolding and Franks (2018) reported only the latter kind of feedforward inhibition in multi-unit recordings from awake mice.

Difference in odor-evoked EPSP kinetics

Our main finding is that odor-evoked firing in HZ cells has a delayed onset, contrasting with the rapid onset in NG cells. We showed that this effect can be at least partly explained by the slower time-to-peak of the odor-evoked compound EPSC in HZ cells (Figure 5C). Surprisingly, however, these kinetic differences cannot be explained

by the properties of short-term synaptic plasticity at LOT synapses, assayed in slices (Figure 5B). What might be alternative explanations?

The LOT is a heterogeneous fiber tract that contains the axons of two distinct types of projection neurons in the olfactory bulb, mitral and tufted cells. Mitral cells have been reported to respond about 2-fold more slowly to odor stimulation than tufted cells (Fukunaga *et al.*, 2012; Igarashi *et al.*, 2012) because of differences in synaptic inhibition in the bulb (Fukunaga *et al.*, 2012; Geramita and Urban, 2017). It is possible that mitral and tufted cell axons preferentially target HZ and NG cells, respectively. Thus, the delayed odor response of HZ cells may originate in the olfactory bulb rather than at synaptic terminals in the PCx. This could be tested anatomically, or by recording from HZ or NG cells *in vivo* while preferentially exciting mitral or tufted cells (Fukunaga *et al.*, 2012; Geramita and Urban, 2017).

A second possible explanation is that HZ cells may receive delayed bulbar input via an intermediary, such as the anterior olfactory nucleus (Haberly and Price, 1978; McGinley and Westbrook, 2011; Kay and Brunjes, 2014). This could again be tested anatomically or by silencing the anterior olfactory nucleus during odor application.

A third possibility is that lateral inhibition from NG cells counteracts early excitation in HZ cells, delaying depolarization. In support of this idea, NG cells profusely innervate HZ cells (Figure 6C) and an odor-evoked hyperpolarization with the right time course can be observed in HZ cells (Figure 6 – Figure Supplement 1). On the other hand, NG cells also receive odor-evoked synaptic inhibition, presumably from other NG cells (Figure 6A), yet do not exhibit delayed odor responses like HZ cells. It is possible that inhibition from NG cells is larger in HZ cells but it was not apparent here because the number of experiments was relatively small.

Functional significance of feedforward inhibition in the PCx

Feedforward inhibition is generally recognized as an input-tracking mechanism which, in the hippocampus and neocortex, can synchronize spike timing (Pouille and Scanziani, 2001; Gabernet *et al.*, 2005) and modulate gain through input normalization (Pouille *et al.*, 2009). The PCx is unusual in that it receives its feedforward inhibitory input onto the distal apical dendrites of principal cells, rather than onto perisomatic regions as in other cortices (Lourenco *et al.*, 2020). What are the consequences of this atypical architecture for the role of feedforward inhibition in the PCx?

One slice study has shown that feedforward inhibition works together with feedback inhibition to provide spatially and temporally modulated inhibition during bursts of olfactory input (Stokes and Isaacson, 2010). Intriguingly, the shift in inhibition reported by Stokes and Isaacson (2010) is in the opposite direction to that found in other brain regions (Pouille and Scanziani, 2004; Silberberg and Markram, 2007). These results suggest that feedforward inhibition in the PCx is functionally important but has unusual features.

More recently, an *in vivo* unit recording study in awake mice showed that odor-evoked spiking in feedforward inhibitory neurons in the PCx increases only slowly and weakly after inhalation, suggesting that these neurons provide tonic inhibition and do not play a major role in sculpting odor-evoked responses (Bolding and Franks, 2018). Modeling confirmed that feedforward inhibition provides modest subtractive normalization in the PCx, in contrast to the divisive normalization it provides in other cortical regions (Stern *et al.*, 2018). An intuitive explanation is that the PCx is a comparatively ‘slow’ processing device. Odor stimuli tend to be slow, and the PCx uses a temporal or rate-based code that is more susceptible to ‘recurrent normalization’ provided by feedback inhibition (Sturgill and Isaacson, 2015; Bolding and Franks,

2018; Stern *et al.*, 2018; Bolding *et al.*, 2020; Pashkovski *et al.*, 2020). Hence, the evidence of Bolding and colleagues suggests that feedforward inhibition plays a minor role in odor processing under the conditions of their experiments.

The results of Bolding and colleagues seem at variance with our finding that many layer 1a interneurons, especially NG cells, respond vigorously to odors. The discrepancy may lie in the different recording conditions, *e.g.* whole-cell *vs* unit recordings, placement of the electrodes (close to or far from the LOT), or our use of anesthesia. Further work is required. However, if feedforward inhibition in the PCx is, indeed, weakly engaged in normal olfactory processing, what other functions might it serve?

The key property of feedforward inhibition in the PCx may be its dendritic localization. By inhibiting the distal apical dendrites of layer 2/3 principal cells, NG and HZ cells have the potential to dampen passive propagation of afferent EPSPs to the soma and to veto dendritic electrogenesis (Larkum *et al.*, 1999; Palmer *et al.*, 2014; Pardi *et al.*, 2020; Anastasiades *et al.*, 2021). Indeed, it has been directly shown *in vitro* that a single layer 1 interneuron can inhibit Ca²⁺ signaling in the distal dendrites of PCx principal cells in a branch-specific fashion (Stokes *et al.*, 2014). Feedforward inhibition might then provide a mechanism for regulating processes that depend on dendritic electrogenesis – including burst-firing (Tseng and Haberly, 1989; Protopapas and Bower, 2001) and spike timing-dependent plasticity (Kanter *et al.*, 1996; Jochenning *et al.*, 2009; Cassenaer and Laurent, 2012) – that may only become apparent during experimental paradigms that engage olfactory learning (Wilson and Stevenson, 2006; Ghosh *et al.*, 2015; Shakhawat *et al.*, 2015; Meissner-Bernard *et al.*, 2019). Future work would need to explore this possibility.

Two types of feedforward inhibition

We have previously shown in slices that layer 1a NG cells and HZ cells generate slow-rising (2-3 ms) and fast-rising (1 ms) feedforward unitary IPSPs, respectively, in layer 2 principal neurons, leading us to suggest that NG cell-mediated feedforward inhibition is slower and more diffuse than that provided by HZ cells (Suzuki and Bekkers, 2012). In the present report, after taking into account the much slower odor-evoked excitatory drive received by HZ cells, we reach the opposite conclusion. Our results are a reminder that the unitary properties of synapses, measured *in vitro*, can be less important than the patterns of concerted synaptic activity occurring *in vivo*.

What might be the value of delivering fast (NG) and slow (HZ) feedforward inhibition? We have shown in a slice simulation (Figure 7) that NG cells can strongly inhibit firing in principal cells immediately after a step application of odor. Although this effect was not observed in the study of Bolding and Franks (2018), there nevertheless appears to be scope for a physiological role for this kind of fast inhibition. For instance, NG cells generate GABA transients that spill out of the synaptic cleft and can activate heterosynaptic GABA_A and GABA_B receptors (Karayannis *et al.*, 2010; Overstreet-Wadiche and McBain, 2015). Future work could explore ways in which this ‘volume transmission’ could enhance computational complexity in the PCx.

The role of HZ cell-mediated feedforward inhibition is more puzzling. Our slice simulation showed that HZ cells generate a delayed, diffuse response with little effect on principal cell excitability (Figure 7). Trained rodents can discriminate odors within a single sniff (<100 ms; Uchida and Mainen, 2003; Abraham *et al.*, 2004; Rinberg *et al.*, 2006; Resulaj and Rinberg, 2015). What could be the relevance of late-firing HZ cells in this scenario? Two unique features of HZ cells suggest that they are potentially important. First, HZ cells are only found close to the LOT (≤ 200 μm), giving them

privileged inhibitory influence over a spatially restricted subset of principal cells (Suzuki and Bekkers, 2010a; Suzuki and Bekkers, 2012). Second, they are the only class of interneuron in the PCx, and one of few in the cerebral cortex, that are profusely spiny (Haberly, 1983). Given the importance of spines for synaptic plasticity (Engert and Bonhoeffer, 1999; Maletic-Savatic *et al.*, 1999; Toni *et al.*, 1999; Matsuzaki *et al.*, 2004; Tonnesen *et al.*, 2014), HZ cells are poised to receive learning-dependent synaptic excitation, which in turn may allow them to exert adjustable inhibitory control over neurons in their neighborhood.

In summary, we have shown that two forms of odor-evoked feedforward inhibition, fast and slow, are expressed in layer 1a of the PCx. Our work reveals a previously unknown multi-functional circuit element in the PCx, and adds to a growing understanding of the role of neurons in layer 1 of the cerebral cortex.

Materials and Methods

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Australian National University and conform to the Australian Code for the Care and Use of Animals for Scientific Purposes, published by the National Health and Medical Research Council of Australia.

Animals and surgery for ‘in vivo’ experiments

Experiments used heterozygous GAD67-GFP (Δ neo) mice bred on a C57BL6/J background (Tamamaki *et al.*, 2003). Animals were aged 35-75 days and weighed 17-24 g. For surgery, an animal was sedated with chlorprothixene (5mg/kg *i.p.*) then injected with urethane (0.7 g/kg *s.c.*) for general anesthesia plus atropine (0.2-0.3 mg/kg *s.c.*) to reduce secretions. The correct level of anesthesia was confirmed by

535 observing regular respiration and the complete absence of a pinch reflex; a topup dose
536 of urethane was sometimes required after 5-6 hours. A local anesthetic (prilocaine,
537 0.2 mg/kg) was applied topically to incision sites during surgery. Access to the PCx
538 was via the cheek and upper mandible, as previously described (Stettler and Axel, 2009;
539 Tantirigama *et al.*, 2017). Briefly, the following surgical procedure was used (total
540 duration ~2 h). After retracting the skin, superficial blood vessels of the cheek were
541 cauterized, then the temporalis muscle was carefully detached and retracted towards its
542 base near the eye, revealing the temporal aspect of the skull. The zygomatic bone and
543 the upper sections of the mandible, including the coronoid and the condyloid processes,
544 were removed, followed by the upper section of the masseter muscle, exposing the
545 basolateral surface of the skull. At this point, the PCx was visible under the translucent
546 skull, identifiable using anatomical landmarks such as the lateral olfactory tract (LOT)
547 and the middle cerebral artery (MCA), which are roughly perpendicular to each other.
548 A metal head post was then glued on top of the skull and the head was stabilized. A
549 craniotomy (~2 mm²) was made with a fine drill (Osada Electric, Nagoya, Japan, or
550 Ram Products Inc, Dayton, NJ) just caudal to the MCA and close to the LOT. This
551 placement of the craniotomy ensured that all recordings were made in the anterior PCx.
552 In many experiments the dura was left intact, but in some the dura was carefully
553 removed using a needle and fine forceps. For the targeted patching a coverglass
554 fragment was glued in place over the craniotomy, leaving a gap at one edge for electrode
555 access. For the calcium imaging a gap was not required, and for 'blind' patch clamping
556 a coverslip was not used. After completion of the surgery a small chamber made from
557 a plastic weighboat and dental cement was constructed around the site. The chamber
558 was filled with phosphate-buffered saline to keep the area hydrated and allow
559 immersion of the microscope objective. For all the above procedures, as well as during

recordings, the animal was placed on an electrically-heated surface at ~37 °C and was kept hydrated by periodic *s.c.* injections of normal saline with 2% dextrose.

Two-photon targeted patch clamping

GFP-positive interneurons in GAD67-GFP (Δ neo) mice were visualized using a 2-photon MOM microscope (Sutter Instrument Company, Novato, CA) with a 40x/0.8 NA water immersion objective (Olympus, Tokyo, Japan) and a Chameleon Ultra Ti:Sapphire laser (Coherent, Santa Clara, CA) tuned to 800-820 nm (Suzuki *et al.*, 2014). Frames (512 x 512 pixels) were acquired simultaneously through a red and green channel at ~5 Hz under the control of ScanImage (Vidrio Technologies, Ashburn, VA). Patch pipettes were pulled with a longer taper than usual and had resistances of 5-8 M Ω when filled with internal solution comprising (in mM) 135 KMeSO₄, 7 NaCl, 0.1 EGTA, 2 Na₂ATP, 2 MgCl₂, 0.3 GTP, 10 HEPES at pH 7.2, supplemented with 0.2-0.4% biocytin (295-300 mOs/kg). This solution had a Cl⁻ concentration of 11 mM and a measured junction potential of -7 mV. For voltage clamp experiments CsMeSO₄ replaced KMeSO₄. These solutions also contained Alexa-594 (20 μ M) for visualizing the electrode in the red channel. Electrical recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices, San Jose, CA). Data were filtered at 10 kHz and sampled at 20-50 kHz using an Instrutech ITC-18 digitizing interface (HEKA, Ludwigshafen, Germany) under the control of Axograph X (Axograph Scientific, Sydney, Australia). The reference electrode was a Ag/AgCl wire inserted under the skin. The patch electrode was advanced rapidly to penetrate the dura, then more slowly to approach the selected cell and obtain a gigaseal whole-cell recording in the usual way (Margrie *et al.*, 2002). For current clamp recordings, bridge balance and capacitance neutralization were adjusted and the cell was allowed to remain at its

resting potential. For voltage clamp recordings, series resistance compensation was not used. Cells were included in the dataset if they had a mean resting potential more hyperpolarized than -50 mV and were stable enough to allow the recording of responses to at least 5 odors. In addition, cells had to be unambiguously identified as either NG or HZ cells according to the criteria given in the Results. At the end of the recording an image stack of the cell was acquired in both the red and green channels.

Blind ‘in vivo’ patch clamping

This method was used to measure the EPSGs used in the *in vitro* simulation in Figure 7B. The dura was removed but a coverslip was not used. Patch electrodes were prepared and filled as for targeted patch clamping, then an electrode was positioned at the surface of the anterior PCx using a dissection microscope. The micromanipulator (MP-285, Sutter Instrument Company, Novato, CA) was zeroed at the surface, then the electrode was advanced rapidly to the search depth (150-280 μ m, corresponding to layer 2) while applying high pressure (25 kPa), after which the pressure was reduced (4-8 kPa) and a whole-cell recording obtained using standard techniques (Margrie *et al.*, 2002; Poo and Isaacson, 2009). Data were acquired as described above. EPSGs were calculated from EPSCs recorded under voltage clamp at a holding potential of -70 mV, close to the chloride reversal potential for these solutions. Layer 2 principal neurons (semilunar [SL] and superficial pyramidal [SP] cells) were identified by their intrinsic electrical properties (Suzuki and Bekkers, 2006) and by the recording depth (SL: 150-200 μ m; SP: 200-280 μ m). Cell identity was also confirmed by fixing the brain at the end of the experiment and recovering the morphology of the recorded neuron as previously described (Suzuki *et al.*, 2014).

610 ***'In vivo' functional Ca²⁺ imaging***

611 Imaging used the red-shifted Ca²⁺ indicator Cal-590 AM (AAT Bioquest,
612 Sunnyvale, CA), which was prepared and injected as previously described (Tischbirek
613 *et al.*, 2015). Briefly, the dura was removed and dye (1 mM) was pressure-injected into
614 the PCx at a depth of ~200 μm using a glass pipette (tip diameter ~10 μm). A coverslip
615 was glued over the PCx and imaging commenced >1 hour after injection. Imaging
616 frames were acquired at 30 Hz using a custom-modified B-scope 2-photon microscope
617 (Thorlabs, Newton, NJ) with a 16x/0.8 NA water immersion objective (Nikon, Tokyo,
618 Japan), resonance-galvanometer scanners and a Chameleon Ultra Ti:Sapphire laser
619 (Coherent, Santa Clara, CA) tuned to 800-820 nm. Cells were included in the dataset
620 if they unambiguously satisfied the fluorescence and soma morphology criteria given in
621 Results, and if they exhibited clear odor responses according to the criteria under *Data*
622 *analysis*, below.

623

624 ***Odor presentation***

625 A custom-built flow-dilution olfactometer was used to deliver up to 15 odors
626 which were diluted to 10% of their saturated vapor pressure in charcoal-filtered medical
627 air (flow rate 1 L/min; Bozza *et al.*, 2004). The odors used in this study were: ethanol,
628 1-pentanol, 1-heptanal, limonene, eugenol, 2-heptanone, acetophenone, butyric acid,
629 benzaldehyde, anisole, lavender oil (Sigma-Aldrich, St Louis, MO), ethyl-n-butyrate,
630 propionic acid (VWR, Poole, United Kingdom), amyl acetate and isoamyl acetate (MP
631 Biomedicals, Sydney, Australia). Odors were presented for 3 s at 60 s intervals.
632 Control experiments used a miniPID photo-ionization device (Aurora Scientific,
633 Aurora, Canada) to confirm that odors were presented in a step-like manner (20-80%
634 risetime in 46 ± 2 ms, $n = 9$ odors; [Figure 3 – Figure Supplement 1](#)). However, there

was a consistent delay of 242 ± 6 ms ($n = 9$) between the switching time of the final valve and the arrival of the odorant at the detector inlet (Figure 3 – Figure Supplement 1). All odor arrival times in the paper have been corrected for this delay. The mouse was freely breathing and its respiration was recorded using a piezoelectric strap (Kent Scientific, Torrington, CT) around the abdomen. Control experiments confirmed that the onset of inhalation coincided with the start of the downward step visible in some respiration traces (e.g. Figure 2, Figure 3 – Figure Supplement 2) and the beginning of exhalation corresponded to the peak of the upward spike. Because the upward spike was a more reliable feature, we estimated inhalation onset by reference to the exhalation. In a subset of experiments in which both features were clear, we measured the mean latency from the peak of the exhalation spike to the start of the following inhalation (239 ± 8 ms; mean time between exhalation spikes, 365 ± 12 ms; $n = 10$ mice). Hence, expressed as a fraction of a respiration cycle, inhalation onset occurred at 0.654 ± 0.007 after the exhalation spike; this was used to estimate the time of onset of inhalation for all experiments. Odor stimulus onset was defined as the time of the first estimated onset of inhalation that occurred following the corrected odor arrival time. To avoid habituation, each odor was presented only once. Cross-habituation between different odors was not observed.

Slice experiments

Coronal slices (300 μ m thick) were obtained from the anterior PCx of GAD67-GFP (Δ neo) mice aged 20-30 days, as previously described (Suzuki and Bekkers, 2006; Suzuki and Bekkers, 2012). Briefly, slices were prepared on a tissue slicer (Campden Instruments, Loughborough, United Kingdom) in ice-cold high-divalent cutting solution comprising (in mM) 125 NaCl, 3 KCl, 0.5 CaCl_2 , 6 MgCl_2 , 25 NaHCO_3 , 1.25

NaH₂PO₄, 2 ascorbate, 3 pyruvate and 10 glucose (osmolarity 305 mOs/kg), bubbled with 5% CO₂/95% O₂ (carbogen). The slices were incubated for 40 min at 34 °C in carbogen-bubbled artificial cerebrospinal fluid (ACSF; composition below) then were held at room temperature until required.

Whole-cell patch-clamp recordings were made from visually identified GFP-positive interneurons using an Olympus BX51WI microscope equipped with infrared differential interference contrast and wide-field fluorescence, as described previously (Suzuki and Bekkers, 2012). Slices were superfused with warmed ACSF containing (in mM) 125 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄ and 25 glucose (310 mOs/kg), bubbled with carbogen and maintained at 33 ± 1°C. The bath solution usually contained 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 µM) and D-aminophosphonovaleric acid (D-APV, 50 µM) to block ionotropic glutamate receptors (Tocris, Abingdon, United Kingdom). Patch electrodes had resistances of 4-6 MΩ when filled with KMeSO₄- or CMeSO₄-based internal solution (same as used *in vivo*). Unless stated otherwise, all compounds were obtained from Sigma-Aldrich.

Recordings were obtained using the same instrumentation and software as for the *in vivo* experiments. The stimulating electrode was made from a low-resistance patch electrode (~1 MΩ) filled with ACSF and coated with conductive paint. For pair recordings, the pre- and postsynaptic electrodes were filled with KMeSO₄- and CsMeSO₄-based internal solutions, respectively, allowing the postsynaptic neuron to be voltage clamped at a holding potential of +50 mV while the presynaptic neuron was allowed to remain at its resting potential in current clamp mode. Cells were included in the dataset if they had a stable resting potential more hyperpolarized than -60 mV (current clamp) or a stable holding current <-100 pA when held at -70 mV (voltage clamp). At the end of the experiment the slice was fixed and processed with an ABC

kit (Vector Laboratories, Burlingame, CA), allowing recovery of the morphologies of the recorded neurons.

Patterned stimulation and dynamic clamp experiments in slices

In vivo-like patterns of inhibitory synaptic stimulation in slice experiments (Figure 7) were adjusted to a common respiration timebase as follows. First, a subset of odor-evoked firing patterns from NG and HZ cells, together with their associated respiration traces, was randomly selected from the full dataset for each cell type. For each subset, one firing/respiration combination was chosen as a reference, and the first upward peak in the reference respiration trace after odor onset was defined as t_0 . Every other odor-evoked firing pattern in that subset was translated in time to align its first respiration peak after odor onset to t_0 . Working backwards and forwards from t_0 , the respiration trace (and associated firing pattern) of each other pattern was linearly warped so the peaks in its respiration trace matched those of the reference respiration trace. These warped stimulus patterns (examples in Figure 7A, red traces) were then used as the trigger to the extracellular stimulator in slice experiments. The same method was used to align the EPSG to the inhibitory stimulation patterns (Figure 7B). In this case the respiration trace for the EPSG was used as the reference timebase.

The EPSG used in Figure 7B was obtained from an EPSC recorded blind *in vivo* from an SP cell in response to a 3 s-long application of ethyl-n-butyrate. For each neuron the conductance magnitude was adjusted to produce a similar firing rate with the extracellular stimulus switched off, then the stimulator was switched on to record the effect of synaptic inhibition. In order to provide a reference firing rate for normalizing the PSTH (Figure 7B), a fixed conductance stimulus was inserted at the

end of the EPSG, well past the odor period (not visible in [Figure 7B](#)). The dynamic clamp was implemented using Igor Pro (Wavemetrics, Lake Oswego, OR).

Data analysis

All analysis was done using Igor Pro, Axograph X, Matlab (MathWorks, Natick, MA), or R (running under RStudio, Boston, MA).

Action potential (AP) properties ([Figure 1D](#)) were measured as previously described for slice experiments (Suzuki and Bekkers, 2010a). Latency to the first AP was the delay from the beginning of the current step to the first AP at rheobase. AP height was the difference between the peak of the first AP at rheobase and its threshold voltage (defined as the membrane potential [V_m] at which dV_m/dt first exceeded 15 V/s). AP halfwidth was the width of the first AP at rheobase measured at half its height. Respiration-correlated oscillations in V_m ([Figure 2](#), [Figure 2 – Figure Supplement 1](#), [Figure 2 – Figure Supplement 2](#)) were characterized as follows. Respiration traces were band-pass filtered at 1-50 Hz and normalized to oscillate between 0 and 1. The time between successive exhalation spikes was used to calculate the mean respiration frequency in three 3 s-long windows (-6 to -3 s, 0 to 3 s, and 16 to 19 s, all with respect to odor onset). The same three windows were used for the analysis of oscillations in V_m . First, V_m was median-filtered to remove APs. Mean peak-to-peak amplitude of the oscillations in V_m was found by excising segments of V_m between successive exhalation spikes, linearly stretching ('warping') them so they had the same time axis, then averaging together all segments within each of the three analysis windows. Mean peak-to-peak amplitude was taken as the difference between the maximum and minimum amplitude of the averaged segment of V_m . For cross-covariance analysis ([Figure 2 – Figure Supplement 1](#), [Figure 2 – Figure Supplement 2](#)), the respiration trace was

replaced by a normalized series of Gaussians representing the upward spikes in the respiration cycle; this was done to eliminate the effect of differences in the amplitude of the recorded respiration trace. The amplitude of the largest positive peak in the covariance was found, as well as the location of this peak expressed as a fraction of the mean time between exhalation spikes for that window.

The odor response indices were found by: (i) calculating the fraction of odors that each cell responded to, then averaging across cells ('cell-averaged index'); and (ii) calculating the fraction of cells each odor activated, then averaging across odors ('odor-averaged index'). Odor responsiveness was determined using a z-score criterion: median-filtered V_m segments demarcated by exhalation spikes were excised as above, and the mean V_m in each of these segments was calculated for the entire odor trial. These values were converted to a z-score by subtracting the mean and dividing by the standard deviation of the list of mean V_m values during the 8 s-long pre-odor baseline period. An odor response was said to occur if this z-score value exceeded 2.5 for any respiration segment during the 3 s-long odor application period (Figure 1 – Figure Supplement 1). Lifetime sparseness was calculated with a modified expression (Pashkovski *et al.*, 2020) that used the peak z-scored response (without thresholding) as the variable.

Individual odor-evoked EPSPs (Figure 4A), EPSCs (Figure 5C) and IPSCs (Figure 6A) were notch-filtered at 2-4 Hz, if required, to remove respiration-associated oscillations prior to averaging (see also Figure 4 – Figure Supplement 1). To estimate the latency to peak and halfwidth, each individual EPSC was fitted to the equation $I(t) = a(1 - e^{-t/\tau_1})^2 e^{-t/\tau_2}$, where a is amplitude and τ_1 and τ_2 are the rising and falling time constants, respectively. The latency and halfwidth were then measured from this fitted curve.

In vivo Ca²⁺ imaging experiments (Figure 3, Figure 3 – Figure Supplement 2) were analyzed by manually drawing regions of interest around the somas of identified neurons in the green channel then measuring mean somatic fluorescence, F , in the red channel for each frame. Baseline fluorescence (F_0) for each neuron was defined as the median of the lower 80% of all values of F measured in all frames acquired prior to odor application. Plots show $\Delta F/F_0 = (F - F_0)/F_0$.

Pair recordings in slices (Figure 6B, C) were analyzed as follows. The peak postsynaptic current was found within a 10 ms-long window starting 2 ms after the time of the presynaptic AP, then the mean peak amplitude (a_{pk}) was found by averaging over a 1 ms long window around the peak and subtracting the averaged baseline over a 10 ms-long window ending 2 ms before the time of the presynaptic AP. The same measurement procedure was then repeated for 200 randomly-chosen times during the 500 ms-long baseline period preceding the time of the presynaptic AP, and the standard deviation (s_b) of these baseline mean amplitudes was calculated. A synaptic connection was identified if $|a_{pk}| > |3s_b|$.

Statistics

All statistical analysis was done using R (Version 3.6.0) running under RStudio. Sample sizes were not predetermined using a statistical test; we established that our sample sizes were sufficient from the size and statistical significance of the results, and our sizes are similar to those commonly used in the field. Data collection and analysis were not blinded or randomized, but analysis was automated whenever possible. Results are presented as mean $\pm$ standard error of the mean (SEM) with associated exact p value (n = number of cells or cell-odor pairs, as indicated). Pairwise comparisons were done using Welch's unpaired 2-tailed t-test ($t.test$ function in R). Multiple paired

comparisons used 1-way ANOVA with Tukey's contrasts (*lmer* and *glht* functions in R) with blocking by cell and odor number. Visual inspection of residual plots did not reveal any large deviations from homoscedasticity. Distributions were compared using the Kolmogorov-Smirnov test (*ks.test*). Significance is indicated on the figures by *ns* (not significant) or by one or more asterisks, following the usual convention in R.

Competing interests

None to declare.

Author contributions

Norimitsu Suzuki, Conceptualization, Data curation, Software, Formal Analysis, Validation, Investigation, Methodology; Malinda L. S. Tantirigama, Conceptualization, Data curation, Software, Formal Analysis, Validation, Investigation, Visualization, Methodology; Helena H.-Y. Huang, Data curation, Validation, Investigation, Methodology; John M. Bekkers, Conceptualization, Resources, Data curation, Software, Formal Analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Project administration.

Ethics

Animal experimentation: This work was performed in strict accordance with the guidelines contained in the Australian Code for the Care and Use of Animals for Scientific Purposes, published by the National Health and Medical Research Council of Australia. All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Australian National University (Protocols A2014/11, A2017/27, A2018/52). All surgeries and experiments were performed under

anesthesia with either urethane or fentanyl/medetomidine, with supplementary
isoflurane as required, and every effort was made to minimize suffering.

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Additional files

Figure Supplements.

Figure Data.

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Figure legends

Figure 1. Layer 1a neurogliaform (NG) and horizontal (HZ) cells are reliably distinguished using two-photon-targeted patch clamping *in vivo*. NG and HZ cells respond differently to odors. **A**, Schematic showing two forms of synaptic inhibition received by principal cells (Pr) in the PCx: feedforward (red, mediated by NG and HZ cells in layer 1a [L1a]) and feedback (blue, mediated by fast-spiking [FS] and regular-spiking [RS] cells in L3). Feedback inhibition from other cell types (*e.g.* bitufted cells, deep NG cells) is not shown. LOT, lateral olfactory tract; *aff*, afferent layer; *assn*, associational layers. **B** (top), 2-photon images from a GAD67-GFP (Δ neo) mouse showing (arrowed, left) the very bright GFP fluorescence in a NG cell and the much weaker GFP in a HZ cell (arrowed, right; arrowhead indicates a nearby NG cell). **B** (bottom), z-projection of the same two cells imaged in the red channel; internal solution contained Alexa Fluor-594. Dashed yellow lines show approximate position of patch electrode. Scale bar (bottom right) applies to all panels. **C**, Responses of NG cell (left) and HZ cell (right) *in vivo* to a depolarizing current step near rheobase. **D**, Comparison of selected properties of action potentials recorded in NG and HZ cells in response to current steps *in vivo*. Points show data from individual neurons, bars show mean $\pm$ SEM. **, $p < 0.01$, $n = 33$ for NG, $n = 11$ for HZ, Welch 2-sample unpaired t-test. **E**, Response of a NG cell to a palette of 15 structurally-diverse odors (name under each trace). Application period is shown by the red bar. Similar results were obtained from a total of 28 NG cells. **F**, Recordings made from a HZ cell. Similar results were obtained from 10 HZ cells. Further details on analyzing these odor responses are in **Figure 1 – Figure Supplement 1**.

Figure 2. Oscillations in membrane potential (V_m) are synchronized to respiration in NG and HZ cells, and the oscillation amplitude is enhanced in NG cells during odors. **A** (top), Response of a NG cell to ethyl-n-butyrate. Gray trace below shows the respiration. **A** (bottom), Indicated time windows are shown expanded, with the traces in 3 consecutive sub-windows shown superimposed: *b*, before odor application; *d*, during; *a*, after. **B**, Same for the response of a HZ cell to ethyl-n-butyrate. **C**, Summary of measurements from the NG cell in panel **A** when applying 12 different odors. Plots show peak-to-peak amplitude of mean V_m (left) and location of peak mean V_m expressed as phase of respiration cycle (right), each averaged over ~10 respiration cycles occurring in a 3 s-long window before, during or after odor application. Lines connect measurements made in the same sweep for each of the 12 odors. **D**, Summary of mean peak-to-peak V_m amplitudes (left) and phase (right) measured as in panel **C**, averaged over $n = 22$ NG cells and $n = 10$ HZ cells. Black, red and blue bars show mean $\pm$ SEM before, during and after odor application, respectively. ***, $p < 0.001$; **, $p < 0.01$; *ns*, not significant; ANOVA. Similar results were obtained using an alternative approach, cross-covariance analysis (**Figure 2 – Figure Supplement 1**). In contrast, respiration-synchronized oscillations in V_m were not observed in an NG cell in the primary somatosensory cortex (**Figure 2 – Figure Supplement 2**).

Figure 3. NG cells tend to fire earlier than HZ cells in response to odors. **A** (top), Spike raster plots for NG cells (left) and HZ cells (right) in our dataset that fired at least one AP before, during or after odor application ($n = 98$ trials in 8 NG cells, $n = 86$ trials in 6 HZ cells). Different colors indicate different cells. Gray

bar indicates period of odor application. **A** (bottom), Peristimulus time histograms (PSTHs) for the raster plots, showing the greater delay to odor-evoked firing in HZ cells. These responses were not limited by the rate of delivery of odorants by our olfactometer (**Figure 3 – Figure Supplement 1**). **B** (top), 2-photon images of GFP (green) and Cal-590 (red) fluorescence for a layer 1a NG cell (left, arrowhead) and a HZ cell (right, arrowhead). **B** (bottom), Averaged $\Delta F/F_0$ plots for the somatic responses of $n = 6$ NG cells (left) and $n = 5$ HZ cells (right) in response to odor. Similar results were observed using alternative anesthesia that produces a more awake-like state (**Figure 3 – Figure Supplement 2**)

Figure 4. Odors cause V_m to depolarize more slowly in HZ cells than in NG cells. **A**, Averaged *in vivo* current clamp recordings of odor-evoked EPSPs measured in NG cells (left, $n = 16$ cells) and HZ cells (right, $n = 6$). Inset (bottom) shows the same traces normalized to their peaks, overlaid and expanded, showing that averaged EPSPs in HZ cells have a slower time to peak. To facilitate comparison, these EPSPs were filtered to remove respiration-linked oscillations (**Figure 4 – Figure Supplement 1**). **B** (top), Response of a NG cell to ethyl-n-butyrate (left) and a HZ cell to 1-heptanal (right). The corresponding respiration trace is shown below (gray). **B** (bottom), z-score-transformed mean V_m amplitude (averaged over each respiration cycle) calculated for the same two neurons as above. Spikes were removed before measuring V_m . Upper horizontal dashed line indicates the detection threshold (z-score = 2.5) for an odor-evoked response. Superimposed red (left) or blue (right) line represents a linear fit to the data points over the 3 s-long odor application period (gray band), giving slopes of -0.70 and

1.36 z-score units/respiration cycle, respectively. **C** (top), Plot of peak V_m z-score during odor application *versus* slope (fitted as in panel **B**, bottom), where each data point represents the odor response of a single cell (red symbols, $n = 310$ responses from 28 NG cells; blue symbols, $n = 188$ responses from 9 HZ cells). Points below the threshold for an odor-evoked response (z-score = 2.5) have been greyed out. **C** (bottom), Probability density function (PDF) of the points plotted in panel **C** (top), excluding the greyed-out values. NG cells (red plot) show a significant skew towards negative slopes, whereas HZ cells (blue) skew towards positive slopes ($p < 0.001$; Kolmogorov-Smirnov test).

Figure 5. Differences in EPSC kinetics partly account for the different odor responsiveness of NG and HZ cells **A**, Schematic showing the circuit dynamics that are proposed to explain the results in Figure 4, *viz.* LOT input onto NG and HZ cells is hypothesized to be depressing and facilitating, respectively. SP, superficial pyramidal cell. **B**, Slice experiments showing EPSPs elicited in NG cells and HZ cells in response to trains of extracellular stimuli applied to the LOT. **B** (top), Six stimuli at 40 Hz elicit facilitating EPSPs in an NG cell (left) but depressing EPSPs in an HZ cell (right), which is opposite to the hypothesized responses (panel **A**). **B** (bottom), A longer train (9 bursts of 40 Hz trains, repeated at 3 Hz), thought to more closely replicate odor-evoked stimuli, elicits a depressing envelope of EPSPs in both an NG cell (left) and an HZ cell (right). Thus, the hypothesis in panel **A** is supported for NG cells but not for HZ cells. Stimulus artefacts are blanked. Similar results were obtained in $n = 6$ experiments of this kind for each cell type. **C**, Averaged *in vivo* voltage clamp recordings of odor-evoked EPSCs measured in NG cells (left, $n = 17$ cell-odor pairs) and HZ

cells (right, $n = 16$ cell-odor pairs). Holding potential -70 mV. Inset (bottom) shows the same traces overlaid and expanded, showing that averaged EPSCs in HZ cells have a slower time to peak.

Figure 6. NG and HZ cells receive odor-evoked feedforward inhibition, predominantly from other NG cells. **A**, Averaged *in vivo* voltage clamp recordings of odor-evoked IPSCs measured in NG cells (left, $n = 14$ cell-odor pairs) and HZ cells (right, $n = 5$ cell-odor pairs). Holding potential $+10$ mV. In separate experiments it was found that synaptic inhibition could also be observed in HZ cells under current clamp (**Figure 6 – Figure Supplement 1**). **B**, Dual recording from a synaptically-connected NG cell and HZ cell pair in a slice. Stimulus was a 20 Hz train of 2 ms-long depolarizing current steps applied to the NG cell. Postsynaptic response is an average of 15 episodes (holding potential, 0 mV). **C**, Summary bar plot showing percentage of NG/HZ cell pairs tested *in vitro* that were synaptically connected. Numbers above bars indicate ‘number of connections/total number tested’. *, $p < 0.05$, Chi square 2×2 contingency test.

Figure 7. NG and HZ cells produce different patterns of synaptic inhibition in postsynaptic SP cells. **A**, Average IPSPs recorded *in vitro* in a SP cell (black traces; stimulus artefacts blanked) in response to extracellular stimulation in layer 1a of the slice, using spiking patterns that had previously been recorded *in vivo* in either NG cells (left) or HZ cells (right). Examples of these spiking patterns are shown in the red traces (top). IPSPs were averaged from $n = 6$ SP cells, to each of which was applied up to 16 different stimulus patterns (NG and HZ). To preserve possible respiration-synchronized effects (Figure 2) when averaging

across different stimulus patterns, each raw IPSP trace was linearly warped to match a reference respiration trace (gray, bottom). **B** (top), Example action potentials recorded in SP cells while using a dynamic clamp to inject an odor-evoked excitatory postsynaptic conductance (EPSP) that had previously been recorded *in vivo*. Responses with the EPSP alone (upper traces, black) were interleaved with responses to the EPSP plus extracellular synaptic stimulation (lower traces, blue; stimulus artefacts blanked) driven by different odor-evoked spiking patterns for NG cells (left) or HZ cells (right). As in panel **A**, stimulus patterns were linearly warped to a reference respiration trace. Asterisks above the upper traces label action potentials that are absent in the lower traces. All slice recordings in panels **A** and **B** were made in the presence of 20 μ M CNQX and 25 μ M D-AP5. **B** (bottom), Mean normalized PSTH plots from $n = 7$ SP cells showing that synaptic inhibition patterned on NG cell activity significantly suppresses early firing in SP cells (left), whereas inhibition patterned on HZ cell firing has a weak, delayed effect (right). Gray bars in panels **A** and **B** indicate the period during which odor was applied in the *in vivo* recordings from NG and HZ cells.

Figure 1 - Figure Supplement 1. Measurement of odor responsiveness.

This example shows the response of a NG cell to ethyl-n-butylate. **A**, Raw membrane potential (V_m) (top), and V_m after median filtering to remove action potentials (middle). Bottom trace is the concurrently-recorded respiration. Peaks in the respiration trace (corresponding to exhalation) were detected (vertical dashed red lines) and the time between consecutive peaks was designated a 'respiration epoch'. **B**, Mean amplitude of the median-filtered V_m , averaged over each respiration epoch. **C**, z-scored version of the data in panel **B**. This was calculated by normalizing each point to the mean and standard deviation of the data points in **B** that occurred during the baseline period, *i.e.* during the 8 seconds prior to odor application. If the z-score exceeded a detection threshold of 2.5 (horizontal dashed blue line) at any time during the odor application period, then an odor response was counted. This cell responded to 14 out of 15 odors so its odor response index was $14/15 = 0.93$. Each odor was applied only once to avoid habituation.

Figure 2 - Figure Supplement 1. Cross-covariance analysis confirms the presence of respiration-locked oscillations in V_m in NG and HZ cells, with the amplitude of these oscillations increasing in NG cells during odors.

A, Same NG cell as in Figure 2A, except that the respiration trace has been replaced by a normalized series of Gaussians representing the peaks in the respiration cycles (bottom). This was done to allow us to compare the cross-covariance between different cells by eliminating differences in the amplitude or shape of the respiration trace. **B**, Cross-covariance between V_m and the Gaussian-normalized respiration for the cell in panel **A**, calculated for 3 s-long windows starting 6 s before, 0.5 s after and 16 s after odor onset, respectively. Asterisks indicate the peak cross-covariance found within ± 0.3 s (approximately ± 1 respiration cycle) of the zero lag time. **C**, Changes in peak cross-covariance amplitude (left) and lag (right) before (b), during (d) and after (a) odor application for the cell in panel **A**. Each triplet of connected points represents data obtained when applying a different odor to this cell. **D**, Summary of average peak cross-covariance amplitude (left) and lag (right) for 22 NG cells and 9 HZ cells. Bars show mean $\pm$ SEM (black, before odor; red, during; blue, after). ***, $p < 0.001$; **, $p < 0.01$; ns, not significantly different; 1-way ANOVA with Tukey.

Figure 2 - Figure Supplement 2. Subthreshold membrane potential (V_m) in NG cells in the primary somatosensory cortex (S1) does not oscillate in phase with respiration and does not respond to odors, in contrast to NG cells in the piriform cortex (PCx).

A, Recording from an S1 NG cell while applying odor (here, lavender; red bar). Trace at bottom is a normalized series of Gaussians representing the peaks in the respiration cycles, as in Figure S2. **B**, Cross-covariance between V_m and the Gaussian-normalized respiration for the cell in panel **A**, calculated for 3 s-long windows starting 6 s before, 0.5 s after and 16 s after odor onset, respectively. Traces are displayed on the same axes as for the piriform NG cell in Figure S2B to facilitate comparison. Note the much weaker cross-covariance for this NG cell in S1. **C**, Average peak cross-covariance amplitude (left) and lag (right) for the S1 NG cell in panels **A** and **B** ($n = 11$ trials) compared with the PCx NG cells ($n = 22$) in Figure S2D. ***, $p < 0.001$; ns, not significantly different; Welch 2-sample unpaired t-test.

Figure 3 - Figure Supplement 1. Response of HZ cells, but not NG cells, is much slower than the speed of odor arrival.

Top trace shows the odor application kinetics for our olfactometer, as measured using a photoionization detector (PID). The brief transients at times 0 and 3 s are due to the opening and closing, respectively, of the valve in the olfactometer. Note the delay to odor onset (242 ± 6 ms, $n = 9$ odors measured), and a similar delay at odor offset. (All data presented in this paper were corrected for this delay.) The 20-80% rise time of the odor arrival was 46 ± 2 ms ($n = 9$). The lower two traces show example recordings from a NG cell and a HZ cell, plotted on the same time scale as the PID trace. The NG cell response rises as rapidly as the odor onset, whereas the HZ response is delayed. These examples are taken from Figure 1 (1-heptanal).

Figure 3 - Figure Supplement 2. *In vivo* 2-photon calcium imaging experiments confirm that NG and HZ cells respond similarly to odors in animals anesthetized with either urethane or fentanyl and medetomidine.

A, *In vivo* two-photon images of GFP (green, arrowhead) and resting Cal-590 fluorescence (red, arrowhead) in a NG cell (left) and HZ cell (right). **B**, Average $\Delta F/F_0$ responses in $n = 3$ NG cells (left) and $n = 5$ HZ cells (right), recorded under fentanyl + medetomidine anesthesia. Odor was applied for the 3 s period indicated. The response kinetics are similar to those recorded under urethane anesthesia (Figure 3B).

Figure 4 - Figure Supplement 1. Procedure for measuring the properties of odor-evoked EPSPs.

Traces show, from top to bottom, the cumulative effect of each step in the analysis. Each row shows the same trace on two different timescales. *Raw trace*, response of a NG cell to lavender. *Median filtered*, same trace after median filtering to remove the action potential. *Notch filtered*, same trace after notch filtering at 2-4 Hz to remove the respiration-synchronized oscillations in V_m . *Averaged and fitted*, average of responses of this cell to 8 different odors, each response filtered as above, then fitted to a smooth function of the form m^2h (superimposed red curve). The mean EPSP time to peak and halfwidth were measured from the fitted curve.

Figure 6 - Figure Supplement 1. Steady depolarization of a HZ cell *in vivo* reveals odor-evoked hyperpolarization and suppression of firing.

Recordings from a HZ cell that has been depolarized by steady current injection then presented with 13 different odors. Top, example showing the transient inhibition of spiking during odor presentation (here, lavender). Middle, raster plots from all 13 odor presentations to this cell. Bottom, PSTH of the data in the middle panel, showing that the steady spiking (~5 Hz) is transiently inhibited by odor. Horizontal dashed line indicates 0 Hz baseline.