ABSTRACT

Intracortical excitation rules in piriform cortex

Marco J. Russo

The cerebral cortex continuously encodes new sensory information and organizes it within an experiential intracortical framework. The cortical integration of internal and external information forms the associations that are the basis for higher order sensory representation, and ultimately, perception. Deciphering the cellular and synaptic principles of sensory-cortical integration requires a system with a simplified interface between the internal and external worlds. The piriform cortex provides a relatively simple substrate for the study of intracortical modulation of sensory coding. Within piriform, primary sensory information from the olfactory bulb converges onto neurons in a single cortical layer, where it directly integrates with intracortical input. The major barrier to studying intracortical influences on sensory representation in piriform has been the inability to isolate single types of intracortical input. Here, we use optogenetic techniques to functionally isolate two important classes of intracortical input to piriform pyramidal neurons, and slice electrophysiology to assess their synaptic properties. We first expressed channelrhodopsin in a small subset of piriform neurons, effectively isolating the recurrent synapses formed onto piriform pyramidal neurons by their peers. Recurrent collaterals form strong excitatory connections that extend throughout piriform without spatial attenuation in strength, linking distant piriform neurons. This extensive recurrent network is constrained by powerful disynaptic inhibition, which can also reduce activation by primary sensory inputs in a timing-dependent manner. Next, we functionally isolated inputs to the piriform from the anterior olfactory nucleus (AON), an early target of olfactory bulb output whose role in olfaction is largely unknown. The AON makes weaker excitatory connections with piriform, but unlike recurrent connections, these inputs do not drive strong disynaptic inhibition. Sequential activation of AON inputs leads to pronounced summation that boosts piriform activation in an NMDA-receptor-
dependent manner, and may enhance plasticity of AON-to-piriform synapses. The AON is a potentially powerful modulator of piriform cortex, whose role in odor information processing merits further study. Our results collectively illustrate critical features of intracortical input classes to piriform cortex, and how these inputs may have distinct roles in shaping odor representations and olfactory learning.
## Contents

### List of Figures

iv

### Introduction

1

#### Overview of the olfactory system

2

#### Representations of odor in piriform cortex

5

#### Synaptic architecture of piriform cortex

7

#### Overview of the anterior olfactory nucleus

10

#### Odor representation in the anterior olfactory nucleus

13

#### Experimental approach to functionally isolate associative inputs to piriform

14

### Results Part I: Functional isolation of piriform recurrent connections

17

#### Sparse and focal expression of channelrhodopsin in piriform cortex

17

#### Spatial extent of channelrhodopsin-expressing neurons

18

#### Recurrent synaptic responses evoked by light

20

#### Distance invariance of piriform-to-piriform recurrent connections

23

#### Long-range recurrent connectivity is not observed in other primary sensory areas

25

#### Quantitation of the number and strength of recurrent excitatory synapses

26

#### Recurrent excitation is coupled to feedback inhibition

29

#### Recurrent activity shapes the response to bulbar synaptic input

31
Results Part II: Anterior olfactory nucleus projections to piriform cortex

Robust, focal expression of channelrhodopsin within the AON

Light-evoked synaptic responses in piriform originating in the AON

Disynaptic inhibition driven by AON afferents

Functional activation of piriform neurons by AON inputs

Location of AON inputs onto piriform pyramidal neurons

AON afferents synapse onto Layer 1a interneurons

Comparison of AON afferents to piriform recurrent connections

Discussion

Possible roles of piriform recurrent connections

Possible roles of AON projections to piriform

Notes on associative plasticity in piriform

Future directions

Methods

Viral vector production and stereotaxic gene delivery

Electrophysiology and data analysis

Histology and post hoc imaging
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification of layer 2 pyramidal neurons</td>
<td>63</td>
</tr>
<tr>
<td>Determination of illumination field</td>
<td>64</td>
</tr>
<tr>
<td>Synapse mapping and laser-scanning photostimulation</td>
<td>64</td>
</tr>
<tr>
<td>Estimation of the number of ChR+ neurons</td>
<td>65</td>
</tr>
<tr>
<td>Figures</td>
<td>67</td>
</tr>
<tr>
<td>References</td>
<td>120</td>
</tr>
</tbody>
</table>
List of figures:

Figure 1. Expression of channelrhodopsin in a sparse, focal subset of piriform neurons. 67
Figure 2. Infection of overlying cortex without infection of piriform cortex does not produce labeled fibers throughout piriform. 69
Figure 3. Recording from ChR-positive cells confirms robust expression in a limited subset of piriform neurons. 71
Figure 4. Targeted patch clamp recordings from YFP+ neurons. 73
Figure 5. Recording light-evoked synaptic responses within piriform pyramidal neurons. 75
Figure 6. Light-evoked recurrent synaptic responses have properties to similar to electrically activated “associational” synapses. 77
Figure 7. Recurrent excitatory synapses extend undiminished across piriform cortex. 79
Figure 8. Recurrent excitatory responses are not spatially extensive in other primary sensory cortices. 81
Figure 9. Piriform recurrent excitatory connectivity is sparse. 83
Figure 10. Quantification of piriform recurrent synaptic release sites. 85
Figure 11. Estimating the number of ChR-positive piriform pyramidal neurons. 87
Figure 12. Recurrent excitation drives local strong, scaled inhibition. 89
Figure 13. Focal illumination indicates laminar organization of excitatory and inhibitory inputs. 91
Figure 14. Feedback inhibition tunes coincidence detection. 93
Figure 15. Inhibition restricts diffuse excitation and epileptiform discharges. 95
Figure 16. Focal expression of channelrhodopsin within the anterior olfactory nucleus. 97
Figure 17. AON afferent synaptic responses recorded in piriform neurons. 99
Figure 18. AON inputs activate disynaptic inhibition onto piriform neurons. 101
Figure 19. Extensive expression of ChR in the AON does not increase disynaptic inhibition in piriform. 103
Figure 20. AON disynaptic inhibition is similar in contralateral piriform cortex. 105
<table>
<thead>
<tr>
<th>Figure 21</th>
<th>Functional consequences of weak disynaptic inhibition.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 22</td>
<td>Boosting of AON EPSP summation with depolarization is NMDA receptor dependent.</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Laminar organization of AON inputs to piriform pyramidal neurons.</td>
</tr>
<tr>
<td>Figure 24</td>
<td>Example of strong excitation by AON input onto piriform basal dendrites.</td>
</tr>
<tr>
<td>Figure 25</td>
<td>Layer 1 interneurons in piriform can be strongly activated by the AON.</td>
</tr>
<tr>
<td>Figure 26</td>
<td>Comparison of AON-to-piriform and piriform-to-piriform connections.</td>
</tr>
</tbody>
</table>
Acknowledgments

I have been humbled by the generous support of the family of mentors, teachers, students, collaborators, technicians, and administrators throughout this process, and will never feel that I could ever adequately repay them for their time and efforts in helping me in both good times and bad.

The lessons that Steve and Richard have taught me through their examples are invaluable and will be the cornerstones of my career. They are deeply devoted to science, and they each pursue the craft in their own unique ways, which clearly have deep personal meaning to each of them. I am grateful that they have welcomed me into the extended family of brilliant young scientists that are drawn to their sides, and from whom I have learned immensely. On a personal note, they have supported me and believed in me through many, many difficult times, which is testament to their steadfast characters and charitable natures. Steve, Richard, thank you for everything.

I must also thank my previous mentors, Marco Martina and Enrico Mugnaini. Had they not taken a risk, and given me unfettered access to an electrophysiology rig and the questions on which to learn independent science, I would never have made it to this point. My time working with Marco and Enrico remains a highlight of my scientific career.
Dedication

To Tatyana, only you truly know what a struggle this has been.

Only you could have led me through.
Introduction

No sensory experience occurs in isolation. Information enters the cortex from the external world and is immediately integrated and organized according to a complex existing pattern of synaptic connectivity and neural activity. Our experience of the world is not a static composite of its physical features, but a dynamic internal construct that results from the interplay of past experience, saliency, and the limitations of sensory transduction or encoding. Often, internal patterning of neural activity based on prior experience constrains or even dominates absolute physical information and distorts reality in favor of ethologically relevant estimation. Visual perception yields striking examples of this, especially in the illusory distortions of color and contrast (Purves et al., 2001). A true understanding of perception requires a detailed examination of the intracortical context that reacts to and shapes sensory information. Study of cortical processing at the interface between external and internal information provides opportunity to access the infinite internal space of the cortex, and to understand general principles of cortical processing. This lofty goal begins with a simpler effort to understand the organization of intracortical connections, and the potential pathways through which intracortical connections might influence sensory input.

The olfactory system provides a uniquely simplified confluence of external and internal information, and is an ideal model for studying intracortical processing. Olfaction is arguably the most important sense in the animal kingdom, though often neglected in favor of those senses that are more central to human experience. The mammalian olfactory system evolved early, and its accompanying cortical circuitry dominated the brains of early mammals, as it does for their contemporary counterparts (Rowe et al., 2011). Olfactory cortex, including the largest olfactory cortical area, piriform cortex, is classified as paleocortex to denote its evolutionary
precedence. Indeed, it is simpler than the neocortex (isocortex) that has more rapidly expanded in recent evolutionary history. Neocortex is six-layer cortex, with at least four distinct layers of cellular processing. Piriform cortex has only three distinct layers, with one relatively homogeneous cellular layer. It resembles the trilaminar structure of dorsal cortex of reptiles, which serves multiple modalities, including vision and touch, in these phylogenetic predecessors to mammals (Luzzati, 2015). Within piriform, odor information and intracortical information come together within a single cell layer. Sensory-cortical integration essentially occurs within single principal neurons in piriform, and is likely influenced by local interneuron microcircuits, subcellular dynamics, and synaptic properties. Despite its relative simplicity, the piriform cortex is constructed from the same basic cellular elements of neocortex, and shares a precise laminar organization (Douglas et al., 2004). By studying intracortical synaptic organization and integration within piriform cortex, we can begin to understand the fundamental ways that intracortical activity modifies sensory representation.

Overview of the olfactory system

We have a detailed and precise knowledge of the molecular and structural organization of the early olfactory system. Olfactory transduction occurs in olfactory sensory neurons (OSNs) that line the nasal epithelium. Each OSN expresses a single type of odorant receptor protein, drawn from a repertoire of approximately one thousand unique odorant receptor genes in the rodent (Buck and Axel, 1991; Chess et al., 1994). Each odorant receptor is highly selective for specific chemical moieties, but is capable of activation by a number of odorant molecules (Malnic et al., 1999). OSNs project to specialized forebrain regions known as the olfactory bulbs (OBs). Axons from all OSNs that express a single receptor type converge onto 2 of ~1800 discrete regions of neuropil, termed glomeruli, on the surface of each the two olfactory bulbs (Mombaerts
et al., 1996). Within each glomerulus, the OSN axons synapse onto the dendrites of 50-100 mitral and tufted (M/T) cells, the principal output neurons of the OB. Mitral and tufted cells project directly to cortical areas, without intervening synapses in the thalamus, as is observed for all other sensory modalities. The glomeruli and their corresponding output tracts form a stereotyped, topographic map of olfactory receptor identity (Rubin and Katz, 1999; Uchida et al., 2000; Meister and Bonhoeffer, 2001; Bozza et al., 2002).

Combinatorial integration of multiple glomerular output channels determines the odor encoding, or “tuning” of cortical neurons (Apicella et al., 2010; Davison and Ehlers, 2011). This is consistent with the anatomic organization of projections from bulb to cortex. Axons projecting from a single glomerulus to the piriform cortex span the entire piriform cortex, such that two piriform neurons separated by millimeters may receive input from the same set of M/T cells (Ghosh et al., 2011; Sosulski et al., 2011). These projections are not stereotyped in any way, but seem to follow a random course across piriform. Stereotyped projections have been observed in other regions (amygdala, for instance), but projections to piriform have no apparent spatial ordering. In complement, retrograde label placed into a small area of piriform will label cells within multiple glomeruli at disparate locations across the bulbar surface (Miyamichi et al., 2011). Thus, the odor profile to which a piriform neuron responds is not likely to correspond to its position in the cortex.

Olfactory cortex is defined as those areas that receive some degree of direct input from the olfactory bulb. The major areas include the piriform cortex, anterior olfactory nucleus, olfactory tubercle, cortical amygdala, and lateral entorhinal cortex (Neville and Haberly, 2004). The piriform cortex (PC) is the largest of the forebrain targets of olfactory bulb projections. It spans the ventral surface of the rodent forebrain and is in close proximity to all other cortical olfactory
areas. Piriform is classically subdivided into an anterior (rostral) and posterior (caudal) portion. The anterior piriform cortex (aPC) is defined by dense input that arrives from the OB via the lateral olfactory tract (LOT). The posterior piriform cortex (pPC) is caudal to the LOT and its input is dominated by projections from the aPC and from higher order brain areas (Haberly and Price, 1978a). It is postulated that the aPC, given its rich direct input from the OB, forms an early representation of odor that evolves with transmission to the pPC for further, higher-order association or linkage to other areas, such as the amygdala complex or entorhinal cortex, representing aspects of sensation, motivation, or internal state.

It must be noted that the deceptively simple, stereotyped glomerular map overlies an extremely complex and sophisticated neural circuit within the olfactory bulb (Shepherd and Grillner, 2010). The olfactory bulb is much more than an entry point for early olfactory information. It has a full complement of excitatory and inhibitory neurons, which communicate via a vast number of reciprocal synapses. It is tempting to attribute many early processes that filter or structure olfactory input to the cortex, but in fact, the bulbar circuitry likely accomplishes much of this “pre-processing”. For instance, lateral inhibition, pattern separation, temporal filtering, and gain modulation are all likely to be performed, at least in part, by the olfactory bulb (reviewed in Murthy, 2011). Also, it is increasingly evident that the temporal pattern of mitral/tufted cell activity carries salient information about odor concentration and identity (Margrie and Schaefer, 2002; Kepecs et al., 2006; Abraham et al., 2010; Cury and Uchida, 2010; Dhawale et al., 2010; Shusterman et al., 2011; Smear et al., 2011). Bulbar circuitry likely contributes to the fidelity of this temporal structuring. Indeed, it has been shown that the cortex is capable of encoding the complex temporal structure of OB output (Haddad et al., 2013). This multidimensional output from the olfactory bulb is a major challenge to understanding the olfactory system that offsets the advantages of relative physical simplicity.
Representations of odor in piriform cortex

Input to the piriform cortex does not recapitulate the ordered topography observed for the olfactory bulb. For instance, mitral or tufted cells from the same glomerulus project throughout the spatial extent of piriform cortex (Miyamichi et al., 2011; Sosulski et al., 2011). There is no apparent spatial organization to the patterns of these afferents or their terminal fields. Rather, sister mitral/tufted cell outputs, and therefore outputs from a single glomerulus, have equal potential or probability to synapse onto any two pyramidal neurons in piriform, regardless of the spatial relationship of these two target neurons. If these distributed projections are driving action potential firing in piriform neurons, they should produce a distributed ensemble of active neurons that approximates a spatially random representation. This is in fact what has been observed in functional studies of the piriform response to odor. Calcium imaging of piriform neurons reveals that odor-responsive neurons are highly distributed across the piriform, with no apparent topography (Stettler and Axel, 2009). Each odor activates a unique ensemble, but each ensemble comprises only 3-15% of the total cells in anterior piriform. Similar distributed ensembles of neurons were observed with immediate early gene labeling (Fos) after odor exposure (Illig and Haberly, 2003). Mixtures of odors resulted in unique ensembles that were not linear combinations of the individual components. Increased concentration or complexity of mixture did not result in an increased number of odor responsive neurons beyond 10-15% (D. Stettler and R. Axel, unpublished observations). Taken together, these observations suggest that odor representations in piriform cortex are seemingly randomly distributed, without discernible spatial order, and that the representations may be shaped by intracortical processes.

Electrophysiological recordings from odor-responsive piriform neurons in vivo support this, and yield additional information about the subthreshold construction of odor representations. Single
neuron responses to odor were measured in anesthetized animals in cell-attached and whole-cell recordings (Poo and Isaacson, 2009). Each odor tested generated suprathreshold, spiking responses in only ~10% of all neurons recorded, which is remarkably consistent with the ensemble size observed with imaging. Subthreshold excitatory events were similarly rare and odor-specific, while subthreshold inhibitory responses were more consistently observed and evoked by multiple odorants. Direct recording from interneurons in response to odor confirmed this broad odor tuning of the interneuron population. Odor representations in piriform are constructed from narrowly tuned subthreshold synaptic responses to individual odorants, interacting with broadly tuned inhibitory responses.

Additional experimental evidence for this distributed representation of odor comes from the creative construction of a synthetic “odor” representation in piriform. Here, the light-activated cation channel, channelrhodopsin (ChR), was expressed in a small number (~500) of piriform neurons (Choi et al., 2011). These neurons were in close proximity to one another, and restricted to a relatively small sphere of tissue within piriform, as a consequence of limited spread of viral vector after injection. The neurons were activated with blue light delivered through an implanted fiber optic. Within the behaving animal, blue light repeatedly paired with an aversive or appetitive stimulus would result in conditioning to the output of the small set of neurons activated by blue light. That is, co-activation of these neurons with an unconditioned stimulus could entrain or associate them, just as if odor had been used as a conditioned stimulus. Therefore, an “odor” representation that can be associated with extrinsic information comprises a random selection of neurons with no spatial relationship. This implies that meaningful (in terms of their associative potential) odor representations in piriform might be constructed from arbitrary collections of neurons.
Synaptic architecture of piriform cortex

Odor activates a sparsely distributed ensemble of active neurons within piriform. Deciphering the synaptic and cellular processes that construct these ensembles is made somewhat easier by the simplified architecture of piriform. Traditional neocortex is six layers, with four dense cell layers. Piriform is only three layers, with one dense cell layer. Interestingly, deletion of a single gene can transform 6-layer neocortex into a 3-layer paleocortex-like structure (Chou et al., 2009). Neocortex may have been a simple evolutionary step from paleocortex, raising the question as to why piriform has retained a 3-layer structure despite the low barrier to increased complexity. This phylogenetic constancy of piriform may indicate that is optimally, or at least sufficiently adapted to its current functions.

The piriform has three distinct laminae (reviewed in Bekkers and Suzuki, 2013). There is a single, dense cell layer (layer 2), which comprises tightly packed pyramidal neurons. These pyramidal neurons have single apical dendrites, which radiate toward the pial surface. The apical dendrites course through a molecular, or plexiform layer (layer 1) that forms the principal input layer to these neurons. Layer 1 can be further subdivided into distal (layer 1a) and proximal (layer 1b) fields. Layer 1a, the distal layer, sits adjacent to the densely myelinated axons of the lateral olfactory tract (LOT). Collaterals from these axons synapse onto the distal apical dendrites in layer 1a. Thus, all olfactory bulb input converges onto the distal apical fields of piriform pyramidal cells and is restricted to this layer (Neville and Haberly, 2004). Associative and commissural input from all other piriform afferents, and from recurrent collaterals within piriform itself, synapse onto pyramidal neurons or their dendrites within layers 1b, layer 2, or layer 3 (Haberly and Price, 1978a, 1978b). Layer 1b corresponds to the proximal apical dendrite of piriform pyramidal neurons. Sensory input from the OB (layer 1a) directly integrates
with associative intracortical input (layer 1b) within a single dendritic compartment in piriform. Layer 3 is a rarefied cell layer, similar to layer 3 of neocortex, that contains the basal dendrites of layer 2 pyramidal neurons, as well as deep layer 3 pyramidal neurons.

The majority of neurons in piriform are by far layer 2 pyramidal neurons. These are similar to layer 2/3 neurons of the neocortex, with short, early branching apical dendrites, and extensive basal dendrites. Electrophysiological recordings from piriform pyramidal cell dendrites indicate that these pyramidal cells are linear integrators of synaptic input (Bathellier et al., 2009). That is, they do not have active dendritic conductances that might provide for dendritic computation, such as supralinear responses to coincident or clustered input. In a way, this is fortuitous, because it adds to the simplicity of the circuit, and implies that neural activity will more directly reflect synaptic properties.

Implicit in the beautiful simplicity of the laminar organization of piriform is the fact that sensory and intracortical (associative) inputs are integrated within a single neuron layer. However, the intracortical synapses are actually a complex mixture of inputs from a number of diverse brain areas. These include the anterior olfactory nucleus, which receives direct input from the OB and transmits its output almost exclusively to piriform (Ferrer, 1969; Haberly and Price, 1978a). Within piriform, recurrent connections originating from collaterals of piriform pyramidal neurons are thought to form a significant fraction of the associative input. The final class of associative input can be thought of as “top-down” input, arising from higher order cortical sites that are often reciprocally connected to piriform. These include the prefrontal cortex (agranular insula and orbitofrontal cortex), amygdala, perirhinal cortex, and entorhinal cortex. This work will focus on two associative input classes whose functional isolation and characterization will support further study of sensory-cortical integration in piriform: (1) piriform recurrent connections; and (2) the
associative inputs from the anterior olfactory nucleus, which provide a unique, disynaptic pathway for bulbar information to enter piriform.

Odor representations within piriform are likely to be shaped by local recurrent collaterals. These recurrent collaterals, formed among similar types of local excitatory projection neurons, are thought to be an essential motif to cortical computation (Abeles, 1991; Rolls and Treves, 1997). Piriform cortex effectively reduces to a single layer of principal neurons that receive distributed afferents, and with putatively dense synaptic interconnections among peer neurons. This simple description evokes similarities to the basic architecture of CA3 of the hippocampus, which has been suggested to function as an autoassociative network (Rolls, 2007). Autoassociative networks are relevant models for sensory processing because they provide a mechanism through which complex patterns of input can be organized into stable representations. Under a Hebbian plasticity paradigm, if cells are consistently synchronously activated by a complex stimulus, an odor mixture for instance, the connections among them will be enhanced. Then, if only a fraction of the cells are activated in the presence of an incomplete stimulus, this active fraction, via the previously strengthened synapses, will recruit the inactive cells to fire, and so reconstruct the original pattern of output. In piriform, this process of pattern completion may serve to stabilize the odor representation despite the turbulent variability of odorant concentration in natural stimuli. A stable ensemble of neural activity in response to an odor is essential to the formation of learned associations in either olfactory cortex or downstream cortical areas. At more sophisticated levels, recurrent connections may bind together simpler odor representations into increasingly complex representations. These representations could carry information about their individual components, but produce unique perceptual meaning for the animal.
Overview of the anterior olfactory nucleus

The anterior olfactory nucleus (AON) contributes to the so-called “associational” inputs to piriform cortex (Haberly and Price, 1978a; Hagiwara et al., 2012). AON afferents have been historically regarded as similar to the other non-bulbar projections to piriform, such as those from entorhinal or frontal cortices. Like recurrent inputs, AON inputs to piriform have only been studied by collective electrical simulation of the associational fiber system. The AON occupies a unique position within the cortical olfactory hierarchy, which makes it an attractive target for further study of its input to piriform. It receives direct projections from both mitral and tufted cells of the olfactory bulb (Haberly and Price, 1977). Of note, tufted cells appear to project only to the AON, anterior piriform, and olfactory tubercle. The AON makes dense, bilateral back-projections to the olfactory bulbs, which can directly depolarize mitral cells (Markopoulos et al., 2012). They also directly activate bulbar interneurons (periglomerular cells), and the strength of AON connections to these neurons may differ between hemispheres, allowing for interhemispheric comparison or computation (M. Russo, unpublished observations). The AON projects forward to the ipsilateral piriform cortex, contralateral piriform, and contralateral AON (Illig and Eudy, 2009). The AON is the only significantly bilateral structure within the olfactory system. Thus, it is a nexus or hub within the olfactory system, with widespread connections to other olfactory regions, but to no known significant structures outside of olfactory areas. The AON might be considered a major auto-associative olfactory node. Interestingly, OB output is parallelized into two distinct channels, one projecting directly to the piriform cortex, and one first synapsing within the AON, where an undetermined transformation occurs. The two channels reconverge within the piriform cortex, where they potentially integrate with unknown result.
Thus, olfactory information leaves the olfactory bulb, and simultaneously enters the piriform cortex and the anterior olfactory nucleus. This information is processed or transformed in an unknown way within the AON, but then is potentially reintegrated with matching information within the piriform. The AON projection to piriform may be thought of as part of an auto-associative loop that recombines two streams of initially similar odor information. It is reminiscent of the parallelization and reintegation that occurs for entorhinal cortex afferents to the hippocampus. Here, direct inputs (perforant path) possibly reintegrate with indirect information, which has been transformed by the dentate gyrus and CA3 circuits, within pyramidal neurons in CA1.

The AON has been largely neglected in olfactory neuroscience. This is possibly due to its diminutive designation as a “nucleus”, rather than a “cortex.” The AON does in fact resemble a cortical structure, and the case has been made that it should be referred to as anterior olfactory cortex (Haberly, 2001; Brunjes et al., 2005). Pyramidal neurons are arranged in radial array in a single, dense cell layer, similar to layer 2 of piriform. Also like piriform, there is a well-defined plexiform layer that corresponds to the superficial input layer. Inputs are segregated along the apical dendrites of principal neurons identically to those in piriform. That is, bulbar inputs impinge on the distal fields, and associational or recurrent inputs on the proximal fields. Unlike piriform, there is not a well-defined layer 3, but rather just the obliterated olfactory ventricle and rostral migratory streams. The lack of three well-defined layers is one reason for the initial designation of nucleus. Another reason is that the AON neurons develop and organize in a superficial-to-deep fashion, in contrast to the deep-to-superficial developmental migration of cortical neurons (Bayer, 1986; Brown and Brunjes, 1990). AON pyramidal neurons have well-developed dendritic spines, with spine densities similar to that observed in neocortex (Brunjes and Kenerson, 2009). In addition to piriform-like pyramidal neurons, the AON has a full
complement of interneurons, with a similarly wide range of interneuron types, as defined by classical phenotypic markers, as the neocortex (Meyer et al., 2006; Brunjes et al., 2011).

The question as to whether AON is nuclear or cortical is an academic one at this point. It is likely that the AON is a cortical entity, with sufficiently sophisticated circuitry to perform cortical operations (for a full discussion, see Brunjes et al., 2005). We cannot say with any precision, however, what exactly a cortical area does, and so to elevate it to a cortical area is also unsatisfying. While it might bolster interest in the AON, it does not enlighten us as to its function. It seems prudent to remain agnostic, to assume that it has an important role in olfactory processing, and to attempt to understand that role regardless of nomenclature.

Inputs to the AON terminate within a single plexiform layer that encompasses the apical dendrites of pyramidal neurons. This input layer is organized identically to layer 1 in piriform cortex: olfactory bulb inputs synapse on the distal apical dendrites, and associative inputs synapse on the proximal apical dendrites. Though it does not have as rich an array of input types as piriform, the AON is itself a likely important site of intracortical and sensory integration.

Within primates, the AON comprises little more than islands of cells scattered among the white matter tracts of the olfactory peduncle (Carmichael et al., 1994). This could be another reason for the AON's relative neglect, as it may have been regarded as a mere vestige of olfactory processing in lower mammals. However, this view only serves an anthropocentric scientific approach. Regardless of definitions of cortical or nuclear structure, or phylogenetic significance, the AON constitutes a sufficiently sophisticated circuit to perform computations normally attributed to cerebral cortex.
The anterior olfactory nucleus (AON) is thought to project to piriform cortex and to significantly contribute to the associational circuitry (Haberly and Price, 1978b; Luskin and Price, 1983a; Hagiwara et al., 2012). It has been largely neglected in studies of olfaction, the AON occupies a central place within the olfactory hierarchy. It receives input from the main olfactory bulb, and projects forward to the ipsilateral and contralateral piriform cortex, olfactory tubercle, and contralateral AON (Price, 1968; Haberly and Price, 1978b; Luskin and Price, 1983b; Kiselycznyk et al., 2006; Illig and Eudy, 2009; Strowbridge, 2009). The AON makes extensive, bilateral backward projections to the olfactory bulbs. Thus, olfactory information leaves the olfactory bulb, and simultaneously enters the piriform cortex and the anterior olfactory nucleus. This information is processed or transformed in an unknown way within the AON, but then is potentially reintegrated with matching information within the piriform. The AON projection to piriform may be thought of as part of an auto-associative loop that recombines two streams of initially similar odor information.

**Odor representation in the anterior olfactory nucleus**

Functional studies of the AON have been limited in number. One important study has characterized the responses of AON neurons to odor in anesthetized animals (Lei et al., 2006). The authors performed single unit and sharp electrode intracellular recordings of AON neurons, and exposed the animals to a diverse panel of ~40 odorants. They demonstrated that the AON has low spontaneous firing rates (<5 Hz), similar to piriform cortex. Single neurons were broadly tuned, and had subthreshold excitatory responses to at least one odor (47 of 83 neurons), but most responsive neurons could be activated by multiple odors (38 of 47 neurons). For comparison, mitral cells in the same preparation were responsive to at most one odorant. Interestingly, some AON neurons had supralinear responses to some odorant mixtures.
(compared to the linear sum of responses to individual components), which may be due to local intracortical contribution to AON activation (though feedforward or intracellular processes are possible). This singular study of odor responses in the AON suggests that, like piriform, AON neurons integrate input from convergent glomeruli and are broadly tuned.

*Experimental approach to functionally isolate associative inputs to piriform*

The piriform cortex is a promising model for study of sensory-cortical integration, and for processes that we suspect are common to all of cortex, such as pattern completion, associative learning, and content-addressable memory. We can only begin to test these hypothesized functions once we have a detailed understanding of the associative circuitry of piriform. Therefore, the goals of the present study are simple: functionally isolate two interesting and significant components of the associative network within piriform so that their unique properties can be assessed. Using newly developed optogenetic methods, we aim to activate either the recurrent collaterals of piriform, or projections from the anterior olfactory nucleus, in isolation. Through selective activation of these inputs with light, we will gain a sense of what their distinct contribution may be to piriform activation, and their unique synaptic organization with respect to the piriform microcircuitry.

As discussed above, the associative, or “associational” inputs to the piriform cortex are neatly segregated from the primary sensory inputs from the olfactory bulb, which synapse exclusively on distal apical dendrites of piriform neurons. Associative inputs are physically intermingled within piriform cortex, and collectively synapse onto similar areas (basal and proximal apical fields) of pyramidal neurons. There is no way to isolate the individual associative inputs, which originate from unique locations, with conventional electrical stimulation. An electrode inserted into layer 1b or layer 3 will activate the entire set of associative inputs onto any neuron, and the
measured post-synaptic response is an amalgam of various afferents with potentially very
different synaptic properties. In order to functionally isolate and characterize any single input
class, a method of activating or inhibiting a single fiber type according to its site of origin.

Recent advances in expressing genetically-encoded light-activated ion channels or pumps,
originally discovered in photosensitive unicellular eukaryotes, in mammalian neurons provide a
powerful method of activating well-defined populations of neurons and their axons and terminals
(Nagel et al., 2003; Boyden et al., 2005; Zhang et al., 2007, 2010). The most effective such
protein tool is channelrhodopsin-2 (ChR), a six-transmembrane-helix protein that forms a cation
conducting pore. The channel is normally closed until absorption of a photon of blue light
(optimally 473-nm wavelength) causes cis-to-trans conversion of retinal chromophore, inducing
conformational rearrangement that opens the pore. At the time of this study, much work had
already been done to optimize ChR for enhanced light sensitivity and mammalian expression
(Gradinaru et al., 2010; Yizhar et al., 2011).

Light-gated opsins can be delivered into any brain region by stereotaxic injection of a viral
vector. With this general method, we can express ChR in any putative afferent of piriform
cortex. After generating acute brain slices, we can perform intracellular recordings on piriform
pyramidal neurons, and activate afferent synapses with brief flashes of blue light, delivered
through the microscope objective. Only those afferents originating from the injected area will
express ChR and so we will have functionally isolated associative inputs to piriform on their
basis of their anatomic origin.

Expression of ChR in a neuron results in localization of the molecule throughout the
somatodendritic compartments, the axons, and the synaptic terminals. This axonal and
synaptic localization allows for direct depolarization of these compartments, regardless of
whether the soma or axon hillock are intact. This enables activation of axon segments and synaptic terminals in a slice preparation, where contiguous connections among brain areas are rarely preserved. Illumination directly above a recorded neuron will activate the full complement of synaptic terminals connected to the neuron (except those lost during slicing), which is not possible with electrical stimulation, which typically requires an intact fasciculation of axons. Therefore, in addition to providing a means for isolating synaptic input from a particular brain area, stimulation via channelrhodopsin enables quantitative estimation of the total input from that area.
Results Part I: Functional isolation of piriform recurrent connections.

Sparse and focal expression of channelrhodopsin in piriform cortex

Optogenetic tools enable functional isolation of the recurrent connections among piriform pyramidal neurons. The light-activated cation channel channelrhodopsin-2 (ChR) must be strongly expressed in order to fully characterize these recurrent connections, but only in a focal and sparse subset of neurons. Expression must be limited to piriform cortex, and to layers 2/3, the principal excitatory cell layers. It is also critical that expression is sparse, generating a limited population of ChR-positive (ChR+) neurons whose inputs onto nearby and distant ChR-negative neurons can be reliably stimulated and recorded. It is not possible to record synaptic responses within ChR+ neurons because the photocurrents are typically orders of magnitude larger than synaptic currents, thus obscuring their measurement.

We generated a focal, sparse subpopulation of ChR-expressing neurons in the anterior piriform cortex through an intersectional approach requiring injection of two viral vectors. Adeno-associated virus (AAV) encoding Cre-dependent, ChR-YFP was co-injected with lentivirus encoding Cre recombinase (Figure 1A). Cre recombinase is expressed in a sparse and focal population of neurons, possibly due to the lentivirus’ low rate of infectivity and limited mobility through the extracellular space. The AAV vector has the additional advantage of high efficiency of infection, providing high copy numbers of the ChR gene for strong expression within the spatially restricted population (Kaspar et al., 2002).

The two viral vectors were injected as a 1:1 mixture into the anterior piriform cortex of a single hemisphere of adult mice (Figure 1B). The expression pattern was initially characterized in fixed brain tissue from a subset of animals sacrificed 14-21 days after injection, which is consistent
with the experimental time of expression. The observed YFP fluorescence, corresponding to ChR+ somata, dendrites, and axons, is well localized to anterior piriform cortex (Figure 1C). A high magnification view of the injection site indicates restriction of Cre expression (revealed by anti-Cre antibody labeling) to a sparse cluster of layer 2/3 cells, just several hundred microns wide, within anterior piriform cortex (Figure 1Di). Cre expression is co-localized with YFP-positive (YFP+) cell bodies, in an area with dense neuropil fluorescence (Figure 1Dii,iii). Examination of a posterior slice, 1 mm caudal to that shown in (D), indicates that (1) the infection is spatially limited, as no Cre-positive cells are seen, and (2) axons from YFP+ cells potentially densely innervate distant piriform sites (Figure 1E).

We were concerned that extensive patterns of axon fluorescence observed throughout piriform may not be due to the small population of Cre-positive cells, but that these fibers might originate from spurious infection or expression sites within or around the injection tract. We turned to a pilot set of injections, in which we injected a simple AAV vector to express only YFP (Figure 2). Many of these injections resulted in YFP expression within piriform cells (Figure 2Ai), with similar patterns of distal YFP+ axons throughout piriform (Figure 2Aii,iii). However, some of these injections were off-target, instead depositing virus just dorsal to piriform cortex, but infecting the same dorsal-ventral tract (Figure 2B). These off-target injections did not result in dense associative fibers within piriform cortex (Figure 2Bii,iii), making it unlikely that the fibers that we observe in ChR-expressing animals originate from outside of piriform due to spurious infection in proximity of the injection tract.

*Spatial extent of channelrhodopsin-expressing neurons*

We prepared acute parasagittal brain slices through piriform cortex from young adult mice (aged 6-8 weeks). Typically, only one brain slice (350-μm thick) per animal included a significant
extent of piriform cortex along the rostrocaudal axis with a focal area of YFP fluorescence (Figure 3A). Visualization of living slices under differential interference contrast (DIC) microscopy and epifluorescence typically showed only a fluorescent cloud or haze (Figure 3B, top). Individual YFP+ cells were difficult to resolve because of the intense fluorescence of the background neuropil. The fluorescence cloud did have a clear point at which the fluorescence intensity was maximal within layer 2 (Figure 3B, bottom). This point of maximum fluorescence intensity is presumed to be the area of maximal infection, and so was marked as the infection center. We used this point as a reference to initially evaluate the spatial extent of our infection and ChR expression.

Whole-cell patch clamp recordings were obtained from multiple layer 2 pyramidal neurons at different distances from the center of the infection site. A 500-ms light pulse delivered through the objective, and centered on the somata of cells within the fluorescent area, evoked robust and sustained currents in a subset of these cells (Figure 3C, top trace). Note that synaptic transmission and action potential generation/propagation were pharmacologically blocked for these recordings. The remaining cells had no response – there were no intermediate current waveforms or non-specific effects of light (Figure 3C, bottom trace). The currents had a time course that is characteristic of channelrhodopsin-dependent photocurrents: rapid activation followed by rapid, partial inactivation, and persistence of a non-inactivating component (Boyden et al., 2005, Zhang et al., 2006). We “blindly” patched within the fluorescent cloud, i.e. without attempting to target fluorescent cells, so as to obtain a functional readout of the ChR+ cell density. At the center of the infection cloud, 35% of neurons were ChR+ (defined by the presence of sustained photocurrent), but the frequency of ChR+ cells diminished dramatically with distance from the infection center (Figure 3D). In contrast, the magnitude of the
photocurrents in ChR+ cells did not vary with distance in or around the injection site, indicating that ChR-expression is relatively homogeneous (Figure 3E).

At rare times, we could resolve individual YFP+ neurons amid the background fluorescence intensity (Figure 4Ai). These YFP+ cells could be targeted for recordings, and filled with red fluorescent dye to confirm successful targeting of the YFP-expressing cell (Figure 4Aii-iv). These neurons reliably fired action potentials in cell-attached mode (without disruption of the membrane by the pipette contents), and always had photocurrents when recording in whole-cell configuration. The amplitudes of measured photocurrents (1.52 ± 0.51 pA, mean ± S.D., n=14) indicate strong ChR expression in layer 2/3 piriform neurons.

Recurrent synaptic responses evoked by light

The expression of ChR in a sparse, focal subset of pyramidal neurons enables us to activate the axons and synapses that form recurrent connections within piriform. We recorded from ChR-negative cells throughout piriform, and assessed their responses to light-evoked synaptic input originating from the restricted subset of ChR+ neurons (Figure 5Ai). We verified that we were recording exclusively from layer 2 pyramidal neurons by their laminar position under DIC (Figure 5Aii), online visualization of morphology by filling with diffusible red fluorescent dye (Figure 5Aiii), and finally by filling with biocytin for post hoc staining and visualization (Figure 5B). This rigorous confirmation of cellular identity is necessary to restrict analysis to layer 2 pyramidal neurons, and exclude less common cell types, such as semilunar cells, layer 3 pyramidal and multipolar neurons, and interneurons – all of which may have different patterns of recurrent connectivity.
Pyramidal neurons that are ChR-negative do not have the sustained photocurrent in response to a long light pulse that is characteristic of ChR-expressing cells (Figure 5Ci). Stimulation with a brief, 2-ms light pulse through the objective, and centered over the soma produces a transient, negative current, consistent with an excitatory post-synaptic current (EPSC, Figure 5Cii, top trace). This transient response is completely sensitive to pharmacologic blockade of AMPA and NMDA glutamate receptors with the drugs NBQX and AP5, respectively (Figure 5Cii, bottom trace).

Repeated stimulation of a single cell with a brief light pulse reliably evokes EPSCs, with little trial-to-trial variability (Figure 5D). The latencies of these responses, measured as the time from stimulus onset to time at 5% of response peak, are consistently on the order of ~2-3 ms. This rapid time course is consistent with fast, monosynaptic transmission. Additionally, the jitter, measured as the standard deviation of the trial-to-trial latencies (i.e. the variability of the latency), is on the order of 100 μs. Again, this is consistent with monosynaptic transmission (Figure 5E). These data indicate that we are recording monosynaptic excitatory inputs onto layer 2 pyramidal neurons.

We wanted to verify that these synaptic responses have properties that are consistent with what has been described for classical “associational” piriform synapses. The term “associational” has historically been used to describe non-bulbar inputs to piriform that have been most typically studied through electrical stimulation of layer 1b or layer 3. They have biophysical and pharmacological properties that distinguish them from bulbar inputs to layer 1a evoked by electrical stimulation of the LOT. Recurrent axons stimulated by light are just a subset of the diverse (in theory) array of fibers that compose the associational (hereafter, abbreviated ASSN)
inputs to piriform pyramidal neurons. It is not obvious, therefore, that recurrent connections should have these same basic properties.

Electrically evoked ASSN responses are typically associated with greater NMDA receptor (NMDAR) content than primary bulbar, or LOT responses (Kanter and Haberly, 1990). This is thought to account for the ease with which long-term potentiation can be induced at ASSN synapses onto piriform pyramidal neurons relative to LOT synapses (Jung et al., 1990; Kanter and Haberly, 1990; but plasticity at LOT synapses is significant in young animals, see Franks and Isaacson, 2005). We observe a similarly high expression of NMDAR-mediated current during light-evoked responses of recurrent synapses. We hold neurons at a sufficiently positive potential of +50 mV to relieve Mg$^{2+}$ blockade and to provide sufficient driving force for NMDAR current. The isolated NMDAR current evoked by each pathway can be compared within a single neuron. Piriform recurrent collaterals drive significantly greater NMDAR current than electrically evoked bulbar inputs, consistent with previous observations for ASSN connections (Figure 6A).

An additional features that has classically distinguished ASSN synapses from LOT synapses is their sensitivity to activation of GABA$\text{B}$ receptors. Application of GABA$\text{B}$ receptor agonists, such as baclofen, consistently reduce the synaptic response to ASSN stimulation (Tang and Hasselmo, 1994), but have no effect on LOT synapses. We repeated this experiment with electrically evoked LOT responses and light-evoked recurrent responses recorded in the same neuron. Baclofen significantly reduces the amplitude of light-evoked recurrent EPSCs, but has no effect on LOT responses, consistent with the prior study (Figure 6B). The GABA$\text{B}$ receptor antagonist CGP55845 reversed the effect of baclofen.

It has also been shown that LOT and ASSN synapses significantly differ in short-term plasticity. Repeat stimulation of the LOT pathway within 100-200 ms of a prior stimulus results in
enhancement of the second response (paired-pulse facilitation). Stimulation of the ASSN pathway, for instance, produces little to no facilitation (Hasselmo and Bower, 1990). We observe a similar lack of facilitation in response to paired light stimuli to that observed for electrically evoked ASSN responses (Figure 6C). This is also further indication that channelrhodopsin does not appreciably alter the dynamics of synaptic release in piriform recurrent synapses in our preparation.

**Distance invariance of piriform-to-piriform recurrent connections**

Focal expression of ChR-YFP produces fluorescent axons throughout the spatial extent of piriform (Figure 1). Moreover, prior studies have indicated that the axons of individual piriform pyramidal neurons can extend for millimeters along the rostro-caudal extent (Datiche et al., 1996, Johnson et al., 2000). Thus, we recorded light-evoked synaptic responses in neurons throughout piriform, with no spatial bias. We elicited synaptic responses in 94 of 95 ChR-negative cells, in slices from 11 animals. The probability of recording a response did not attenuate with distance from the injection site. Again, the center $\Delta x = 0$ is defined as the point of maximum fluorescence intensity (Figure 7A,B, see also Figure 3B). For instance, we were able to observe synaptic responses in 100% of cells recorded at nearly 2 mm from the infection focus. The EPSC amplitude was largely independent of the distance of the recorded cell from the infection site, and the amplitudes of recorded EPSCs generally only attenuate in cells at distances $\geq 1$ mm (Figure 7C). We established exponential fits to the relationships between normalized EPSC amplitude and relative recording distances from injection sites according to the following simple equation:

\[
EPSC \text{ (norm.)} = e^{-\frac{x}{\lambda}}
\]

A single exponential fit to EPSC amplitude versus cell position indicates a decrease to 1/e of the
maximum amplitude after 1.6 mm distance in the slice, and provides a useful “decay” or attenuation constant for the distance-dependence of responses. In other slices (Figure 1D), there was no measurable attenuation of EPSC amplitude with distance. Large amplitude EPSCs (here, ~1 nA) were recorded at 1808 μm from the injection site. A single exponential of was forced to the data, but constrained to \( \lambda \leq 3\text{mm} \) by assuming a reasonable maximum \( \lambda \), and programmatically setting this parameter. We assume that EPSC amplitude does not increase with distance. It is possible that synaptic connectivity is enhanced at greater distances, due to underlying constraints on axon path-finding and wiring during development (e.g. a minimum growth distance before synapse formation), but we do not further address this in the present study.

Recurrent synaptic excitation could lead to recruitment of additional piriform neurons, and this polysynaptic activation could account for the large synaptic responses we record at long distances. If this is the case, we would expect that both the average latency of each response, and the trial-to-trial variability in onset (jitter) should increase with distance. However, we observe no appreciable change in latency or jitter with recording site distance (Figure 7E). Furthermore, we can eliminate action potential propagation altogether, and thus potential for polysynaptic recruitment, by adding TTX and 4-AP to the bath to block voltage-gated sodium channels and voltage-gated potassium channels, respectively. Channelrhodopsin-dependent synaptic responses can still be evoked by light, presumably because of direct depolarization of synaptic terminals and induction of transmitter release (Jackman et al., 2014). With TTX/4-AP in the bath, we observe that EPSC amplitudes attenuate at distances similar to those measured when action potential propagation was intact (\( \lambda = 3.6\text{ mm} \), Figure 7F).
Prior work has sought to understand the differences in the organization of associational or recurrent circuitry between the anterior piriform cortex and the posterior piriform cortex. For instance, current-source density (CSD) analysis indicates that recurrent synapses are much more strongly activated in the posterior piriform after strongly electrically stimulating the LOT (Rodriguez and Haberly, 1989). Thus, recurrent connections may have a strong bias in strength or number from anterior to posterior piriform. We did not systematically explore anterior versus posterior piriform differences among recurrent connections, but in one experiment, we focally expressed ChR in the posterior piriform (caudal to the termination of the lateral olfactory tract). We then recorded light-evoked responses in anterior piriform cortex, at millimeter distances, across the posterior-to-anterior transition area (Figure 7G). While these responses were generally weaker (209.1 ± 185.3 pA, n=10) than those in anterior piriform cortex (difficult to interpret in one animal), there was no appreciable decline with distance. In fact, the maximum posterior-to-anterior response (682 pA) was recorded at 1150 μm from the injection site in posterior piriform.

*Long-range recurrent connectivity is not observed in other primary sensory areas*

We wondered whether the observed long-range connections were unique to the piriform cortex, or whether it was a general feature of other cortical areas receiving early sensory information. We injected equal volumes and titers of the lentivirus-AAV mixture into layer 2/3 of primary visual area (V1) and barrel field of the primary somatosensory area (S1). We achieved similar focal, sparse channelrhodopsin expression, indicated by fluorescence limited to several hundred microns (Figure 8A, inset). Recording at various distances in an acute V1 slice revealed large EPSCs within several hundred microns of the injection site, but consistently small EPSCs at nearly 1mm. In V1, a cell near the injection site (Δx = 241 μm) has a large, fast EPSC,
consistent with monosynaptic excitation, followed by a tail of EPSCs suggesting highly reverberatory recurrent activity. However, in this slice, cells at a distance of 651 and 1152 μm display only small monosynaptic excitation (Figure 8Ai). When a single exponential is fit to the EPSC amplitude vs. distance data, the spatial decay constant is only \( \lambda = 165 \) μm.

A similar spatial dependence of EPSC amplitude is observed in the barrel cortex (Figure 8B, \( \lambda = 117 \) μm). Repeated experiments in V1 (4 slices) and S1 (5 slices) indicate that sharp attenuation of recurrent excitation with distance is a consistent pattern these areas (Figure 8C). Moreover, comparison of this spatial dependence among these cortical areas reveals a fundamentally different organization for piriform cortex recurrent connections from that of visual or barrel cortices (Figure 8D, \( \lambda \) mean ± S.D., piriform 1.94 ± 0.90 mm, V1 0.144 ± 0.052 mm; and S1 0.255 ± 0.226 mm).

Quantitation of the number and strength of recurrent excitatory synapses

We next obtained a quantitative estimate of the number and strength of the intrinsic excitatory inputs onto a given piriform neuron. By activating via the objective directly over the neuron, we assume that we are activating the full complement of ChR+ terminals onto this neuron, minus any synapses/dendritic segments lost during slicing. This is supported by the trial-to-trial consistency of EPSC amplitudes using this method. Thus, wide-field illumination over the cell provides an estimate of total recurrent synapse strength. The amplitudes of light-evoked responses were large, but somewhat variable within a given animal (441 ± 334 pA, mean ± S.D.; C.V. 0.76; n = 95 cells from 11 animals/slices, Figure 9A). Again, these maximal responses are presumably comprised of the sum total of ChR+ axons and terminals, each of which contributes a unitary response. A unitary response is defined here as the total synaptic response that results when a single axon is excited. A single axon will drive at least one, but
possibly several terminal release sites. By determining the unitary response, we can estimate the average number of recurrent connections made onto pyramidal neurons.

Recording light-evoked unitary responses requires a modified stimulation strategy. We decreased the light intensity, and restricted the spatial extent of illumination with an aperture diaphragm behind the objective. By minimizing the illumination stimulus, we were able to activate a single axon at near threshold. Threshold stimulation is recognized as an EPSC response of discrete amplitude interleaved with response failures, such that we are recording responses approximately 50% of the time. The amplitude of this response is the unitary EPSC (uEPSC). Using this method, the mean uEPSC amplitude was 36.2 pA (±20.3 pA, S.D.; range: 16-74 pA, n=10, Figure 9B). These measurements may be biased towards slightly larger, more easily resolved responses (responses ~10pA become difficult to resolve from baseline). The average success rate of responses across neurons (0.52 ± 0.047; n=10) provides a lower bound on the probability of synaptic vesicle release at recurrent synapses. However, the minimal trial-to-trial variability of maximal EPSCs evoked by light suggests that release probability is closer to 1, at least under these conditions. Also, it is important to note that after patching each neuron, we moved the objective several hundred microns from the soma to eliminate any confounding effects of direct ChR-dependent depolarization and activation of synaptic terminals.

We next determined the number of synaptic contacts each ChR+ axon makes onto a given layer 2 pyramidal neuron by measuring quantal responses (qESPC) evoked by replacing extracellular Ca\(^{2+}\) with Sr\(^{2+}\) to desynchronize synaptic release. In slices bathed in Sr\(^{2+}\), light pulses evoked an early, large synchronous response with a tail of many small events that are thought to represent quantal synaptic currents (Dodge et al., 1969; Goda and Stevens, 1994; Figure 10Ai). The similar amplitude of the light-evoked uEPSCs and qEPSCs (25 pA ± 10 pA, SD; n=11;
Figure 10Aii, iv) suggests that a recurrent axon typically makes single, en passant synaptic contacts with a given pyramidal cell in piriform cortex, consistent with anatomical predictions (Datiche et al., 1996; Johnson et al., 2000). Moreover, at this contact, a presynaptic action potential evokes release of, at most, a single quantum of transmitter. The light-evoked qEPSCs were larger and had faster kinetics than qEPSCs evoked from electrical stimulation of mitral and tufted cell axons in the lateral olfactory tract (LOT) in the same cells (14 pA ± 4.0 pA, n=9, Figure 10Aiii, iv). The amplitudes of qEPSCs from afferent and recurrent inputs are consistent with the range of amplitudes of miniature EPSCs (mESPCs) we recorded in TTX (17.3 ± 7.1 pA, mean ± S.D.; n = 562 events, 9 cells, Figure 10B). The difference in the size of the afferent and recurrent qEPSCs may reflect differences in their biophysical properties (Schikorski and Stevens, 1999) or may simply reflect greater dendritic filtering of the more distal LOT inputs.

The ratio between the average EPSC (500 pA) evoked with a saturating light intensity that activates all ChR+ inputs (see Figure 9A) and the unitary ESPC (25 pA) suggests that a cell receives, on average, 20 active inputs from the population of ChR+ neurons. From the distribution of ChR+ cells determined from patching numerous cells in and around the center of infection (see Figure 3D), we estimate that we infected about 8000 excitatory neurons per animal (Figure 11). This implies that the connectivity between any two pyramidal cells is less than 1%, and this value is largely independent of the distance between two piriform cells. Moreover, given that we infected less than 1% of all piriform pyramidal neurons (8000 neurons out of a total of an estimated $10^6$ pyramidal cells in the piriform), our observation of 20 activated ChR+ inputs per cell implies that that each neuron receives, at least, 2000 recurrent excitatory inputs. In contrast, pyramidal cells are thought to receive only about 200 afferent inputs from the bulb (Davison and Ehlers, 2011). These inputs, however, are multiquantal and can be quite large, with each axon typically making ~5 contacts per cell (Franks and Isaacson, 2006;
Individual pyramidal cells may therefore receive strong inputs from 200 mitral/tufted cells in the bulb and weak inputs from more than 2000 pyramidal cells across piriform cortex.

*Recurrent excitation is coupled to feedback inhibition*

The distributed recurrent network would result in runaway excitation in response to odor unless its activity is constrained by inhibition. We isolated the inhibition coupled to recurrent excitation in order to investigate the role of inhibition in modulating the activity of the recurrent excitatory network. We accomplished this by recording responses while holding the neuron at a voltage near the equilibrium potential for EPSCs (determined empirically to be $V_m = +5$ mV), effectively isolating the inhibitory synaptic current. We first recorded from ChR-negative cells close to the infection site in the presence of NBQX and AP5 to block glutamatergic transmission. Under these conditions, light pulses evoked outward currents that were blocked by the GABA$_A$-receptor antagonist SR-95531 (Figure 12A), indicating that these were inhibitory post-synaptic currents (IPSCs) originating directly from ChR+ GABAergic neurons. Though all cells in or near the infection site showed direct IPSCs, direct inhibition rapidly decayed at distances $>300$ μm beyond the edge of the infected area, indicating that the direct inhibition is local (Figure 12D).

In other recorded neurons, we observed large outward currents that were reversibly abolished by blockers of fast excitatory transmission (Figure 12B,C) and GABA$_A$ receptors (not shown). The onsets of these outward currents were typically delayed with respect to the onset of the corresponding EPSC by $1.6 \pm 0.12$ ms (n=21). These are therefore *indirect* disynaptic IPSCs coupled to activation of recurrent collaterals. In contrast to the local *direct* inhibition, when inhibitory currents were recorded with excitatory transmission intact, we observed large IPSCs in almost every neuron, regardless of distance from the site of infection (85/87 neurons, Figure
Because direct inhibition is local, inhibitory currents distant from the site of infection must result from the activation of long-range excitatory ChR+ axons that synapse onto and activate local inhibitory interneurons. Our methodology enables us to selectively isolate disynaptic inhibition by recording from cells far from the infection site where the light-evoked IPSC is not contaminated by direct inputs from ChR+ inhibitory neurons.

A comparison of the magnitudes of excitatory and disynaptic inhibitory currents (conductances) in a given cell revealed that the inhibitory response was much larger than the excitatory response (Figure 12E). We compared the input-output relationship of excitation versus inhibition by recording the excitatory and inhibitory responses to a series of light pulses of increasing intensity (Figure 12F, top). Increasing the intensity of the light pulse increased the excitatory responses from a level at which we failed to observe any synaptic response to a level at which the EPSC amplitudes saturated and failed to increase with increasing light intensity. The IPSC scaled with, and dominated, the EPSC across the entire range of stimulus intensities (Figure 12F, bottom).

We also determined the laminar organization of the recurrent excitatory and inhibitory synaptic inputs onto layer 2 pyramidal neurons using focal illumination along the cell’s apical-basal axis in the presence of TTX and 4-AP to eliminate action potential propagation. Thus, any recorded responses were due to direct depolarization and activation of synaptic terminals at the site of contact. These experiments indicate that pyramidal neurons receive the majority of their recurrent excitatory input onto their proximal apical dendrites in layer 1b, whereas feedback inhibition is preferentially recruited by their axons and collaterals projecting through layer 3 (Figure 13).
Recurrent activity shapes the response to bulbar synaptic input

How does the recurrent network shape the response of piriform neurons to bulbar inputs? We paired a brief train of electrical LOT stimuli that mimics the burst firing of mitral cells to odorant stimulation (Margrie and Schaefer, 2002; Cang and Isaacson, 2003) with a brief train of light pulses in piriform cortex (both stimuli, 5 pulses at 40 Hz; i.e. a 100-ms burst) and recorded the responses in pyramidal cells in current clamp. The stimulus strengths were adjusted to evoke spiking in 10% of the trials when either stimulus was presented alone (probability of spiking was 0.10 ± 0.38 following electrical stimulation of the LOT and was 0.10 ± 0.054 with light-activation of piriform, n=6). In contrast to the low probability of spiking when LOT or piriform was activated alone, action potentials were evoked in 90% of the trials (0.90 ± 0.056) when the two inputs were presented simultaneously (Figure 14A).

We next examined the effect of altering the temporal relationship between the pairing of bulbar and recurrent inputs. No increase in spiking was observed when the onsets of the two 100-ms-long bursts of stimuli were 150 ms apart. However, when the LOT train was delivered 100 ms before the piriform train, such that the last LOT-evoked input coincided with the first light-evoked input, the cell fired action potentials in 75% of the trials (0.75 ± 0.098, Figure 14B,C). In contrast, no enhancement in spike firing was observed when the piriform train arrived 100 ms before the LOT input (0.20 ± 0.073; unpaired t-test vs. LOT alone, p=0.423; vs. piriform alone, p=0.315, Figure 14B,C).

We then examined the role of inhibition in this pairing paradigm by repeating these experiments in the presence of SR-95531 and the GABA\textsubscript{B} antagonist, CGP55845. Blocking inhibition broadened the time window over which spiking could be enhanced by pairing the inputs (Figure 14C, red bars). Furthermore, the efficacy with which the pairing of the inputs enhanced the
response was less dependent on the order in which the two inputs were presented (skew of control distribution, 0.64 ± 0.17, n=6; skew of distribution in SR/CGP, 0.21 ± 0.04, n=4; unpaired t-test, p < 0.05). This result implies that much of the asymmetry we observed in the efficacy of pairing order is a consequence of inhibition. We hypothesized that the response to LOT inputs might be suppressed by prior activation of the cortical circuitry because of the recruitment of strong feedback inhibition. This prediction was tested by delivering a short train of LOT stimulation (3 pulses at 40 Hz) to achieve spiking on half the trials (0.56 ± 0.042). Indeed, when a similar train of piriform stimuli (3 pulses at 40 Hz; probability of spiking; 0.36 ± 0.16) preceded the LOT input by 100 ms, we observed an 18% reduction in the probability of spiking. (LOT train following PIR train, 0.46 ± 0.049; n=9 cells; paired t-test comparing two LOT trains, p = 0.017; Figure 14D).

Two forms of inhibition have been described in the piriform cortex. Feedforward inhibition is mediated by interneurons in layer 1 that receive direct input from the LOT and synapse on apical dendrites of pyramidal cells, whereas feedback inhibition is mediated by the layer 2/3 interneurons that are activated by pyramidal cells and synapse onto pyramidal cell bodies (Neville and Haberly, 2004; Luna and Schoppa, 2008; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2012). Two experimental approaches were employed to demonstrate that feedback inhibition is significantly stronger than feedforward inhibition. We observed a dramatically greater effect of SR-95531 on synaptic responses following subthreshold recurrent stimulation versus LOT stimulation (Figure 14E). We also determined the lowest stimulation intensities of either the LOT or recurrent inputs that reliably drove spiking when inhibition was blocked. LOT stimulation at this intensity could still generate spiking when inhibition was intact (Figure 14F), consistent with a relatively small role for feedforward inhibition. In contrast, piriform stimulation at this intensity always failed to evoke spikes in downstream piriform neurons when inhibition
was intact. These data support a dominant role for feedback versus feedforward inhibition in controlling the activation of piriform cortex pyramidal cells.

A primary role of recurrent collaterals may be to drive feedback inhibition, and constrain further excitation as a function of piriform output. The piriform cortex is a known epileptogenic area (Löscher and Ebert, 1996). We asked whether epileptiform activity could be triggered by pyramidal cells in a distal part of the piriform cortex, given that it is extensively connected by long-range recurrent collaterals. Using the same intersectional strategy for sparse and focal virus infection, we expressed the light-activated chloride pump halorhodopsin (NpHR) in a subset of layer 2 pyramidal cells and verified that yellow light (590 nm) evokes outward, hyperpolarizing currents in infected neurons (Figure 15A). With inhibition blocked, we presented a train of weak electrical stimuli to the LOT – mimicking mitral cell bursting (6 pulses at 40 Hz) – to produce a series of EPSPs in layer 2 pyramidal cells (Figure 15B,C). Increasing the stimulus intensity resulted in intermittent, large epileptiform bursts, which became reliable when stimulus strength was increased still further. In order to examine the role of recurrent connections we interleaved control trials with trials in which yellow light was used to suppress layer 2 neurons in a distant (377±29 μm, n=6) region of piriform cortex. These bursts were markedly suppressed when the stimuli were presented in the presence of yellow light, even at stimulation intensities that always produced bursting activity under control conditions. Bursting could sometimes be evoked in the presence of yellow light when LOT stimulation intensity was increased significantly, though the envelope of excitation was always suppressed by light (not shown).

Recurrent excitation can be eliminated by baclofen (50 μM), which suppresses transmitter release at recurrent but not LOT inputs (Figure 15D). In baclofen, LOT stimulation never produced bursting, even with much stronger LOT stimulation (n=3; Figure 15D). With inhibition
intact, trains of LOT stimulation never evoked bursting under, and suppression of distant layer 2 cells had no detectable effect on the magnitude of the postsynaptic response (n=5; Figure 15E). This configuration of widespread excitation across piriform cortex coupled to strong inhibition implies that activation of layer 2 pyramidal cells produces powerful inhibition that can regulate the activity of the entire network.
Results Part II: Anterior olfactory nucleus projections to piriform cortex

Robust, focal expression of channelrhodopsin within the AON

The anterior olfactory nucleus (AON) sits within the olfactory peduncle, the thin stalk of tissue that joins the olfactory bulb to forebrain. It is nearly continuous with the olfactory bulb, the piriform cortex, the orbitofrontal cortical areas, and abuts the dense fasciculation of axons of the lateral olfactory tract as they course from bulb to forebrain. We used the dual viral vector approach to achieve highly localized, specific expression of ChR in AON neurons, and to avoid any confound from infection of these neighboring areas (Figure 16A). We used identical volumes and titers of these vectors, and identical injection procedures, as for our assay of piriform recurrent circuitry.

Co-injection of lentivirus and AAV vectors into the AON results in focal, sparse expression of ChR, as observed for infection of piriform (Figure 16B). Again, pan-neuronal promoters were used in both viruses, so it is assumed that the ChR+ population comprises both excitatory and inhibitory neurons. Focal expression of ChR-YFP in the AON produces YFP+ axons that course throughout the rostrocaudal extent of the anterior piriform cortex (that portion of piriform directly bordered by the LOT), but their density tapers as they enter the posterior piriform (Figure 16C).

The observed pattern of AON projections is consistent with prior tract-tracing studies that show a similar sharp attenuation of labeled AON axons at the border of posterior piriform (Haberly and Price, 1978b). Additionally, YFP+ AON axons are consistently seen in the olfactory bulb, as well as the contralateral olfactory bulb, contralateral AON, and contralateral anterior piriform (not shown).
Within piriform, AON axons fasciculate within layer 1b, similarly to recurrent associational axons from piriform pyramidal neurons (Figure Di, ii). However, AON axons appear to ramify throughout layer 3, with higher density than was observed for piriform axons. There are also YFP+ axons within layer 1a, where bulbar axons synapse on the distal dendrites of piriform pyramidal neurons. This is also the location of interneurons that receive bulbar input, and mediate feedforward inhibition onto piriform neurons (Luna and Schoppa, 2008; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2012). Though sparse, these layer 1a fibers are consistently seen when ChR-YFP is expressed in the AON, and have also been observed using traditional tract-tracing techniques (Haberly and Price, 1978b).

We recorded from YFP+ neurons in the AON to assess the strength of ChR expression. In cell-attached mode, trains of blue light pulses reliably evoked action potential firing in YFP+ neurons (Figure 16Ei). Whole-cell recordings revealed large, characteristic photocurrents, similar in magnitude to those observed in piriform neurons (1.81 ± 0.86 nA, mean ± S.D., n=7, Figure 16Eii).

**Light-evoked synaptic responses in piriform originating in the AON**

Confident that we achieved robust, selective channelrhodopsin expression in the AON, we turned to assessment of AON afferents to piriform cortex. While recording from layer 2 pyramidal neurons, brief (2 ms) light pulses evoked rapid inward currents (Figure 17A). These inward currents were highly consistent from trial to trial. The median latency and jitter for these responses were 3.0 ms and 0.16 ms, respectively, consistent with single-synapse transmission. Light-evoked responses were consistently and reversibly abolished by the addition of blockers of fast glutamatergic transmission (Figure 17B). AON afferents form monosynaptic, glutamatergic synapses with piriform pyramidal neurons.
Like piriform recurrent collaterals, AON projections are part of the larger class of “associational” connections, which have been studied by *en masse* electrical stimulation. Consequently, it is unclear which of these pathways are responsible for the characteristics normally attributed to the associational connections. We have seen that piriform recurrent synapses in isolation may account for these features, including baclofen-sensitivity, NMDA-receptor content, and lack of synaptic facilitation or depression.

We first assessed whether activation of AON inputs is associated with appreciable NMDA-receptor (NMDAR) current, which has been observed for associational (ASSN) synapses (Kanter and Haberly, 1990). Also, recent optogenetic analysis of AON projections to piriform cortex in the rat indicates that AON synapses onto piriform pyramidal neurons have high NMDA receptor content (Hagiwara et al., 2012). Our results are consistent with these recent observations. AON synapses onto pyramidal neurons can indeed have high NMDAR current, as measured by holding the neurons at +40 to +50 mV and recording responses to light (Figure 17C, inhibition blocked with SR-95531). The responses were highly variable from cell to cell, but there was significant NMDAR current associated with AON afferents (mean NMDA:AMPA conductance 7.5 ± 10.9 nS; n=16).

The short-term plasticity, or synaptic facilitation/depression, did not differ significantly from either what is observed for associational synapses, or what we observed for piriform recurrent synapses. AON responses were weakly facilitating (~25%) when preceded by a pulse within 50 ms (Figure 17D). Thus, AON synapses have similarly high NMDA content, and limited short-term dynamics, which is consistent with what has been described for electrically evoked associational synapses. We did not assess sensitivity to baclofen or GABAB activation at AON
synapses, but it is assumed that AON axons are also baclofen sensitive, as baclofen typically completely abolishes ASSN-evoked EPSCs.

We recorded from a total of 121 piriform pyramidal neurons (layer 2) from 16 animals in which ChR was expressed in the AON. Morphology and identity of the recorded neurons was verified by direct visualization and post hoc staining, as previously discussed for the study of recurrent connectivity. We stimulated with wide-field illumination, with the objective positioned over the somata, so as to evoke maximal EPSC responses. Large EPSCs were observed (786 pA, Figure 17D, top trace), but were more typically smaller (65.9 pA, middle trace), or even on the order of a single synaptic release site (11.2 pA, bottom trace). The median response was 47.1 pA (range 4.5 - 786 pA) and the mean response was 96.2 ± 146 pA (mean ± S.D., n=66). Of the 121 neurons, only 66 (55%) demonstrated any response to light, in contrast to the 99% probability of observing a response due to recurrent excitation.

It should be noted that when we did occasionally record from semilunar neurons or layer 3 pyramidal neurons, and these neurons did receive input from the AON. The mean EPSC recorded in semilunar cells was 85.2 ± 38.3 pA (mean ± sd, n = 4), and in layer 3 pyramidal neurons was 101.9 ± 125 pA (mean ± sd, n=4). It is likely that these cell types also receive input from the AON (but see Hagiwara et al., 2012). However, these classes of piriform neurons were excluded from analysis to maintain consistency with our investigation of recurrent connections among layer 2 pyramidal neurons.

By assessing the unitary response amplitude (uEPSC) for these AON afferent synapses, we can estimate the average number of synapses that are activated during maximal stimulation.

Stimulation with minimal light power and spatially restricted illumination elicits discrete responses with alternating successes and failures (peri-threshold stimulation). The uESPCs
recorded with this method were 14.9 ± 5.0 pA (mean ± sd, n = 9, Figure 17F). This is on the 
order of the strength of a single release site, suggesting that AON afferents make single 
terminal or *en passant* synapses onto piriform pyramidal neurons. We did not further assess 
the quantal composition of unitary responses, as was done for piriform recurrent synapses. For 
comparison, this unitary response is somewhat weaker and is approximately 40% of that 
obscerved for piriform recurrent synapses (36.2 ± 20.3 pA).

We did not systematically examine the distance dependence or spatial organization of AON 
afferents to piriform. We did note that we observed responses of varying magnitudes 
throughout the extent of anterior piriform cortex, and that there was not an increased likelihood 
of observing larger responses within the rostral-most portions of anterior piriform. As seen in 
Figure 17G, which is pooled data from 3 slices/animals, and where zero microns represents the 
AON-piriform junction, large EPSCs are observed 1-2 mm into the anterior piriform. We did not 
observe responses in any of 18 neurons patched in posterior piriform (not shown). Given this 
result, and the observation of very few YFP+ fibers posterior piriform of injected animals, we 
chose to confine our analysis exclusively to the anterior piriform cortex.

*Disynaptic inhibition driven by AON afferents*

The AON provides relatively weak excitatory inputs to the piriform cortex. It is not known 
whether the AON drives disynaptic, feed-forward inhibition onto piriform neurons, or whether this 
inhibition dominates excitation, as it does for piriform recurrent connections. We performed 
voltage clamp recordings of piriform neurons at -70 mV and +5 mV to assess the relative 
strengths of excitatory and inhibitory responses to light activation, respectively. A brief light 
pulse evokes a negative EPSC at -70 mV, as expected. While holding at +5 mV in the same 
neuron, we consistently observe a positive response to light, with delayed onset relative to the
EPSC (Figure 18Ai). The median latency and jitter for these positive responses, recorded in 14 neurons, were 4 ms and 0.25 ms, respectively, which is consistent with a polysynaptic process (Figure 18Aii). Moreover, addition of blockers of fast excitatory synaptic transmission (CNQX and AP5), completely and reversibly blocked these positive responses (Figure 18B). The responses recorded at +5 mV were also abolished by GABA_A-receptor blockade with SR-95331 (data not shown).

AON afferents reliably drive disynaptic inhibition, but what is the relative balance of excitation to inhibition? We recorded maximal excitation (at -80 mV) and inhibition (at +5 mV) in individual cells, and plotted maximal inhibitory conductance as a function of maximal excitatory conductance for each cell (Figure 18C). Unlike the strong bias toward inhibition observed for piriform recurrent connections, the AON excitation does not drive strong inhibition. In fact, even for the largest measured excitatory responses (see several points at approximately 10 nS), very little inhibition was activated. A crude linear fit to the data indicates the bias toward excitation (slope = 0.16 ± 0.07).

These initial experiments suggest that AON afferents, while weaker than piriform recurrent collaterals, are coupled to only weak inhibition. However, it is possible that our method of focal and sparse expression of ChR enables only excitation of a limited number of inputs, and even maximal stimulation does not provide enough coincident activation to maximally drive inhibitory interneurons. That is, AON afferents may be contacting the same interneurons that are mediating the dominant inhibition observed for piriform recursents, but these interneurons may require integration of multiple AON inputs to become maximally active. We therefore sought a method to increase the total excitation provided by stimulation of AON afferents. Because our initial approach limited infection to a focal, sparse subset of neurons, the new strategy was to
increase the number of ChR+ neurons within the AON (Figure 19A). We achieved this extensive infection in either of two ways: (1) with an AAV1 vector expressing ChR-YFP under control of the CAG promoter; or (2) with an AAV9 vector expressing ChR-YFP via a CaMKII promoter. Both of these methods provide wider area and higher density of infection than the lentivirus/AAV method (Figure 19B). Our early efforts utilized the AAV1 vector, but we eventually relied exclusively on AAV9. The AAV9 serotype uniquely lacks tropism for mitral and tufted cells within the olfactory bulb, and thus eliminates concern for potential contamination of inputs from the nearby bulb (Hagiwara et al., 2012, Figure 19C). Additionally, ChR expression via AAV9 has been shown, by an unknown mechanism, to more faithfully recapitulate the synaptic properties measured by electrical stimulation than when expressed in other serotypes (Jackman et al., 2014).

Extensive ChR expression in the AON does, in fact, lead to larger observed EPSCs in the piriform (Figure 19Di). The probability of recording a response of any amplitude increased to 82% (Figure 19Diii, n = 58 total attempts). The median EPSC amplitude with extensive expression was 139.9 pA (compare to 47.1 pA with focal expression), with similar distribution, suggesting uniform increase in excitatory inputs (Figure 19Diii). Thus, by extensive ChR expression, we have enabled strong AON-driven excitation of piriform neurons.

We then asked whether this increased excitation would increase the coupled inhibition, suggesting integration within individual interneurons or recruitment of additional interneurons. Again, inhibitory conductance was determined as a function of excitatory conductance for each recorded neuron (Figure 19E). In this set of experiments, there were several neurons with substantial excitation (~10 nS) and one with ~20 nS excitation, but with similar proportion of inhibition as observed for focal ChR expression. There is a clear bias toward excitation
throughout the neurons (slope 0.18 ± 0.08). As a final confirmation that we were maximally activating inhibition, we determined the stimulus-response relationships for both excitation and inhibition in individual neurons (Figure 19F, left). Light intensity was logarithmically scaled until the peak amplitudes of both responses were consistently maximal. From the resultant stimulus-response curves, we see that excitation and inhibition scale proportionally, and that both saturate at similar light intensities (Figure 19F, right).

The AON makes bilateral projections to piriform cortex. It occurred to us that AON projections might drive different levels of inhibition, or interact with different interneurons altogether, depending on whether they terminated in ipsilateral or contralateral piriform cortex. Such asymmetric inhibition across hemispheres could serve to normalize the odorant information from disparate nares, or may even enhance interhemispheric differences, signaling odor laterality to the animal. There is some precedence for olfactory asymmetry in the pars externa of the AON (a small, specialized portion of the AON not activated in the present study). It was shown that these neurons are differentially excited or inhibited depending on to which nares odor was presented (Kikuta et al., 2010). We recorded AON-driven excitatory and inhibitory responses in ipsilateral and contralateral piriform. Contralateral responses were less frequently observed, and did not consistently differ in amplitude from ipsilateral projections (Figure 20A,B). This is similar to previous observations of AON connectivity (Hagiwara et al., 2012). The data were highly variable, but inhibition did not significantly differ across the hemispheres. AON does make projections to contralateral piriform cortex, but these appear similar in character to ipsilateral projections.
AON excitatory input to the piriform is coupled to relatively weakly scaling inhibition. The next step was to assess the functional consequences of these synaptic properties, and to ultimately understand the AON’s potential to drive piriform neurons to fire action potentials. We performed current-clamp recordings of piriform pyramidal neurons in response to AON stimulation. All current-clamp experiments were performed in animals with extensive ChR expression in the AON, and therefore stronger excitation. Trains of 5 light pulses at 20 Hz produced reliable summation of depolarizing EPSPs in piriform neurons (Figure 21A, compare to Figure 14A for piriform). Frequency of stimulation throughout our experiments ranged from 20-50Hz, similar to the spike frequencies of AON neurons in response to odors in anesthetized animals (Lei et al., 2006). Summation resulted in a near doubling of depolarization from beginning to end of the train (1st peak 0.98 ± 0.64 mV; 5th peak 1.95 ± 1.0 mV; n=9 neurons). A dramatic effect of this summation was observed in one neuron (Figure 21B) in which AON inputs were alone sufficient to drive action potential firing in a piriform neuron from a resting potential of -65 mV. Action potentials were more frequently evoked late in the train, indicating that summation was required to reach threshold (Figure 21B, right).

The observed summation of AON inputs could be attributable to the relatively weak disynaptic inhibition. Unlike piriform recurrent EPSPs, which are truncated by short-latency, large amplitude inhibition, AON EPSPs follow a more gradual return to baseline. A simple test of the effect of inhibition is to adjust the resting potential. The theoretical reversal potential for chloride in our preparation is close to -80 mV. Therefore, stepping to -80 mV should reduce the chloride driving force, and minimize any effects of inhibition, while stepping to -60 mV should amplify the effects of inhibition. We adjust the resting potential with small current injections in this manner,
while delivering trains of light pulses (Figure 21C). Interestingly, summation was greatest at -60 mV and diminished at -80 mV, the opposite of what is expected if inhibition is significantly influencing summation.

We recorded single light-evoked AON EPSPs and electrically evoked associational (ASSN) EPSPs (with an electrode in layer 1b) in the same neuron to more closely examine the effects of resting potential on EPSP time course. The ASSN pathway is known to couple to large inhibition, so this provides a comparison for the expected effects of inhibition on EPSP time course (Ketchum and Haberly, 1993). As suggested by the enhanced summation at -60 mV, the decay of the AON EPSP was maximal in duration at -60 mV and minimal at -80 mV (Figure 21D). The increased duration of decay allows for addition of subsequent EPSPs, and so explains enhanced summation at -60 mV. Within the ASSN pathway, the inhibition markedly attenuates the EPSP at -60 mV, but has little effect at -80 mV, as predicted. The voltage dependence of the decay time course can be quantified by measuring the decay time constant, \( \tau_{\text{off}} \) (time to decrease to 1/e peak amplitude). There is a consistent increase in \( \tau_{\text{off}} \) of the AON as voltage becomes more depolarized from -80 mV to -60 mV, which is in contrast to the steep drop in \( \tau_{\text{off}} \) of the ASSN pathway (Figure 21E). Unlike the ASSN pathway, inhibition does not markedly shape the EPSP time course at depolarized potentials. As a final demonstration of this, we recorded trains of EPSPs elicited by either pathway in the same neuron, in the presence and absence of SR95331 to block fast inhibition (Figure 21F). Blockade of inhibition allows for dramatic enhancement of summation of the ASSN responses. In contrast, AON responses are only slightly increased, with little effect on overall summation. Thus, disynaptic inhibition does not restrict AON responses as is observed for piriform recurrent synapses.
The increase in decay time with depolarization suggests a voltage-dependent biophysical mechanism for prolongation of the AON EPSP. One such mechanism could be dendritic segregation or gradation of active, voltage-dependent conductances. For example, AON inputs could synapse onto dendritic compartments that are rich in hyperpolarization-activated cation-selective conductances (I_h currents), mediated by HCN1 channels. Depolarization by current injection (though here limited by somatic access) would cause closure of HCN1 channels, increased local input resistance, delayed charge dissipation, and thus prolonged post-synaptic potentials (Magee, 1998; Magee and Magee, 1999). However, recordings by ourselves and others indicate that piriform pyramidal neurons do not have appreciable I_h current, as indicated by measurement of somatic voltage sag in response to hyperpolarization, or direct recordings from pyramidal cell dendrites (Bathellier et al., 2009; McGinley and Westbrook, 2010). We did not assess the role of I_h current in shaping EPSP time course, but turned to other possible mechanisms.

Another plausible and immediately testable explanation for the voltage-dependence of EPSP time course is that AON afferents form synapses that are enriched with NMDA receptors. The depolarization dependence and slower kinetics of NMDA receptors could account for the enhanced summation and delayed decay of AON EPSPs at depolarized voltages. We recorded AON EPSPs in the presence and absence of the NMDA receptor blocker AP5. Blockade of NMDA receptors had an appreciable effect on the amplitude of single EPSPs at -60 mV (Figure 22A,B). It also caused a small but consistent reduction in the decay time constant and area measured for single pulses (Figure 22B). The role of NMDA receptors is most dramatically illustrated by application of AP5 after stimulation with a 40-Hz train of pulses: NMDA receptor blockade nearly abolishes summation in a reversible manner (Figure 22C). Thus, NMDA receptors subtly increase the time course of EPSPs in a voltage-dependent fashion, which
results in significant enhancement of synaptic summation of AON inputs. Combined with the limited activation of disynaptic inhibition, AON synapses have the potential to significantly depolarize and activate piriform principal neurons.

**Location of AON inputs onto piriform pyramidal neurons**

The topography of AON afferents to the AON were previously studied with classical tract tracing techniques (Haberly and Price, 1978b). These results are consistent with the patterns of fluorescent fibers that we observe originating from the ChR-YFP expressing cells in the AON, That is, there is predominant fiber density in layers 1b and layer 3 (see Figure 16D). We considered whether there might be a finer compartmentalization or segregation of AON inputs onto piriform pyramidal neurons, which could additionally account for the unique properties of post-synaptic response. Our first approach to assess topography was to perform an experiment similar to that performed for piriform recurrent connections. We reduced the illumination field with an aperture, and added TTX and 4-AP to the bath to block action potential propagation (Figure 23A, and see Figure 13). By stimulating at 100-μm increments across the apical-basal axis of the pyramidal neuron, we measured the strength of excitation according to the apical-basal position of the neuron. As we had done for piriform, we deconvolved the result from the point-spread function of the illumination field. The average of 8 neurons indicates peak AON excitation over and around the soma, similar to piriform recurrent connections.

One concern for this method is that it naturally biases for excitation near the soma, especially in neurons with the dendritic geometry of piriform pyramidal cells. These neurons have apical dendrites that branch early and fan outward from the apical-basal axis. The basal dendrites form a similar splayed cone in the basal field. As we move along the apical/basal dendrites with our light source, in a single line, we will illuminate more synapses as the dendrites funnel in
toward the soma. Therefore, this method does not adequately illuminate synapses that form at more distal points along dendritic segments.

A potential solution to this problem is to scan the entire field, including distal and lateral regions, with a much more focal light source. This approach has been utilized to efficiently map neocortical callosal projections onto layer 5 pyramidal neurons (Petreanu et al., 2009). Using a 470-nm laser directed by fast scanning mirrors, we repeated the above experiment by scanning and activating the entire field, including all layers of piriform, while recording responses in piriform neurons (Figure 23B,C). Again, TTX and 4-AP were included in the bath to block action potential propagation. An exemplary set of responses is shown in Figure 23D, and the corresponding intensity profile is shown in (E). We then generated maps of the location and amplitude of each recorded EPSC, and aligned these maps so that data from 12 neurons could be combined into an average intensity profile. Piriform neurons were most strongly activated by stimulation of AON inputs over the proximal apical field (Figure 23F). One neuron, however, was reliably activated by a small area within deep layer 3, suggesting that synaptic contacts onto the basal dendrites by AON afferents are possible, if less common (Figure 24). Note that this neuron was scanned with a higher magnification objective (20X) than the experiments above, requiring two adjacent scans to cover layers 1 and layer 3. Due to this difference in protocol and scan pattern, this neuron was not included in the pooled data to form the composite activation map. Nevertheless, it raises the possibility that some piriform pyramidal neurons may receive basal contacts from the AON, and this may in part account for the dense AON axons observed in layer 3.
**AON afferents synapse onto Layer 1a interneurons**

While trying to further understand the exact loci of AON connectivity within piriform, we were intrigued by the sparse, but consistently observed fibers that course through layer 1a. These appear to emerge as collaterals from the dense fibers in layers 1b and 3, and do not appear to descend from the LOT. Furthermore, they are even present in animals that were injected with the AAV9 vector, which appears to reliably prevent mitral/tufted cell labeling. Layer 1a itself contains few synaptic targets: presynaptic bulbar terminals, distal apical dendrites, and interneurons.

We recorded from layer 1a interneurons in animals expressing ChR in the AON (Figure 25A). Surprisingly, we observed responses to light stimulation in 6 of 7 layer 1a interneurons. We did not target a specific morphology interneuron under DIC, nor were we able to correlate the positive responses to a particular interneuron type within this small subset (see filled neurons in 25A). We initially recorded in cell-attached configuration, and three separate interneurons could be robustly driven to fire action potentials with light stimulation (Figure 25B). Whole-cell recordings demonstrated massive inward currents in response to light that did not saturate in amplitude, and often led to unclamped action potentials (Figure 25C).

These interneurons are likely those that mediate feedforward inhibition driven by bulbar inputs (Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010, 2012). Stimulation (in current-clamp) of one interneuron with light pulses and with electrical stimulation of the LOT revealed that this cell receives input from both the LOT and the AON. Thus, AON afferents could potentially modulate the activity of interneurons that mediate bulbar feedforward inhibition.
Comparison of AON afferents to piriform recurrent connections

When ChR is expressed in the AON neurons with similar procedures as those used to express ChR in a focal subset of piriform neurons, weaker total excitatory responses are measured in piriform neurons than were measured for recurrent connections. The probability of observing a response due to AON excitation was less than for piriform recurrent inputs (55% vs. 99%, Figure 26Ai). The distributions of responses, while similar in shape to piriform recurrents, was biased toward lower amplitude EPSCs (median AON response 47.1 pA vs. piriform recurrent median response 305 pA, Figure 26Aii,iii). The smaller amplitude of AON responses is in part attributable to the weaker strength of unitary inputs. We measured unitary responses in the AON to be 14.9 ± 5 pA (vs. 36.2 ± 20.4 pA for piriform recurrent unitary strength Figure 26Aiv).

Though weaker, these AON projections to piriform do not drive powerful disynaptic inhibition like piriform recurrent collaterals. Piriform recurrent activation consistently generates an inhibitory conductance, and this conductance is typically much greater than the accompanying excitatory conductance (Figure 26B). The AON, on the other hand, can frequently generate excitation with little or no inhibition (10 of 48 neurons), or inhibition that is proportional in a one-to-one manner. That is, the ratio of inhibitory conductance to excitatory conductance (G_{inhibitory} / G_{excitatory}) for AON afferents is 0.89 ± 0.20 (n=48), which is significantly different from a ratio of 1.9 ± 0.22 (n=22) observed for piriform recurrent connections (Figure 26Biii, p=0.006). In other words, individual AON excitatory afferents appear weaker than piriform recurrent collaterals, but synchronous or convergent AON input has potential to strongly activate piriform neurons without inhibitory constraint.
Discussion

The piriform cortex presents a tractable model for sensory-cortical integration, and provides opportunity for systems level study of the sculpting of a sensory representation by cortical dynamics. A prerequisite to this is a detailed characterization of the associative inputs to piriform cortex, and their coupling to the local microcircuitry. By selective expression of channelrhodopsin, we have functionally isolated two important contributors to the intracortical associative circuitry of piriform: the intrinsic recurrent circuitry, and early sensory input from the anterior olfactory nucleus. These two classes of associative synapse interact with two distinct, non-overlapping interneuron populations, and are thus poised to play very different roles in activating or suppressing piriform cortex in response to odor.

Possible roles of piriform recurrent connections

The specificity of an odorant in piriform cortex is represented by a unique ensemble of neurons that is distributed without discernable spatial order. These cells also make extensive recurrent connections with other excitatory and inhibitory neurons that may shape the odor representation. We have shown that axons of layer 2/3 pyramidal neurons project across piriform cortex where they make excitatory synaptic contacts with other pyramidal neurons. The likelihood that any two pyramidal neurons are synaptically connected is very small, but remains roughly constant over remarkably long distances compared to neocortical sensory areas, namely the visual and barrel cortices. However, because there are a massive number of piriform pyramidal neurons, that are seemingly functionally uniform with respect to bulbar and recurrent connectivity, each pyramidal neuron receives excitatory inputs from at least 2000 of its peers. Recurrent collaterals also activate GABAergic interneurons that form powerful inhibitory synapses onto nearby pyramidal cells to counter and often overwhelm the recurrent excitation.
The recurrent circuitry in piriform cortex thus produces global excitation that recruits strong local inhibition, which scales with the excitatory drive. This allows temporal pairing of bulbar input with activation of the recurrent network to alter piriform responses, thereby shaping the odor representation.

Projections from individual glomeruli are distributed throughout piriform cortex without any obvious topographic order, and individual pyramidal cells receive convergent input from a random collection of glomeruli. This afferent information is then redistributed across piriform by the diffuse and apparently random recurrent network. Nevertheless, an odor will consistently activate the same ensemble of piriform neurons in an individual (Poo and Isaacson, 2009; Stettler and Axel, 2009).

We consider two distinct models for the activation of a cortical odor ensemble. In one model, an odorant may activate a sufficient number of mitral and tufted cell inputs to generate a direct, suprathreshold synaptic response in all of the odor-responsive piriform neurons. In this case, the long-range recurrent excitation would mainly serve to recruit inhibitory neurons to generate a strong, diffuse feedback inhibition. Alternatively, an odorant may evoke suprathreshold input from the olfactory bulb in a small subset of odor-responsive neurons. This small fraction of spiking piriform cells would then generate sufficient recurrent excitation to recruit a larger population of neurons that receive subthreshold afferent input. The strong feedback inhibition resulting from activation of this larger population of neurons would then suppress further spiking and prevent runaway recurrent excitation. In the extreme, some cells could receive enough recurrent input to fire action potentials without receiving afferent input.
Two recent studies lend support to the second model. First, Davison and Ehlers (2011) observed robust responses in piriform neurons upon activation of a set of glomeruli that were not synaptically connected to the recorded cell. Second, Poo and Isaacson (2011) observe that, in a subpopulation of cells, afferent, LOT input only accounts for a small fraction of the odor-evoked excitatory drive onto a subset of layer 2 pyramidal cells. Our studies demonstrate that pairing weak bulbar inputs with recurrent inputs can dramatically increase the activation of piriform neurons. These effects are observed even though we expressed ChR in less than 1% of piriform neurons. Thus the spiking of only a small fraction of piriform cells by direct input from the bulb could activate the recurrent circuitry to recruit the ensemble of odor-responsive neurons. Recurrent input could therefore contribute significantly to the activation of a piriform ensemble, though these data do not exclude models in which piriform pyramidal cells are driven largely by bulbar input.

Our results indicate that the effect of recurrent input on the ability of olfactory bulb input to drive spiking is highly dependent on the relative timing of the two sets of inputs. When piriform axons are activated simultaneously with or slightly after stimulation of the LOT, the firing of piriform neurons is significantly enhanced. However, when piriform is activated prior to stimulation of the LOT, the firing of piriform neurons in response to LOT inputs is suppressed. This dynamic circuitry is poised to generate a homogenous, associative network that can potentially explain a number of features of olfactory processing observed in piriform. For example, the number of odor-responsive neurons in piriform is only weakly dependent on odorant concentration (Stettler and Axel, 2009), even though both the number of activated glomeruli (Rubin and Katz, 1999) and the amount of excitatory input to individual piriform pyramidal cells (Poo and Isaacson, 2009) increases with odorant concentration. A diffuse recurrent cortical network with scaled inhibition affords a normalization mechanism that can maintain a constant level of piriform
activation. The recurrent piriform network may also explain the observation that the number of piriform neurons activated by a mixture of odorants is far less than the sum of the neurons activated by individual odorant components. Rather, odorant mixtures tend to suppress activity in cells responsive to individual odorants presented alone (Stettler and Axel, 2009). Thus, the pattern of active neurons in response to a mixture of odorants differs from the representation of individual components. A highly interconnected recurrent network might accommodate these computations (Haberly and Bower, 1984; Wilson and Bower, 1992; Barkai et al., 1994; Haberly, 2001).

We find that the recurrent circuitry in piriform cortex exhibits organizational properties that are different from those of neocortical sensory areas. In vision, touch and hearing, spatial information in the peripheral sense organ is maintained in the cortex. In sensory neocortex, cells responsive to similar stimulus features tend to be clustered. In these cortices, recurrent circuitry is primarily local and serves to connect cells with similar receptive fields (Braitenberg and Schüz, 1998; Ko et al., 2011). As a consequence, this circuitry is thought to increase signal-to-noise (Douglas et al., 1995) and sharpen the tuning of neurons to specific features of the stimulus (Anderson et al., 2000; Wehr and Zador, 2003; Wilent and Contreras, 2005; Murphy and Miller, 2009) Longer-range parasagittal connections in neocortex are specific and connect areas that respond to similar features (Gilbert, 1992). In piriform cortex, pyramidal cells receive random, convergent input from multiple glomeruli, and an odor activates an ensemble of neurons distributed across the cortex. Recurrent projections in piriform are long-range, span the entire cortex, and exhibit no apparent topography. This extensive recurrent circuitry may therefore enable an ensemble of active piriform neurons to function as a highly associative, homogenous network.
**Possible roles of AON projections to piriform**

The AON makes glutamatergic excitatory connections onto pyramidal neurons of the piriform cortex. These projections have properties similar to the electrically evoked associational projections. They synapses onto proximal apical dendrites and potentially synapse onto the basal dendrites of pyramidal neurons, which is consistent with observations of the laminar distributions of AON afferents in piriform. The AON seems to make fewer excitatory synapses onto piriform cells than recurrent collaterals, as indicated by lower probability of observing responses, and smaller average EPSC amplitudes, which is in part accounted for by the weaker unitary strengths of AON inputs than those of piriform recurrent collaterals. However, it is unclear how valid a comparison of connectivity is between the two input classes. We did not rigorously estimate the number of ChR-expressing neurons within AON, as we did for piriform cortex. We injected identical volumes and titers of virus into the AON as for piriform experiments, but the AON has an obvious difference in neuron organization and density. The density of AON neurons is roughly half of what we estimated for piriform cortex (190 cells/mm³, with ~60,000 neurons per hemisphere, Brunjes et al., 2011). Therefore, we do not speculate as to the relative connectivity of AON and recurrent connections.

When the number of ChR-expressing AON neurons was increased with extensive infection, we observed a nearly uniform increase in the strength of responses, and the probability of observing a response approached 100%. Interestingly, as was observed for piriform recurrent responses, there were outlier neurons that demonstrated maximal responses an order-of-magnitude above the mean, suggesting a high number of inputs. While this is likely a simple consequence of distributing a large number of inputs across a population of neurons (true stochastic outliers), it raises an intriguing question as to whether they function as hubs or nodes.
in a scale-free-like network. One can imagine such neurons linking together a larger number of odor representations, enabling diffuse reactivation or recall. There is indirect evidence that certain neurons, in both the AON and piriform, make disproportionate contributions to electrically evoked intracortical synaptic responses (McGinley and Westbrook, 2013).

The critical difference between AON projections to piriform and recurrent collaterals is their interaction with local interneuron populations. Piriform recurrent excitation is dominated by inhibition, which is likely mediated by perisomatic inhibitory synapses formed by fast-spiking, parvalbumin-positive interneurons in layers 2/3. AON projections are coupled to a different interneuron population. Maximal activation of AON inputs causes inhibition to saturate at proportional levels. The observed weaker AON-driven inhibition is not due to inadequate activation of the same interneurons that mediate recurrent inhibition. More likely, the AON synapses onto a unique interneuron population that produces weaker inhibition. We have made the intriguing preliminary observation that the AON is capable of synapsing onto and driving firing in layer 1a interneurons, possibly the same interneurons that mediate feedforward inhibition from the OB. These interneurons likely synapse onto distal dendritic compartments of the piriform pyramidal cells, and dendritic filtering could account, in part, for the relatively weak inhibitory responses recorded in the somata. These observations require rigorous follow-up, but if correct suggest that the AON may directly influence the primary input to piriform. It should also be mentioned that in numerous experiments in which electrical stimulation of the LOT was paired with light-evoked AON activation, no non-linear effects were observed that would indicate AON modulation of feedforward inhibition (data not shown).

Synapses from the AON and piriform appear to have similar levels of NMDA receptor content. However, AON synapses are more likely to experience depolarized potentials because they are
not coactive with shunting inhibition. Depolarization of AON synapses causes concomitant activation of local NMDA receptors, which results in added depolarization, prolongation of EPSPs, and enhanced summation. Sequential bursts of AON inputs, even weak EPSPs, nearly always result in pronounced summation and depolarization. In some neurons, summation is sufficient to induce firing. This unrestrained NMDA receptor activation has several important possible consequences: (1) the AON may directly evoke firing in some piriform neurons; (2) it may directly integrate with LOT inputs without the strict timing dependence of piriform-LOT integration; (3) AON synapses may easily undergo NMDAR-dependent long-term potentiation; and (4) given that they have similar synaptic loci, the AON may provide the depolarization and/or calcium influx to facilitate plasticity at coincidentally active piriform recurrent synapses.

It is possible that AON-to-piriform projections follow a determinate topography, and that this may account for the sparse connectivity observed between the AON and piriform neurons. For instance, it is not known whether AON neurons that receive inputs from a particular set of glomeruli project to piriform neurons that receive inputs from a similar set of glomeruli. It is not difficult to imagine a developmental process whereby coincident activation of AON and piriform neurons by an odor results in preservation of synapses between simultaneously active AON and piriform neurons. A future question is whether interconnected AON neurons and piriform neurons are responsive to similar odorants.

One intriguing hypothesis for the olfactory system is that through parallel distribution of OB output, cortical areas construct a hierarchical odor object (Haberly, 2001). This odor object is thought to coalesce with the posterior piriform cortex, where multiple olfactory and auxiliary areas send their inputs, which combine with a representation that evolves from anterior piriform cortex. Unlike other sensory systems, the hierarchical odor object is constructed not from
concrete physical feature representations, but from early abstractions of odor identity. Each area orthogonalizes or categorizes OB output along a different, arbitrary dimension. The utility of this is that learning and recall of odors occurs along ethologically relevant dimensions. That is, odors are not categorized by chemical structure, but by context, affective state, or learned experience. The AON has a privileged position as an early node within this hierarchical processing structure. Moreover, its output immediately reintegrates with the incipient odor representation in the piriform cortex. Thus, the AON may integrate glomerular information along a different dimension than piriform, and AON projections to piriform could select for, or bias the representation according to this early categorization.

An alternative hypothesis is that the AON may play very specific role in biasing the piriform representation according to a highly specific signal or state of the animal. For instance, the AON contains vasopressin neurons that respond to social odor cues (Wacker et al., 2011). Vasopressin signaling in the AON may alter the signal projected to piriform, and bias or shift the representation according to the presence of social stimuli.

**Notes on associative plasticity in piriform**

Many of the hypothesized roles for an extensive recurrent network within piriform rely on associative plasticity to link neurons that participate in a common odor representation. Dynamic associations among neurons within and between representative odor ensembles would enable the network to achieve perceptual stability, pattern completion, high-level categorization and associative learning. The simplest model requires that neurons undergo Hebbian plasticity at their recurrent synapses, such that neurons that are frequently co-activated by an odor, a combination of odors, or more general “top-down” contextual input will strengthen the recurrent synapse(s) among them. Thus, any one of these neurons (or more realistically, small fraction of
these neurons), when active, will increase the likelihood that the remainder of the representation will be recruited with time or additional input. We now know, however, that recurrent synapses are dominated by inhibition, to such an extent that for sufficient post-synaptic depolarization to occur to induce plasticity, coincident bulbar input is likely required. Accordingly, associative plasticity, through spike-timing dependent plasticity or heterosynaptic associative plasticity have only been achieved through blanket blockade of inhibitory neurotransmission (Johenning et al., 2009), or through bath application of high concentration neuromodulator (Barkai and Hasselmo, 1997). Plasticity at recurrent synapses does not appear as facile as expected for an associative network. Rather, it seems to require a specific set of conditions that dampen inhibition, and/or alter intrinsic excitability or synaptic properties via neuromodulation to allow associations to form. We did not block inhibition, and were not able to induce either homosynaptic plasticity or heterosynaptic plasticity, by pairing with LOT stimulation, at either piriform recurrent or AON synapses (data not shown).

Future directions

It is our hope that the results detailed herein inspire and guide further thinking as to the functional roles of associative input to the piriform cortex, from both the anterior olfactory nucleus and recurrent collaterals. We have provided significant evidence that these two classes of associative input are distinct and interface with the piriform microcircuitry in distinct ways. The greater goal is to understand how these inputs modulate the odor representation in piriform, and ultimately how they drive learning and behavior. The next step is to functionally activate and inhibit these pathways in the living animal, and observe the changes in the electrically or optically measured responses of piriform neurons. Isolation of piriform-to-piriform recurrent collaterals will require a similarly deft approach as our dual-vector method for modulation of a
subset of the network. The AON is geographically distinct, and may be more immediately
amenable to optogenetic manipulation. Also, mice are available (CRH-Cre, Gensat) that could
further restrict expression to a large, CRH-expressing subset of the AON. With an optogenetic
method of reliably inhibiting (and/or activating) recurrents or the AON, initial experiments should
assess any alteration of the odor response in piriform when these pathways are inactivated.
Additional experiments, most appropriately done in parallel, should assess the behavioral
consequences of their inactivation. It is possible that odor learning, discrimination, or
association, or even more complex perceptual learning and pattern completion will be affected
by loss of recurrent or AON inputs.

An additional set of experiments would address whether recurrent collaterals enable the piriform
to function as a true auto-associative network. The extensive recurrent network in piriform could
serve to link neurons that are consistently activated by similar sets of glomeruli. This linkage
would occur through a canonical Hebbian process, with or without additional neuromodulatory
influences. These directed associative connections could perform pattern completion within
neural ensemble representing a single odor profile. Here, we have determined the average
weights of recurrent synapses derived from a randomly selected (ChR+) subset of piriform
neurons. However, if we could isolate all piriform neurons that participate in a single odor
representation, we could study the strength of connectivity between functionally related neurons
versus those that are a part of unrelated representations. It is possible that ongoing Hebbian
processes, in addition to developmental sculpting, has produced a number of recurrent sub-
networks that provide for consistent activation of odor representations and thus, perceptual
constancy.
Methods

All experiments followed approved national and institutional guidelines of the Columbia University Medical Center, and were reviewed and approved by the Institutional Animal Care and Use Committee. All animals were C57Bl/6J mice obtained from Jackson Laboratories.

Viral vector production and stereotaxic gene delivery

A Cre recombinase/GFP cassette (Le et al., 1999) was cloned into a lentivirus vector, with expression driven by the human synapsin promoter. High-titer lentivirus was either prepared using established protocols (Zhang et al., 2010) or produced commercially (System Biosciences, 8.55 x 10^8 IFU/ml). Adeno-associated viruses (AAV) were generated from pAAV-EF1a-DIO-hChR(H134R)-EYFP-WPRE-pA and pAAV-EF1a-DIO-eNpHR3.0-EYFP plasmids (gifts from Karl Deisseroth) and produced commercially (UNC Vector; serotype 2/1; 4 x 10^{12} IFU/ml). Cre-independent vectors were obtained similarly: CamKIla.hChR(H134R)-eYFP.WPRE.hGH (Penn Vector, serotype 2/9, titer 1.98 x 10^{13}), and CAG.hChR(H134R)-eYFP.WPRE.hGH (Penn Vector, serotype 2/1). Viruses were separated into 4 μL aliquots and stored at -80°C.

Young adult C57Bl/6J mice (4-8 weeks old) were anaesthetized with ketamine/xylazine (100/10 mg/kg, respectively, intraperitoneal) and head-fixed in a stereotaxic device. Virus was injected with a pulled glass pipette using standard procedures (Cetin et al., 2006). Briefly, an incision was made in the scalp and a small craniotomy (~1mm diameter) was drilled above the anterior olfactory nucleus or piriform cortex using stereotaxic coordinates optimized by fluorescent bead injection (FluoSpheres, Invitrogen). Individual aliquots of lentivirus and AAV were thawed, mixed (1:1), and slowly injected with a Nanoject II (Drummond Scientific) through a glass pipette.
(tip size, ~20 μm; 681 ± 64 nl, range 200-1250 nl). The pipette was left in place for 10 minutes before being slowly retracted. The same procedure was used for injections into somatosensory cortex, visual cortex and olfactory bulb, except that AAV2/5-Syn-hChR(H134R)-EYFP (produced from the plasmid pAAV2/5-Syn-hChR(H134R)-EYFP-WPRE-pA, gift from Karl Deisseroth) was injected into olfactory bulb. Off-target injections resulted in no expression or expression that was largely absent from the AON or piriform cortex, and argue against spurious activation from other brain regions that were sometimes infected following virus injection. Animals with off-target infection were not used.

**Electrophysiology and data analysis**

Eighteen ± 1 days (range 13-28) after virus injection, mice were anesthetized with isoflurane and decapitated. The cortex was quickly removed in ice-cold artificial CSF (aCSF). Parasagittal brain slices (350 μm) were cut using a vibrating microtome (Leica) in a solution containing (in mM): 10 NaCl, 2.5 KCl, 0.5 CaCl₂, 7 MgSO₄, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 glucose, and 195 sucrose, equilibrated with 95% O₂ and 5% CO₂. Slices were incubated at 34°C for 30 min in aCSF containing: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 25 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂, 2 NaPyruvate. Slices were then maintained at room temperature until transfer to a recording chamber on an upright microscope (Olympus Optical) equipped with a 40x objective (LUMPLFN 40XW, 0.8 N.A.). Patch electrodes (3-6 MΩ) contained: 130 D-Gluconic acid, 130 CsOH, 5 mM NaCl, 10 HEPES, 12 phosphocreatine, 3 MgATP, 0.2 NaGTP, 10 EGTA, 0.05 AlexaFluor 594 cadaverine, 0.15% biocytin. For current-clamp recordings, electrodes contained: 130 K-Methylsulfonate, 5 mM NaCl, 10 HEPES, 12 phosphocreatine, 3 MgATP, 0.2 NaGTP, 0.1 EGTA, 0.05 AlexaFluor 594 cadaverine, 0.15% biocytin. Voltage- and current-clamp responses were recorded with a Multiclamp 700A amplifier,
filtered at 2-4 kHz, and digitized at 10 kHz (Instrutech). Series resistance was typically 8-12 MΩ, always <20 MΩ, and was compensated at 80%–95%. Experiments were discontinued if there was an increase in series resistance >25%. The bridge was balanced using the automated Multiclamp function in current clamp recordings. Data were collected and analyzed off-line using a combination of Axograph X, IGOR Pro (Wavemetrics), MATLAB (Mathworks), and Prism (GraphPad).

Short, collimated light pulses from a 470 nm LED (LEDC5, Thor Labs; 0-250 µW measured at the sample) were delivered to the tissue through the objective every 10-15 seconds. In most experiments we used a 2-ms, 250-µW pulse. In some experiments, an aperture in the light path was closed restricting illumination to a focal point (see Figure 13A). In a subset of experiments, we noted Δx for each recorded cell (166 cells from 11 slices/11 animals). We first determined whether these cells were ChR+ or ChR-negative, and whether ChR-negative cells exhibited light-evoked EPSCs at -70 mV (n=96) or IPSCs at +5 mV (n=87). Some cells (n=71) were recorded at +5 mV in the presence of NBQX/APV to test for direct IPSCs. The cells from all experiments were pooled and binned with respect to Δx (200 µm bins), from which the observed probabilities (Figures 1E, 1G and 3B) were determined. NBQX or CNQX (10 µM), D-AP5 (50 µM), SR95531 (10 µM), TTX (1 µM) and 4-AP (100 µM) were all obtained from Tocris. We did not correct for liquid junction potentials. All experiments were done at 34°C. Traces typically represent averages of 6-10 trials. Unless stated otherwise, data are presented as mean ± sem.

Histology and post hoc imaging

Animals were anesthetized with ketamine/xylazine and perfused through the heart with cold PBS followed by 4% paraformaldehyde. Brains were removed and post-fixed overnight. Tissue
was embedded in a 5% gelatin block and 100 µm-thick coronal sections were cut on a vibrating microtome (Leica). Slices were permeabilized (0.3% PBS-T) and incubated at 4°C overnight in chicken anti-GFP (Invitrogen, 1:1000) and rabbit anti-Cre (Novagen, 1:1000) antibodies. Slices were rinsed and then incubated in donkey Alexa-488 anti-chicken and Alexa-555 anti-rabbit antibodies (1:1000) and NeuroTrace 640 (1:500; all Invitrogen) at 4°C for 2-4 hours. Rinsed slices were mounted with Vectashield and visualized with a Zeiss 710 confocal microscope. For visualizing patched neurons, slices were removed from the recording chamber after recording and placed in 4% PFA overnight. Slices were permeabilized (0.25% PBS-T) and incubated at for 24-72 hours at 4°C in a rabbit anti-GFP antibody (Invitrogen, 1:1000) and Alexa-555 conjugated Streptavidin (Invitrogen, 1:200). Slices were rinsed and incubated in donkey Alexa-488 anti-rabbit antibody and NeuroTrace 640 (Invitrogen, 1:500) 4°C overnight. Rinsed slices were mounted with VectaShield (Vector Labs) and visualized on a fluorescent microscope.

Identification of layer 2 pyramidal neurons

Neurons were patched in deep layer 2 under DIC optics and visualized at 590 nm with a monochrometer and cooled CCD camera (TILL Photonics). Pyramidal cells were identified based on morphological and electrophysiological indicators (Haberly, 2005; Suzuki & Bekkers, 2009), including one or two spiny apical dendrites that branched extensively and extended to layer 1a and basal dendrites that extended into layer 3, and input resistances of 100-200 MΩ (124 ± 8.4 MΩ). Recordings from semilunar cells or high input resistance layer 2 GABAergic neurons could readily be distinguished from pyramidal cells and were discontinued. After recording, slices were fixed and processed to visualize the biocytin label, confirming that patched cells were layer 2 pyramidal cells (see Figure 11).
Determination of illumination field

We measured the size of the illumination field by recording from ChR\(^+\) neurons in TTX, NBQX, AP5 and SR95531 to isolate photoc currents. Light pulses were presented at different distances from the cell, normal to the apical-basal axis of the dendrites. An aperture in the LED light path was either open or closed, for wide-field illumination or focal illumination, respectively. The relationship between distance-dependence the photocurrent amplitude and distance from the recorded cell evoked with wide-field illumination was well fit by a Gaussian (width, 368 µm). The distance-dependence following focal illumination was fit with a single exponential function with a length constant of 87 µm (see Figure 13).

Synapse mapping and laser-scanning photostimulation

For localizing synaptic inputs, responses were recorded in TTX to block action potentials and 4-AP to enhance photoactivation of ChR\(^+\) synaptic terminals. The light spot was centered on the soma and light pulses were presented at 50 µm steps along a straight line extending from 400 µm apical to the soma to 400 µm basal to the soma. The pial surface was 308 ± 23 µm (n=10) apical to the soma. Responses evoked at each location (6-10) were averaged and normalized to the maximal average response recorded in that cell. Normalized response profiles from all cells were averaged and deconvolved in MATLAB using the Lucy-Richardson algorithm with PSF=87 µm. For localizing excitatory and inhibitory synaptic inputs, responses were recorded in GBZ or NBQX and AP5, respectively. For localizing the origin of disynaptic inhibition, the same procedure was used except that responses were evoked with no drugs present and with \(\Delta x > 1,000 \mu m\). Slices were placed in the recording chamber with the pial surface/LOT at the top of the chamber and apical-basal axis of layer 2 pyramidal cells was roughly aligned along the
vertical axis. Misalignment of this axis, lateral spread of dendrites and dendritic filtering all bias the efficacy of somatic measurements, which were not corrected.

Laser-scanning photostimulation was performed by scanning the slice surface with a pair of galvanometer-controlled scan mirrors (Till Photonics), controlled by custom software written in C and LabView (National Instruments). The 470-nm laser (100 mW) was power modulated with an AOTF (AA Optoelectronic) and fiber coupled to the scan mirrors. Planar deflection was achieved with a scan lens (Till Photonics) behind the objective. The beam diameter (1/e²) of the illumination spot was either ~30 μm (20X objective) or ~15 μm (40X objective). The order of illumination sites was initially designed so as to maximize the time between neighboring illumination sites, thus minimizing artifact from inactivation by neighboring illumination. However, serial trials with conventional grid illumination pattern (left-to-right, top-to-bottom) showed no such neighborhood effects. All data were analyzed with MATLAB.

Estimation of the number of ChR+ neurons

Variability in the number of ChR+ cells from animal to animal required that we determine the number of ChR+ cells from the same animals in which we performed quantitative electrophysiological experiments. We were not able to resolve individual ChR+ cells in the 300-μm slices used in electrophysiology experiments, or reliably resection these slices for post hoc analysis. We therefore estimated the average number of layer 2 ChR+ neurons per animal from the observed distribution of ChR+ cells with distance from the injection center and an estimate of the total layer 2 cell density. Confocal images of Nissl-stained sections (NeuroTrace 640; 1:500; 10 μm optical sections) were used to count the number of layer 2 nuclei in a region of anterior piriform cortex. Only nuclei with well-defined boundaries in the plane of focus were
counted. Small, heterochromatic nuclei with intense label were not counted, as these were likely to be glia (Nauta and Feirtag, 1986). We counted 166 ± 20 layer 2 nuclei in delimited area of layer 2 of 0.044 ± 0.005 mm² (18 sections from two animals). We corrected for the estimated 10% of inhibitory layer 2 neurons (Mugnaini and Oertel, 1985; Löscher et al., 1998). The sample space for each count was simplified to a rectangular cuboid with sides 440 µm (width of each image) x 100 µm (height of layer 2) x 10 µm (depth defined by optical section), in which we determined a volume density of layer 2 excitatory neurons of 342 ± 45 x 10³ cells/mm³.

The probability of patching a ChR+ cell with distance from the injection site follows a normal distribution, and so we assume that the probability density for ChR+ neurons within the sheet of layer 2 cells can be described by a simple two-dimensional Gaussian curve, given as:

\[ p(x, y) = A \cdot \exp \left( \frac{(x)^2}{2\sigma^2} + \frac{(y)^2}{2\sigma^2} \right) \]

where \( A = 0.37 \) (fraction of ChR+ cells at injection center) and \( \sigma^2 = 223 \). The total number of ChR+ neurons was then determined by integrating this probability density function over the estimated number of neurons in a 2 mm x 2 mm x 0.1 mm sheet of cells, discretized into 1,600 columns (0.1 mm x 0.1 mm x 0.1 mm), with the injection site at the center of the sheet. With this method, we estimate that we express ChR in 7,889 excitatory layer 2 neurons.
**Figure 1**

A.

Lentivirus

5' hSyn1 Cre GFP 3'

+ AAV1

EF1α YFP ChR2 ChR2

loxP / lox2722

transcription

B.

LV (Cre)

AAV1 (flex-ChR2-YFP)

C.

injection site

posterior section

Di

Cre

ii

YFP

iii

Comb.

Ei

Cre

ii

YFP

iii

Comb.
Figure 1. Expression of channelrhodopsin in a sparse, focal subset of piriform neurons.

(A) Strategy for sparse and focal ChR expression. High-titer AAV, used to express Cre-dependent ChR-YFP (within a flipped-excision, or ‘flex’ cassette), was co-injected into piriform with a lentivirus driving neuronal expression of Cre recombinase. Lentivirus infects a fraction of the neurons and does not spread beyond a small area, providing focal and sparse ChR expression.

(B) Diagram with coronal brain schema to indicate approximate location of piriform injections.

(C) Confocal image of a virally infected brain showing infection restricted to anterior piriform, with dorsoventral extension of axons (scale bar = 1 mm).

(D) Higher magnification confocal images of the injection site within anterior piriform. (i) Anti-Cre antibody staining (red) reveals a sparse pattern of Cre expression in layers 2/3 of piriform cortex. (ii) YFP fluorescence (green) corresponding to membrane-bound localized channelrhodopsin. Note the sparse cellular fluorescence, as well as locally intense staining of the neuropil in all layers. (iii) Combined images with background nuclear stain (blue) to show relationship to layer 2 (scale bar = 100 µm).

(E) High magnification images as in (D), but of a site 1 mm posterior to the injection site. (i) No Cre expression is observed distant to the infection site. YFP fluorescence (ii and iii) shows a dense fasciculation of fibers in layer 1b, with sparser collaterals in layer 3.
Figure 2

Well-targeted injection

Poorly targeted injection

Ai

Bi

Aii

Bii

Aiii

Biii

injection site

1mm rostral

detail

Well-targeted injection

Poorly targeted injection

Ai

Bi

Aii

Bii

Aiii

Biii

injection site

1mm rostral

detail
Figure 2. Infection of overlying cortex without infection of piriform cortex does not produce labeled fibers throughout piriform.

(A) Coronal section from a brain in which an AAV1-hSyn-YFP vector was injected into anterior piriform cortex from the dorsal surface of the brain. (i) The injection tract is visible as a thin dorsoventral column of fluorescence, and is often unavoidable (scale bar = 1 mm). (ii) Coronal section 1 mm rostral to the injection site (scale bar = 1 mm). (iii) Magnified view of the boxed area in (ii), which shows dense fibers throughout layer 1b of piriform, as we observe with the dual vector method for channelrhodopsin-expression (scale bar = 50 μm).

(B) Coronal section at the site of a poorly targeted injection. (i) Here, the vector was delivered just dorsal to piriform, with infection of overlying cortical structures, but no infection of piriform (scale bar = 1 mm). (ii) Section rostral to injection site (scale bar = 1 mm). (iii) Magnified view of the boxed area in (Bii), which shows no fibers in piriform as observed in (Aiii). This indicates that fibers observed throughout piriform in well-targeted injections are not due to projections from spuriously infected cells in the overlying cortex.
Figure 3

A

B

Bi

Bii

C

D

E

ChR+

ChR−

Fluorescence (norm.)

Fraction ChR+

Amplitude (nA)

Distance (µm)

Distance (µm)

Distance (µm)

Distance (µm)
Figure 3. Recording from ChR-positive cells confirms robust expression in a limited subset of piriform neurons.

(A) Parasagittal section of mouse brain injected with lentivirus and AAV. Typically, infection was limited to a single 350-μm section per animal (top, scale bar = 1 mm), and to a single intensely YFP-fluorescent area within that section (bottom, scale bar = 500 μm).

(B) Differential interference contrast (DIC) image with overlay of the YFP fluorescence (scale bar = 100 μm). (i) Fluorescent cloud is visible under epifluorescent illumination of the YFP, with a well-defined point of maximum fluorescence intensity in layer 2 (ii) Fluorescence intensity profile across the dotted line in (Bi) that shows the point of maximal intensity at zero microns.

(C) Whole-cell recordings from neurons within the fluorescent cloud. Illumination with 500-ms light pulse (blue bar) produces characteristic inward photocurrents in ChR-positive cells (top trace), while ChR-negative cells do not respond to illumination. These two cells were <50 μm apart, and recordings were obtained in the presence of NBQX, AP5, SR95531, TTX, and 4-AP to isolate photocurrents.

(D) Probability of recording from a ChR+ cell as function of distance, fit by a normal distribution (width ± S.D., 368 μm ± 20.2 μm; n=166 cells from 11 slices). The origin (Δx = 0) is defined as point of maximal fluorescence intensity as in (B).

(E) Size of sustained photo-activated currents from 22 cells from one slice as a function of distance from site of infection (200-μm bins).
Figure 4

A

B

cell attached

20 pA

C

whole cell

300 pA

300 ms

D

Photocurrent (nA)

0.0

0.5

1.0

1.5

2.0

2.5

0.0

0.5

1.0

1.5

2.0

2.5
Figure 4. Targeted patch clamp recordings from YFP+ neurons.

(A) YFP-expressing cells (i) could occasionally be identified under epifluorescence (FITC) at the edge of the intensely fluorescent cloud (scale bars = 10 μm). Differential interference contrast (ii), intracellular Alexa 594 cadaverine dye (iii), and the merged image (iv) confirm that we are recording from the YFP+ cell.

(B) Cell-attached recording from a YFP+ neuron. Weak (25 μW) light pulses reliably evoke firing in YFP+ neurons, indicated by rapid capacitive transients.

(C) Whole-cell recording from the neuron in (B). A 500-ms light pulse (blue bar) produces a rapid inward current followed by a characteristic steady-state photocurrent. Note that the rapid component was so large that a transient current associated with an un-clamped action potential is evident (not included in determination of average).

(D) Plot of amplitude of all photocurrents recorded in YFP+ neurons (1.52 ± 0.51 pA, mean ± S.D., n=14). Amplitude is measured as the peak inward current during the early rapid phase of the photocurrent.
Figure 5. Recording light-evoked synaptic responses within piriform pyramidal neurons.

(A) Recording from a piriform cortex neurons distant (~1 mm) from the infection site. Image (i) shows location of recording pipette and cell relative to infection site (scale bar = 100 μm). Recorded cells were located within layer 2, which was identifiable under DIC (ii). Cells were confirmed to be pyramidal cells during experiment by filling with Alexa Fluor 594 intracellular dye (iii, scale bar = 10 μm).

(B) Final confirmation of cellular identity through post-hoc staining of intracellularly loaded biocytin.

(C) Voltage clamp recording from a ChR-negative neuron far from site of infection (Δx, 1260 μm). A 500-ms light pulse (blue bar) evokes a rapid inward current, but no sustained, steady state photocurrent (top). Stimulation with a 2-ms light pulse results in a rapid, transient inward current (middle). Addition of blockers of excitatory synaptic transmission (NBQX and AP5) to the bath eliminates the light response.

(D) Overlay of 10 independent trials (grey) of photostimulation to a single cell. The black trace is the average of these 10 responses. Light pulses were delivered every 20 seconds.

(E) Plots of the latency (time from stimulus onset to 5% rise) and jitter (standard deviation of trial-to-trial latency) for 45 piriform neurons. Median latency: 2.74 ms (range 1.8-3.9ms). Median jitter: 0.11 ms (range 0.04-0.42 ms). Boxes: 25th/75th quartiles; whiskers: 10th/90th percentiles.
Figure 6

A

LOT

PIR

50 ms

B

LOT

PIR

10 ms

Ci

ii

iii

PPR

0 200 400 600

ISI (ms)

 LOT

 PRI 

 + Baclofen

 + CGP55845

 PIR

 LOT

 ▪ PIR

 ○ LOT
Figure 6. Light-evoked recurrent synaptic responses have properties to similar to electrically activated “associational” synapses.

(A) Voltage-clamp recordings of a piriform pyramidal neuron. When voltage is held at -70 mV, electrical stimulation or light pulses evoke negative, inward currents of similar magnitude in the LOT or piriform recurrent (PIR) pathways, respectively. At +50 mV, and in the presence of SR95331 to block fast GABAergic inhibition, stimulation evokes outward, positive currents with slower kinetics, consistent with NMDA-receptor-mediated current. More NMDA current is evoked stimulation of piriform recurrent synapses.

(B) LOT (left) and piriform (right) responses at -70 mV. Application of the GABA<sub>B</sub>-receptor agonist baclofen (red trace) reduces the light-evoked inward current, but not the LOT response. Addition of CGP55845 rescues the inward current through blockade of baclofen-activated GABA<sub>B</sub> receptors.

(C) Short-term plasticity at LOT and piriform recurrent synapses. (i) Responses to paired LOT pulses at 50-, 100-, and 200-ms intervals. (ii) Responses in the same neuron to light pulses at similar intervals. (iii) Plot of paired-pulse ratio (PPR), the ratio of amplitude of the second pulse to the first for LOT and piriform responses. LOT synapses facilitate significantly, while piriform recurrent synapses do not appreciably facilitate or depress.
Figure 7

A

B

C

D

E

F

G

EPSCs

Response
probability

Distance, ∆x (µm)

1.0

0.0

0

1000

2000

EPSC amplitude (pA)

EPSC amplitude (pA)

Latency (ms)

Jitter (ms)

λ = 3.6 mm

λ > 3 mm

λ = 1.6 mm

248 µm 700 µm 1242 µm

1 nA 100 ms

381 µm 645 µm 1808 µm

500 pA 100 ms

0 1000 2000

0 1000 2000

0 1000 2000

0 1000 2000

0 1000 2000

posterior piriform anterior

> 3 mm

TTX + 4-AP

TTX + 4-AP

TTX + 4-AP

TTX + 4-AP

TTX + 4-AP

TTX + 4-AP

EPSC amplitude (pA)

EPSC amplitude (pA)

EPSC amplitude (pA)

EPSC amplitude (pA)

EPSC amplitude (pA)

EPSC amplitude (pA)
Figure 7. Recurrent excitatory synapses extend undiminished across piriform cortex.

(A) Focal ChR expression in layer 2/3 neurons in an acute brain slice. DIC image with fluorescent-image overlay. Recording sites are measured relative to the point of maximal fluorescence intensity (Δx = 0), here indicated by the asterisk (scale bar = 200 μm).

(B) Fraction of ChR-negative neurons exhibiting a light-evoked EPSC as a function of distance from the site of infection. Solid line, linear fit. Dotted line is the probability of recording a ChR+ cell as a function of distance (directly from Figure 2D, provided for reference).

(C) Top, representative EPSC recordings from neurons at different positions in a single slice (V<sub>hold</sub> = -70 mV). Bottom, plot indicates EPSC amplitudes from 16 cells recorded at different Δx in one slice. EPSC amplitudes from all cells (≥ 5 cells/slice) were scaled to the largest response, and a single exponential fit with a length constant (λ) was forced to the data (here, λ = 1.6 mm). with an imposed ceiling of λ = 3 mm.

(D) Slice in which EPSC amplitude does not attenuate with distance. Top, representative EPSC recordings at indicated positions. Bottom, plot of EPSC amplitudes of 11 cells with distance in one slice. There is no attenuation with distance on the experimental scale, and λ > 3 mm.

(E) EPSC latency (top) and jitter (bottom) do not vary with distance from injection site (n=45 cells, 6 animals).

(F) EPSC recordings of 9 cells at different positions in a single slice, in the presence of blockers of action potential transmission, TTX and 4-AP (λ = 3.6 mm).

(G) Expression of ChR in the posterior piriform, and EPSC recordings from neurons at various distances strictly rostral to the injection site, toward anterior piriform (n=9 cells). Line is linear fit (slope = 0.17) to highlight trend.
Figure 8

Visual cortex (V1)

Barrel cortex (S1)

A

241 µm 651 µm 1152 µm

100 pA

100 ms

λ = 165 µm

B

154 µm 288 µm 523 µm

1 nA

200 ms

λ = 117 µm

Ai

Amplitude (norm.)

Δx (µm)

Bi

Amplitude (norm.)

Δx (µm)

Aii

λ = 165 µm

Bii

λ = 117 µm

C

V1

S1

Amplitude (norm.)

Δx (µm)

D

λ (mm)

V1

S1

Δx (µm)
Figure 8. Recurrent excitatory responses are not spatially extensive in other primary sensory cortices.

(A) Voltage clamp recordings of light-evoked EPSCs in primary visual cortex. Inset, coronal brain section illustrating dual-vector injection site within V1 visual cortex. (i) Representative EPSCs recorded at various distances from the injection center in visual cortex. Note the train of EPSCs following the initial EPSC at 241 μm, potentially due to reverberant local recurrent activity. (ii) Plot of EPSC amplitude with distance for 6 cells in single slice. Line is a single exponential fit to the data, $\lambda = 165 \mu m$.

(B) Recordings of light-evoked EPSCs in the barrel field of somatosensory cortex. (i) EPSCs recorded at various distance from the injection center in S1. (ii) Plot of EPSC amplitude with distance for 8 cells in a single slice of S1. Exponential fit, $\lambda = 117 \mu m$.

(C) Summary of spatial dependence of EPSCs in visual cortex (left) and somatosensory cortex (right). Single exponential fits for independent experiments are plotted together (V1, 4 slices/4 animals; S1 5 slices/5 animals). Each color represents a different slice/experiment.

(D) Spatial decay constants ($\lambda$) for piriform, visual, and somatosensory cortices. Piriform cortex $\lambda$ values greater than 3 mm are clustered separately. Open circles: $\lambda$s for each slice; red circles, $\lambda$s measured in TTX/4-AP; filled circles: mean ± S.D.; piriform, 1.94 ± 0.90 mm, n=6; V1, 0.144 ± 0.052 mm n=4; S1, 0.255 ± 0.226 mm, n=5.
Figure 9
Figure 9. Piriform recurrent excitatory connectivity is sparse.

(A) Variability of light-evoked EPSC amplitudes at sites far from viral infection. Left: sequential recording from three cells; Δx: top, 910 mm; middle, 702 mm; bottom, 692 mm. Right: distribution of saturating, light-evoked EPSC amplitudes recorded from 95 cells in 11 slices (one slice per animal).

(B) Light-evoked unitary EPSCs (uEPSCs) recorded at -70 mV in ChR-negative layer 2 pyramidal cells. Left: examples of responses and failures from two cells that were evoked with low-intensity focal light pulses distant from the recorded cell (range: 245-408 μm), showing “all-or-none” responses, presumably caused by threshold firing of single ChR+ axonal inputs. Right: individual uEPSC amplitudes (open circles) and mean uEPSC amplitude ± S.D. (filled circle, 36.2 ± 20.3, n=11 cells).
Figure 10

Ai

PIR

LOT

Aii

Aiii

Aiv

Bi

Bii

Piriform

TTX
4-AP
SR-95331
Figure 10. Quantification of piriform recurrent synaptic release sites.

(Ai) Quantal EPSCs (qEPSCs) evoked by light (blue boxes) or LOT stimulation (red boxes) when extracellular Ca\(^{2+}\) was replaced with Sr\(^{2+}\). Insets at an expanded scale correspond to boxed regions in the upper trace. Asterisks (*) indicate qEPSCs. (ii) Top: 50 individual traces (gray) and ensemble average (blue) of quantal events evoked by the light pulse. Bottom: the distribution of qEPSC amplitudes (filled blue bars) and noise (open bars) in this cell. (iii) Same as (ii), but for events following electrical stimulation of the LOT (red). (iv) Inset: normalized average qEPSCs from light-evoked (blue trace) and LOT-evoked (red trace) stimuli. Mean amplitude of light-evoked (n = 11 cells) and LOT-evoked (n = 9 cells) qEPSCs for each cell (open circles) and for the population (filled circles). Error bars indicate S.D.

(B) Spontaneous miniature EPSCs (mEPSCs) recorded in piriform neurons, in the presence of TTX/4-AP and SR-95331. (i) Histogram of mEPSC amplitudes (bin size 2.5 pA). (ii) Box and whiskers plot of spontaneous mESPCs; median mEPSC 15.7 pA (range 5.7 to 45.8 pA). Box: 25\(^{th}\)/75\(^{th}\) percentiles; whiskers: 10\(^{th}\)/90\(^{th}\) percentiles.
Figure 11

A

B

C

D

No. ChR+ cells
200

0

Cells/slice

Density 10^3 cells/mm^3

0

200

400

1000

500

0

-500

-1000

x direction (µm)

y direction (µm)

No. ChR+ cells

100

50

0

-1000

-500

0

500

1000
Figure 11. Estimating the number of ChR-positive piriform pyramidal neurons.

(A) Nissl-stained section (gray-scale image) of anterior piriform cortex (scale = 50 μm).

(B) Inverted, contrast-enhanced area from image (A) used to count individual layer 2 nuclei (red circles).

(C) Number of counted cells (mean ± S.D., 166 ± 20) and extrapolated density of cells (342 ± 45 x10^3 cells/mm^3; 18 sections from 2 animals).

(D) Distribution of ChR+ excitatory neurons estimated from the total density of layer 2 neurons and the probability of patching a ChR+ cell at a given distance from the injection center.
Figure 12

A

Δx = 250 µm
(in NBQX/AP5)

+5 mV

+SR95331

B

Δx = 1260 µm

-70 mV

+5 mV

+NBQX/AP5

C

i. -70 mV ii. +5 mV iii. CNQX/AP5 iv. wash v. SR95331

D

○ Direct IPSC (CNQX/AP5)
● Disynaptic IPSC

--- ChR+ Responses

E

Inhibitory conductance (nS)

Excitatory conductance (nS)

F

500 pA

30 ms

+5 mV

-80 mV

Excitation

Inhibition

Norm. charge

Stimulus Strength (µW)
Figure 12. Recurrent excitation drives local strong, scaled inhibition.

(A) A light-activated IPSC recorded at +5 mV from a ChR-negative layer 2 pyramidal cell near the site of infection (Δx, 250 µm). Responses were blocked by SR but not NBQX/AP5, indicating IPSCs were caused by direct inhibitory input from ChR+ GABAergic interneurons.

(B) EPSCs (recorded at -70 mV) and IPSCs (recorded at +5 mV) recorded in a pyramidal cell far from site of infection (Δx = 1260 µm). Blue bar, 2-ms light pulse. Both responses were blocked by NBQX/AP5 (overlaid red traces at -70 mV and +5 mV), indicating that these synaptic IPSCs were evoked by activating excitatory ChR+ axons that, in turn, recruited ChR-negative interneurons. Inset: Individual traces at an expanded scale showing that EPSCs consistently preceded IPSCs.

(C) Time course of pharmacologic dissection of synaptic inhibition with synaptic responses (top) and amplitude vs. time (bottom). Note recovery of IPSCs after washing CNQX/AP5.

(D) Probability of observing direct (open circles, sigmoid fit) or disynaptic (filled circles, linear fit) IPSCs as a function of distance from site of infection. Dashed line, distribution of ChR+ neurons from (1E). Indirect inhibition at short distances was disambiguated from direct inhibition in the same cell by determination of the IPSC component sensitive to NBQX/AP5.

(E) Relationship of excitatory (-70 mV) and disynaptic inhibitory (+5 mV) responses in each cell. Graph shows peak conductance (slope, 2.45; r = 0.55). Dashed line, relation if inhibitory and excitatory conductances were equal. All cells recorded at Δx > 800 µm.

(F) EPSCs and disynaptic IPSCs evoked in a cell following 2-ms light pulses at graded intensities. Summary input/output relationship of EPSQs and IPSQs (n=11) showing that inhibition scales with excitation. Responses are normalized to those at highest light intensity.
Figure 13

Ai

Wide-field

Δx

Focal

B

TTX / 4-AP

Soma

Focal

200 µm

400 µm

50 pA

100 ms

Ci

Focal

+200 µm

+50 µm

-50 µm

-200 µm

+500 pA

100 ms

ii

Fractional response

Wide-field

Focal

0 µm

100 µm

400 pA

250 ms

iii

1.0

0.8

0.6

0.4

0.2

0.0

Fractional response

Wide-field

Focal

0

Δx (µm)

1000

iv

+TTX/4-AP

-200 µm

+50 µm

+TTX/4-AP

Δx < 300 µm

+TTX/4-AP

Δx > 800 µm

Norm. charge

Distance (µm)

Norm. charge

Norm. charge

Norm. charge
Figure 13. Focal illumination indicates laminar organization of excitatory and inhibitory inputs.

(A) (i) Strategy for determining size of the excitation field. (ii) The amplitude of the isolated photocurrent in ChR+ cells was measured following light pulses presented at different distances from the soma along layer 2, perpendicular to the apical basal axis of the cell. Wide-field or focal light pulses were evoked with an aperture in the light path either open (solid line) or closed (dashed line), respectively. (iii) Photocurrent amplitudes as a function of light distance from the soma with wide-field illumination (filled circle, n=5) or focal illumination (open circle, n=5). The size of the full-field response was approximated by a normal distribution (width: 370 µm). The distance-dependence of the photocurrent amplitude was fit to a single exponential (87 µm).

(B) In the presence of TTX/4-AP and with focal illumination, EPSCs are evoked with the objective over the soma (left trace), but not at 200 µm (middle trace), or 400 µm from the cell.

(C) (i) Relative EPSC amplitude evoked by focal illumination along the apical-basal axis of the cell to determine site of innervation of ChR+ synapses (soma = 0 µm, dashed line). Positive values are toward the pia, or the apical field of the neuron. Negative values are toward the basal field. (ii) Normalized EPSC amplitudes following viral expression of ChR in either piriform (circles; n=10) or olfactory bulb mitral cells (shaded area, n=2) recorded at -70 mV in SR, TTX and 4-AP. (iii) Site of inhibitory connections was determined by recording responses close to the center of infection (Δx < 400 µm) at +5 mV in NBQX, AP5, TTX and 4-AP. (iv) Locus of feedback inhibition, evoked by photo-activation of recurrent collaterals, was determined by recording at +5 mV in cells far from the infection site (Δx > 800 µm) in the absence of any receptor antagonists or channel blockers.
Figure 14

A

LOT PIR
-80 mV

Trial

10

0

B

LOT PIR
-80 mV

Trial

20

0

C

Δt: ±100 ms

Δt (ms)

LOT PIR
-200 0 200

Spike probability

0

1

D

LOT PIR
-70 mV

Trial

200

0

E

LOT PIR
-79 mV

Control +SR95531 Difference

10 mv 100 ms

F

LOT PIR
-70 mV

Control +SR

10 mv 200 ms
Figure 14. Feedback inhibition tunes coincidence detection.

(A) Current-clamp recordings in a ChR-negative pyramidal cell following subthreshold trains (5 pulses at 40 Hz) of electrical stimulation to LOT (arrowheads) to evoke EPSPs from bulbar inputs or light pulses (blue bars) to evoke recurrent piriform inputs. Top: single, truncated traces; bottom: raster plots. Stimulus trains were presented alone (left two trains) and together (right). Coincident stimulation in both pathways reliably drove spiking (right).

(B) Left: presentation of the LOT stimulus train 100 ms before the piriform train. Right: presentation of the piriform train 100 ms before the LOT stimulus train.

(C) Probability of evoking a spike for unpaired LOT and piriform stimuli (left), and with pairing at different intervals (right, $\Delta t =$ onset of piriform - onset of LOT). Experiments were performed under control conditions (grey bars, n=6) or with inhibition blocked (red bars, n=4). Line above plot highlights dramatic difference between pairing at $\Delta t = -100$ ms vs. +100 ms.

(D) Stronger LOT stimuli evoked spikes on 56% of trials when presented alone, but spiking was suppressed by preceding, subthreshold piriform stimulation ($\Delta t = 100$ ms; n=6). Data shown as raw (left, paired t-test, $p = 0.017$) and normalized to unpaired LOT response (right, $p=0.022$).

(E) Left: EPSPs evoked from LOT stimulation or light pulses before (black traces) and after (red traces) SR-95531 application. Right: ratio of the integrated EPSPs (300 ms) with and without SR-95531. Open circles represent recordings from each cell; filled circles are the average across cells, showing greater suppression by feedback inhibition (n=5; p = 0.026).

(F) Recording showing response to same LOT and piriform stimuli before and after blocking inhibition with SR-95531. Responses were compared at the lowest stimulus intensity that reliably drove spiking when inhibition was blocked.
Figure 15

A

NpHR+

NpHR–

B

LOT

C

550 µA

580 µA

600 µA

D

+ Baclofen

650 µA

E

Amplitude (Paired/Unpaired)
Figure 15. Inhibition restricts diffuse excitation and epileptiform discharges.

(A) Outward photocurrents recorded in cells expressing light-activated chloride pump halorhodopsin (NpHR+, top trace), but not in neighboring NpHR-negative cells (bottom trace).

(B) Experimental configuration: recordings from layer 2 pyramidal cells following electrical LOT stimulation, and with yellow light (590 nm) illumination of a remote (>300 µm) part of piriform cortex expressing NpHR.

(C) Example of individual current clamp recordings in one cell following LOT stimulation at different intensities. Control trials (left) were interleaved with trials in which LOT stimulation was presented with yellow light (yellow bar, right). At increasing LOT stimulation intensities, epileptiform bursts of activity were observed, but these were suppressed by NpHR3.

(D) These bursts were sensitive to baclofen, and responses with and without NpHR3 activation were identical.

(E) Summary showing the ratio of the light-paired and unpaired responses to weak (subthreshold for epileptiform bursts, <550 µA) and strong (supratreshold for epileptiform bursts, >550 µA) LOT stimulation. With inhibition intact (open circles, n=5) suppression of recurrent inputs had little effect on excitation to weak or strong LOT stimulation. With fast inhibition blocked (SR-95531, yellow circles, n=6) strong LOT stimulation recruited recurrent inputs that could be suppressed by NpHR3, but not in the presence of baclofen (n=3, not shown).
Figure 16. Focal expression of channelrhodopsin within the anterior olfactory nucleus.

(A) Left: viral vectors used for strong, localized expression of ChR in the AON. Strategy was identical to that used for piriform injections: lentivirus for Cre recombinase expression in a sparse, focal set of cells, and AAV for expression of Cre-dependent ChR-YFP. Right: injections were targeted to the center of AON within olfactory peduncle.

(B) Parasagittal section showing ChR-YFP expression well localized to the AON (scale bar = 1 mm).

(C) Medial-lateral series of parasagittal sections from an injected brain. YFP+ axons course from the AON medially to the piriform ventrolaterally. Axon density tapers sharply after anterior piriform, and these axons do not densely innervate the posterior piriform (scale bar = 1 mm).

(D) Laminar distribution of AON afferents. (i) Magnified view (rotated 90°) of the boxed area in (C), of YFP+ axons in the anterior piriform cortex (scale bar = 100 μm). (ii) Binary mask of the fluorescence pattern in (D) to highlight the laminar distribution of AON fibers in piriform. Fibers are densest in layer 1b, with considerable staining in layer 1a (though not LOT) and layer 3.

(E) Verification of channelrhodopsin expression in AON neurons. (i) Cell-attached voltage-clamp recording from a YFP+ neuron within the AON. Trains of brief (2 ms) light pulses at 20 Hz drive robust firing in these neurons. (ii, left) Whole-cell voltage clamp recording from the cell in (i). A 500-ms light pulse evokes a characteristic ChR photocurrent. (ii, right) Summary of photocurrent amplitudes (early fast component) recorded in AON neurons (1.81 ± 0.86 nA, mean ± S.D., n=7).
Figure 17. AON afferent synaptic responses recorded in piriform neurons.

(A) Epifluorescence image of an acute brain slice, showing patched, dye-filled piriform neurons (red) among layer 1b axon (green) originating in the AON (scale bar = 100 μm).

(B) Left: Voltage-clamp recordings (-70 mV) from piriform pyramidal neuron and responses to brief (2 ms) light pulses. Twelve consecutive stimulation trials are overlaid (gray traces), with the average response (black trace). Right: plot of latency and jitter for 7 responses. Median latency: 3.0 ms (range 2.4 - 4.6); median jitter: 0.16 ms (range 0.07 - 0.67).

(C) Pair of 2-ms light pulses to stimulate AON-to-piriform synapses in the absence (top) and presence (bottom) of blockers of excitatory synaptic transmission CNQX and AP5.

(D) Distribution of maximal AON inputs to piriform neurons. (i) Synaptic responses within three different neurons to show distribution of total EPSC size in these neurons. Peak (negative) amplitudes: top, 11.2 pA; middle, 65.9 pA; bottom, 788 pA. Right: histogram of maximum EPSC responses recorded in 66 piriform neurons. Median response: 47.1 pA (range 4.51 - 786); mean response 96.2 ± 146 (±S.D.). Inset: fraction of recorded neurons that had any magnitude of response.

(E) Unitary responses to AON inputs onto piriform cells. Axons were stimulated with illumination at a site distant (~400 μm) from recorded cells. Left: representative overlay of 20 trials, in which response failures occurred on approximately 50% of the trials. Right: plot of unitary responses in 9 cells (14.9 ± 5.0 pA, mean ± S.D.).

(F) Maximal EPSC responses to AON inputs within anterior piriform as a function of distance from the AON-piriform junction (pooled data from 3 slices/animals). There was no obvious distance dependence of response amplitude within the anterior piriform cortex.
Figure 18

Ai

- AON
- PIR
- +5 mV
- -70 mV
- 50 pA
- 2 ms

Aii

- Latency (ms)
- Jitter (ms)
- 0 0.2 0.4 0.6 0.8 1.0
- 0 2 4 6 8

B

- Control
- CNQX/AP5
- +5 mV
- -70 mV
- 100 pA
- 100 ms
- 200 pA
- 25 ms

C

- Inhibitory conductance (nS)
- Excitatory conductance (nS)
- 0 5 10 15
- 0 2 4 6
Figure 18. AON inputs activate disynaptic inhibition onto piriform neurons.

(A) Voltage clamp recordings (i) of piriform neurons at -70 mV (bottom) and +5 mV (top) in response to AON stimulation; overlay of 8 trials at each holding voltage. Black traces are mean responses. Inset: magnified timescale of the responses at -70 mV and +5 mV. (ii) Plot of latency and jitter for responses recorded at +5 mV. Median latency: 4.0 ms (range 2.1 - 7.3); median jitter: 0.25 (range 0.07 - 0.89).

(B) Response to a pair of light pulses under control conditions at -70 mV (top trace), and responses at +5 mV in the presence of blockers of synaptic transmission, CNQX and AP5. Responses return after washing out of drugs (bottom trace).

(C) Left: maximal EPSCs (-80 mV) and IPSCs (+5 mV) in a single cell. Right: total inhibitory conductance versus total excitatory conductance in each cell. Linear fit: conductance slope = 0.16 ± 0.07, $R^2 = 0.15$, dashed line; n=29).
Figure 19

A

extensive infection

CAG GFPChR2
AAV1

or

AAV9
CaMKII GFPChR2

B

C

D

1 nA
100 ms

E

+5 mV
-80 mV

500 pA
50 ms

F

+5 mV
-80 mV

500 pA
10 ms

103
Figure 19. Extensive expression of ChR in the AON does not increase disynaptic inhibition in piriform.

(A) Strategy for extensive expression of channelrhodopsin in AON neurons. Animals were injected with either (top) AAV1 CMV early enhancer/chicken β-actin promoter driven ChR-GFP, or (bottom) AAV9 CaMKII driven ChR-GFP. Both are strong promoters, and the CaMKII limits expression to glutamatergic projection neurons.

(B) Parasagittal section through the AON of an animal injected with AAV9-CaMKII-ChR-YFP vector. ChR is expressed throughout greater extent of AON than for focal method (scale bar = 500 μm).

(C) Tissue section through the olfactory bulb adjacent to the AON. The AAV9 serotype has limited/no tropism for olfactory bulb projection neurons. Only centrifugal fibers within the granule cell layer are visible – no YFP+ mitral or tufted cells were observed (scale bar = 100 μm).

(D) Extensive infection and ChR-expression in the AON leads to larger recorded EPSCs in piriform neurons. (i) Two examples of large AON EPSCs in piriform neurons. (ii) Histogram of maximal EPSCs recorded in piriform neurons after extensive AON infection; median EPSC: 139.9 pA (range: 4.5 pA to 1508 pA, n = 47).

(E) Left: Recording of EPSCs (-70 mV) and IPSCs (+5 mV) in piriform neurons in response to AON stimulation. Right: Relationship of excitatory (-70 mV) and disynaptic inhibitory (+5 mV) responses in each cell. Graph shows peak conductance (slope, 0.21 ± 0.14; R² = 0.27; dashed line, open circles) charge transfer (measured in 50 ms after light pulses; slope, 0.36 ± 0.23; R² = 0.13; solid line, filled circles).

(F) Determining the input-output relationship of AON-drive disynaptic inhibition. Left: EPSCs
(-70 mV) and IPSCs (+5-10 mV) were evoked with increasing light intensities in single neurons (2.9-173 μW). Right: Excitatory (open circles) and inhibitory (closed circles) PSC amplitudes plotted against light stimulus intensity, normalized to the value recorded at maximum intensity.
Figure 20

A. Response probability vs. Ipsilateral EPSC amplitude. Solid circles for Focal, open circles for Extensive.

B. Contralateral EPSC amplitude vs. Ipsilateral EPSC amplitude. Error bars indicate standard deviation.

C. Comparison of $G_{\text{inhibitory}}$ and $G_{\text{excitatory}}$ between Ipsilateral and Contra conditions.
Figure 20. AON disynaptic inhibition is similar in contralateral piriform cortex.

(A) Probability of observing responses in the ipsilateral or contralateral piriform after focal (closed circles) or extensive (open circles) expression of ChR in the AON (ipsi vs. contra, focal: 0.67 vs. 0.36; extensive 1.0 vs. 0.41; n = 3 animals each).

(B) Amplitude of excitation in contralateral vs. ipsilateral piriform for 5 animals. Each point represents average of all ipsilateral and contralateral neurons within a single animal. Note that excitation is typically stronger in ipsilateral piriform than contralateral, except for one animal in which mean EPSC amplitude in contralateral neurons is ~400pA.

(C) Ratio of AON synaptic inhibitory conductance to excitatory conductance for ipsilateral (black bar) and contralateral (white bar) piriform neurons. Data are highly variable, but there is no trend toward differing ipsilateral and contralateral inhibition driven by the AON (p=0.79, unpaired t-test, n=5 neurons).
Figure 21

A

B

C

D

E

F

ASSN

AON

SR95531

-60 mV

-60 mV

-60 mV

-60 mV

-60 mV

-60 mV

108
Figure 21. Functional consequences of weak disynaptic inhibition.

(A) Current-clamp recordings of piriform pyramidal neurons in response to AON stimulation. Left: train of 5 light pulses (5x2ms) at 20 Hz. Right: peak voltage amplitudes were measured for 5-pulse trains in 9 neurons (1st peak 0.98 ± 0.64 mV; 5th peak 1.95 ± 1.0 mV; mean ± S.D.; n=9).

(B) AON EPSP summation can drive piriform neurons to fire action potentials. Left: overlaid trials in which light trains (5x2ms, 20 Hz) drove a piriform neuron to fire (APs in 9/20 trials). Right: raster plot of action potentials in this neuron. Note that firing occurs more frequently late in the train.

(C) Representative traces in which membrane voltage was varied from -60 mV, -70 mV, and -80 mV while delivering a stimulus train. Note increased summation and depolarization at -60 mV.

(D) Left: single light pulses to evoke single AON EPSPs at varying voltages. Right: single pulses of electrical stimulation delivered via a glass microelectrode in layer 1b of piriform, to activate the collective “associational” inputs for comparison.

(E) Quantification of the time course of single EPSPs evoked at different membrane potentials. The time constant (τoff) was measured from a single exponential fit to the decay, or return-to-baseline phase of EPSPs. ASSN (open circles) EPSP time constants are attenuated at depolarized potentials, but AON (closed circles) time constants are consistently increased.

(F) Blockade of inhibition with SR-95531 (green traces) markedly enhances ASSN summation and depolarization, but has little effect on AON EPSPs.
Figure 22

A

B

C

Control AP5

2.5 3.0 3.5 4.0 4.5 5.0

EPSP amplitude (mV)

Control AP5

10 15 20 25 30

Area (mV/s)

4 mV 100 ms

Control AP5 Wash

C

Control AP5 Wash
Figure 22. Boosting of AON EPSP summation with depolarization is NMDA receptor dependent.

(A) AON EPSPs are sensitive to blockade of NMDA receptors. Single AON EPSP in the absence (black trace) and presence (blue trace) of AP5, a blocker of NMDA glutamate receptors, recorded at -60mV.

(B) EPSP amplitudes (left) and decay time constant (middle) and area (right) in the presence/absence of AP5 for 5 neurons (amplitude: control 4.6 ± 0.18 mV, AP5 3.4 ± 0.48 mV; time constant: control 23.3 ± 3.63 ms, AP5 17.2 ± 4.15 ms; area: control 0.17 ± 0.05 mV·s, AP5 0.10 ± 0.03 mV·s; n = 5).

(C) Effect of AP5 on summation of AON EPSPs. Train of light pulses (5x2ms, 50 Hz) under control (left, black trace) and with AP5 (middle, blue trace). Summation returns with wash of AP5 from the bath (right).
Figure 23

A) Schematic diagram of a neuron with apical and basal aspects. The diagram includes a graph showing normalized charge versus distance.

B) Diagram showing a 470-nm laser with scan mirrors. TTX and 4-AP are indicated.

C) Images showing a pattern of Δy (µm) and Δx (µm) with scale bars.

D) Graph showing raw and deconvolved data with a color scale indicating pA levels.

E) Heatmap showing Δy (µm) and Δx (µm) with scaled intensity.

F) Similar heatmap to E with norm. values.
Figure 23. Laminar organization of AON inputs to piriform pyramidal neurons.

(A) Determining the localization of AON inputs onto piriform neurons using the previously employed method of restricting light diameter to a focal ~100 μm spot, and moving the objective along the apical-basal axis in the presence of TTX and 4-AP. Representative traces are shown for EPSCs evoked at 100-μm increments from the soma in either the apical (top) or basal (bottom) directions. Right: summary of responses in 8 neurons. The responses were normalized to the maximal response, and deconvolved according to the activation point spread function previously derived (see Figure 8).

(B) More precise method of determining laminar distribution of AON inputs to piriform cortex. The schematic outlines strategy for laser scanning activation of individual synaptic boutons, in the presence of TTX and 4-AP using a scanning mirror and lens system.

(C) DIC image (10X) showing a typical laser scanning activation preparation. Stage position is adjusted so that the cell is at the center of the scan field. The scan field (blue spots, overlay) is chosen so as to cover the major input layers to the piriform cell (8x11 positions, Δxy 80 μm, scale bar = 100 μm). Bottom: red fluorescent image of Alexa 594 dye-filled cell in (C) to confirm identity and soma position.

(D) Single scan of the cell in (C). Response locations are indicated by their position in the grid. Response amplitude is indicated by color, according to the color bar in upper right. Relative position of cell body is indicated by gray triangle.

(E) Interpolated intensity map of the synaptic inputs in (D). Soma indicated by asterisk.

(F) Average of intensity maps for all neurons tested. Each map was normalized to the maximal response for each neuron, and these were aligned and averaged to produce the map shown (n=12 neurons).
Figure 24. Example of strong excitation by AON input onto piriform basal dendrites.

(A) DIC image of excitation field, stitched from two adjacent 40X images (scale bar = 50 μm).
Blue spots indicate stimulation sites (16x11 positions, 2 separate scans, Δxy = 20 μm) relative to horizontal layers of piriform.

(B) Single scan of the cell in (A). Response locations are indicated by their position in the grid. Response amplitude is indicated by color, according to the color bar in upper right. Relative position of cell body is indicated by gray triangle.

(C) Interpolated intensity map showing areas of greatest activation relative to soma. Dashed line indicates artifact discontinuity where two maps (corresponding to two fields, but same scale) are joined. Note the ~200 pA response elicited by stimulation at basal dendrites in deep layer 3.
Figure 25

A

AON

Layer 1 IN

B

Cell 1

50 ms

Cell 2

50 ms

Cell 3

50 ms

C

LOT

L1

L2

D

AON

LOT

1 mV

50 ms
Figure 25. Layer 1 interneurons in piriform can be strongly activated by the AON.

(A) Recording from layer 1a (L1a) interneurons within piriform. Responses to stimulation of AON fibers were observed in 6 out of 7 L1a interneurons. Left: DIC image with red fluorescent overlay showing Alexa Fluor 594 fill of an interneuron near the LOT (scale bar = 20 μm). Right: series of fluorescent images of dye filled L1a interneurons that were responsive to activation of AON axons.

(B) Cell-attached recordings of L1a interneurons. Three separate AON neurons that are consistently driven to fire by brief light activation of AON inputs.

(C) Whole-cell voltage clamp recording from cell 1 in (B). Brief (2 ms) light pulses at increasing light intensity until the response in the interneuron is no longer clamped, and an action-potential associated inward current is observed.

(D) Whole-cell current clamp recording of voltage responses to light (i) and LOT electrode stimulation (ii). This L1a interneuron receives input from both the AON and LOT.
Figure 26

Ai

% Responding

Piriform | AON

Aii

EPSC amplitude (pA)

AON | Piriform

Ai

Inhibitory conductance (nS)

G_{inhibitory} / G_{excitatory}

Bi

PIR

AON

Bii

Excitatory conductance (nS)

Biii

unitary EPSC (pA)
Figure 26. Comparison of AON-to-piriform and piriform-to-piriform connections.

(A) (i) Frequency of observing either piriform recurrent responses or AON responses after focal expression of ChR (99 vs. 55%, piriform vs. AON, 95 and 121 neurons, respectively). (ii) EPSC amplitude distributions for AON (grey) and piriform recurrents (white) plotted together. (iii) Bar and whisker plot of the distributions in (ii). Note that the piriform has much larger median response (median 305 pA, range 18.00 - 1688) than AON (median 47.1 pA, range 4.505 - 786.3). (iv) Unitary responses for piriform (36.2 ± 20.4, mean ± S.D., n=12) and AON (14.9 ± 5.0, mean ± S.D., n=9) synapses are significantly different (p = 0.007, unpaired t test).

(B) Comparison of disynaptic inhibition in each pathway. (i) Traces showing the inhibitory current (positive trace) evoked with excitatory current (negative trace) at either piriform-to-piriform (top) or AON-to-piriform synapses (bottom). (ii) Plot of inhibitory conductance vs. excitatory conductance for each recorded neuron. Responses measured after extensive ChR expression in the AON were included. Inset shows smaller data points for easier visualization. (iii) Comparison of ratios of inhibitory conductance to excitatory conductance for piriform (white, 1.9 ± 0.29, mean ± S.D., n=22) and AON (gray, 0.89 ± 0.20, mean ± S.D., n=48). Piriform-to-piriform connections activate significantly greater disynaptic inhibition than the AON-to-piriform (p <0.0001, unpaired t test).
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