Neural circuits of the mouse olfactory cortex: linking neural connectivity to behavior

Inês Vieira

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Neural circuits of the mouse olfactory cortex: linking neural connectivity to behavior

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Thèse de doctorat de neuroscience

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"I learned that courage was not the absence of fear, but the triumph over it. The brave man is not he who does not feel afraid, but he who conquers that fear."

Nelson Mandela

The House of Congress speech, USA, 1989

“...when the brain is released from the constraints of reality, it can generate any sound, image, or smell in its repertoire, sometimes in complex and "impossible" combinations.”

Oliver Sacks

Hallucinations, 1995
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LIST OF ABBREVIATIONS

AAV: Adeno-associated virus
AON: Anterior olfactory nucleus
ArchT: Archaerhodopsin
CAV2-Cre: Canine Adenovirus 2 expressing Cre recombinase
ChR2: Channelrhodopsin 2
CNO: Clozapine N-oxide
CoA: cortical amygdala
CS: conditioned stimulus
DREADDs: designer receptors exclusively activated by designer drugs
EYFP: Enhanced yellow fluorescent protein
fMRI: functional magnetic resonance imaging
GCamp: Genetically encoded calcium indicator
GFP: Green fluorescent protein
GPCRs: G protein–coupled receptors
hM3Dq: human M3 muscarinic DREADD receptor coupled to Gq signaling pathway
hM4Di: human M4 muscarinic DREADD receptor coupled to Gi signaling pathway
IC: insular cortex
ITI: Intertrial interval
lENT: lateral entorhinal cortex
LOT: Lateral Olfactory tract
LV: Lentivirus
M/T cells: Mitral and tufted cells
mPFC: medial Prefrontal Cortex
NpHR: Halorhodopsin
OB: Olfactory bulb
OR: Odorant receptor
OSNs: Olfactory sensory neurons
OT: Olfactory tubercle
PV: Parvalbumin
SST: Somatostatin
US: unconditioned stimulus
VIP: Vasoactive intestinal polypeptide
RÉSUMÉ

Comment les odeurs contrôlent-elles le comportement animal et comment l'expérience passée change-t-elle la perception des odeurs? Dans ma thèse, j'ai utilisé des manipulations optogénétique et chimiogénétique in vivo de l'activité neurale combinées à des analyses comportementales pour explorer l'organisation de circuits cérébraux impliqués dans des comportements olfactifs chez la souris.

Dans la première partie de la thèse, j'ai mis au point un test de conditionnement aversif olfactif indépendant de l'intensité des odeurs. J'ai démontré que les souris pouvaient généraliser une réponse aversive en présentant différentes concentrations d'odeurs. J'ai ensuite testé si les souris pouvaient apprendre cette tâche en inactivant les interneurones exprimant la parvalbumine dans le cortex olfactif (piriforme). Ces derniers constituent une population cellulaire candidate pour la médiation de l'invariance de la concentration d'odeurs. J'ai trouvé que l'inactivation des cellules PV, par inhibition opto- et chimiogénétique, n'était pas suffisante pour abolir l'aversion aux odeurs acquise, ce qui suggère que des composants de circuits neuronaux supplémentaires contribuent à la perception de l'odeur indépendamment de sa concentration.

Ensuite, j'ai tenté de comprendre la constitution relative des différentes voies neurales du piriforme dans ce comportement d'aversion apprise. À l'aide d'outils génétiques et viraux, j'ai ciblé des sous-populations distinctes de neurones piriformes, et j'ai constaté que l'activité neurale induite par la lumière dans les cellules principales du piriforme, mais pas dans les neurones inhibiteurs, conduit à une réponse comportementale. De plus, j'ai testé si des sous-populations de neurones de projection du piriforme étaient suffisantes pour générer une aversion acquise. J'ai caractérisé des voies de sortie du piriforme distinctes avec des cibles de projection non chevauchantes, et j'ai montré que la photo-stimulation de ces populations active des sous-réseaux distincts de neurones dans le piriforme. Enfin, j'ai constaté que la photo-stimulation des neurones du piriforme, situés dans les couches profondes du cortex piriforme, projetant vers le bulbe olfactif et vers le cortex préfrontal, était suffisante pour supporter le conditionnement aversif. En revanche, les neurones du piriforme qui projettent vers l'amygdale corticale et vers le cortex entorhinal latéral, situés dans les couches superficielles du cortex piriforme, ne conduisent pas à l'aversion acquise dans les mêmes conditions expérimentales.

Ensemble, ces résultats contribuent à mieux comprendre les propriétés fonctionnelles des circuits neuronaux corticaux pour l'olfaction.
SUMMARY

How do odors control animal behavior, and how does past experience change odor perception? In my thesis, I have used in vivo optogenetic and chemogenetic manipulations of neural activity combined with behavioral analyses to explore the organization of brain circuits involved in olfactory behaviors in mice.

In the first part of the thesis, I established an odor intensity-independent olfactory conditioning task. I demonstrated that mice were able to generalize a learned escape behavior across a range of different odor concentrations. I then tested if by silencing Parvalbumin-expressing interneurons in the olfactory (piriform) cortex, a candidate cell population for mediating odor concentration invariance, mice would fail to learn the task. I found that silencing PV cells, using both opto- and chemogenetic inhibition, was not sufficient to abolish learned aversion, suggesting that additional neural circuit components contribute to concentration-invariant odor perception.

Next, I asked whether different piriform neural output pathways differed in their ability to support learned aversion. Using intersectional viral-genetic tools, I targeted distinct subpopulations of piriform neurons, and I found that light-induced neural activity in piriform principle cells, but not in inhibitory neurons, could drive a behavioral response. Furthermore, I tested the sufficiency of subpopulations of piriform projection neurons to drive learned aversion. I characterized distinct piriform output pathways with largely non-overlapping projection targets, and I showed that photostimulation of these distinct populations activated distinct subnetworks of neurons in the piriform. Finally, I found that photostimulation of olfactory bulb- and prefrontal cortex-projecting piriform neurons, located in the deep layers of piriform cortex, was sufficient to support aversive conditioning. In contrast, cortical amygdala- and lateral entorhinal cortex-projecting piriform neurons, located in the superficial layers of piriform cortex, failed to drive learned aversion under the same experimental conditions.

Together, these results provide new insights into the functional properties of cortical neural circuits for olfaction.
1. INTRODUCTION
1.1 Olfaction

1.1.1 Odors, emotions, and behavior

Odors strongly influence our emotions and behaviors. Some odors elicit instinctive responses, which are largely independent of experience and often similar between different individuals. For example, the smell of fire is often perceived as an alarm signal, while the smell of a rose is perceived as pleasant. However, odor perception is also strongly shaped by past experience, and behavioral responses to odors often reflect the learned association of a smell with a particular environment or event. For example, even the smell of perfume, when smelled in a claustrophobic context of a stuck elevator, can be associated with a negative emotional response, anxiety. The smell of the perfume then becomes a conditioned stimulus for that traumatic experience and acquires the ability to elicit anxiety when encountered in the future. On the other hand, the smell of chlorine smelled while enjoying the first dives in the swimming pool on a sunny spring day can bring joy to a gray rainy winter day, even when smelled years later. Such experience-dependent, associative learning can explain how odors can be linked with a wide range of emotions and behaviors.

Animals, like humans, can experience odors in instinctive as well as experience-dependent ways. For mice, for example, the smell of a cat will evoke an instinctive escape behavior. On the other hand, an otherwise neutral odor can be associated with positive and negative experience and elicit an experience-dependent, or learned behavior. Odor memories are very robust and can last for the livetime of an animal.

In my thesis, I will focus on how neural networks in the olfactory cortex can associate an odor stimulus with experience to control behavior.

1.1.2 The organization of the mammalian olfactory system

Odorant molecules bind to and activate odorant receptors in the nasal cavity. Odor-evoked neural activity is transmitted first to the olfactory bulb, and then to several higher olfactory centers in the cortex. Olfactory sensory neurons form synapses onto the distal dendritic tufts of mitral and tufted (M/T) cells in the olfactory bulb. M/T cells are glutamatergic cells and the major output neurons of the olfactory bulb. M/T cell axons converge below the mitral cell layer to form the lateral olfactory tract (LOT), which sends olfactory information to the olfactory cortex (Figure 1).
Figure 1. Schematic of a rat brain exposing the ventral surface. The olfactory bulb (OB) receives inputs from olfactory sensory neurons (OSNs) located in the nasal epithelium. Mitral cells within the OB send their axons via the lateral olfactory tract (LOT) to the anterior and posterior piriform cortex (APC and PPC). rs: rhinal sulcus. OT: olfactory tubercle.

In the olfactory bulb (OB), the distal dendrites of approximately 25-50 M/T cells and the axons of olfactory sensory neurons (OSN) that innervate them form dense regions of neuropil called “glomeruli” (Ma and Shepherd, 2000a). Molecular studies have revealed that OSNs in the nasal epithelium express ~1000 types of G-protein coupled odorant receptors (Buck and Axel, 1991; Chess et al., 1994), and that individual OSNs express only a single type of these receptors. The axons of OSNs expressing the same odorant receptors converge selectively onto only one or two defined glomeruli in the OB (Mombaerts et al., 1996; Vassar et al., 1994). Individual M/T cells project a single dendrite to a single glomerulus. Thus, an individual glomerulus and its contingent of M/T cells is thought to be a functional unit representing a single type of odorant receptor (Figure 2).

In addition to receiving sensory inputs from the olfactory epithelium, neurons in the OB also receive dense feedback excitatory projections from olfactory cortex pyramidal cells (de Olmos et al., 1978; Haberly and Price, 1978; Luskin and Price, 1983). These cortical fibers predominantly terminate in the granule cell layer of the OB, potentially recruiting granule cells to provide inhibition onto M/T cells (Nakashima et al., 1978; Banerjee et al., 2015). Granule cells are the major source of inhibition in the OB. Inhibition from granule cells is proposed to mediate a sharpening of M/T response properties (Gao and Strowbridge, 2009; Balu et al., 2007) contributing to odor-discrimination in behaving animals (Abraham et al., 2010).
**Figure 2. Anatomy of the principal olfactory system.** Olfactory sensory neurons (OSNs) are distributed across the nasal epithelium and express a single odorant receptor (OR). Every OSN expressing a particular OR sends its axon to a spatially stereotyped location on the surface of the olfactory bulb, termed a glomerulus (dashed circles). The bulb contains a number of interneuron types (yellow) including periglomerular and granule cells. Mitral and the more superficial tufted cells (M/T) send their dendrites into a single glomerulus. Their axons fasciculate to form the lateral olfactory tract (LOT), which projects to the olfactory cortex.

### 1.1.3 Odor-evoked neural activity and odor information coding in the olfactory bulb

The patterns of odor-evoked activity in the olfactory bulb accurately reflect the sites of convergence of axons from neurons expressing a specific odorant receptor. Thus, the quality of an olfactory stimulus can be encoded by the specific combination of glomeruli activated by a given odorant in the OB, a model referred to as a “chemotopic spatial map”. M/T cells then process these stimuli and transmit information to higher olfactory centers in the brain.

How can odor concentration changes be detected in the OB? The specificity of odorant-receptor binding depends on the concentration of an odor (Jiang et al., 2015; Malnic et al., 1999), and, as a consequence, patterns of odor-evoked glomerular activity change with changing odorant concentration (Bozza et al., 2004; Meister and Bonhoeffer, 2001). When odorants bind olfactory receptors in OSNs, they initiate a series of intracellular events producing second-messenger signals that gate ion channels. Depending on the input strength, OSNs spike frequency, number of spikes, and latency to first spike are modulated so that weak signals can be detected and strong signals normalized (Klene, 1997).

Odors detected by odorant receptors in OSNs are transformed into spikes, which are relayed to higher brain centers. Spikes are digital signals whose number, timing and distribution across neural assemblies contain all of the stimulus information available to the nervous system. A variety of mechanisms have been proposed for the encoding of odor intensity at different stages in the
olfactory pathway, based on observed changes in neural activity as a function of odorant concentration. The temporal patterns of the output neurons of the OB, M/T cells, depends on odor concentration (Hopfield, 1995; Schaefer and Margrie, 2012). In addition, odors activate different OB glomeruli and M/T cells at specific phases of the respiration cycle. Although some M/T cells respond to odor stimuli with increased excitation at higher odorant concentration, others are inhibited or respond with more complex temporal patterns of mixed excitation and inhibition that may change with concentration. Furthermore, odors elicit sniff-locked rhythmic spike bursting and/or spike inhibition with a diverse phase distribution over the sniff cycle. Cells that display immediate excitatory responses could selectively convey rate codes of intensity. However, cells that are excited by some odorants may be inhibited by other odorants via lateral inhibition, complicating the readout (Rinberg et al., 2006). At higher odor concentration, spike latencies of mitral cell excitatory responses are reduced (Cang and Isaacson, 2003). If the reduction occurred uniformly across inputs, then patterns of relative latency between glomeruli could act as concentration-invariant codes for odor quality (Hopfield, 1995; Schaefer and Margrie, 2012). This latency code mechanism could account for fast discrimination of simple odor pairs in single sniffs of less than 200 ms in duration. However, strong lateral inhibition in response to the activity of OSNs in the presence of different odors can pose a problem for the latency code. Thus, current models to precisely describe how odor concentration is encoded in the OB will require future refinement.

1.1.4 How is odor information transmitted to cortical areas?

The olfactory system is unusual among sensory systems in the brain in that the first stages of information processing bypass the thalamus (Haberly and Price, 1978). The axons of M/T cells project directly to a number of cortical areas including the olfactory tubercle (OT), the anterior olfactory nucleus (AON), the entorhinal cortex (Ent), the prefrontal cortex (PFC), and the cortical amygdala (CoA). The largest olfactory area receiving projections from the OB is called the piriform cortex (primary olfactory cortex), which is a phylogenetically old brain region and part of the paleocortex (Illig and Haberly, 2003; Laurent, 2002). The olfactory cortex is generally believed to be important for the generation of odor perception and memory (Haberly, 2001; Wilson and Stevenson, 2003).

The piriform cortex projects to several areas of the brain such as the olfactory bulb (OB), the anterior olfactory nucleus (AON), the medial prefrontal cortex (mPFC), the olfactory tubercle (OT), the cortical amygdala (CoA), the lateral entorhinal cortex (lEnt) and the agranular insular cortex (IC) (Haberly and Price, 1978; Diodato et al., 2016) (Figure 3).
Figure 3. Association (cortico-cortical) axons from two neighboring superficial pyramidal cells in piriform cortex. The black spot indicates the position of the cell bodies. The circles at top right denote typical diameters of pyramidal cell dendritic trees at the depths where they are contacted by association fibers (proximal apical dendrites in layer Ib and basal dendrites in layer III). The borders of piriform cortex and the insular-perirhinal border are indicated by solid lines; the dashed line outlines the lateral olfactory tract and the dotted line is the rhinal sulcus.

Lesioning piriform cortex in rodents leads to deficits in the learning of odorants and discrimination of complex odor mixtures (Staubli et al., 1987a). Associating the activation of a small ensemble of piriform neurons (300 neurons, correspondent to approximately 0.5% of the number of piriform neurons activated by an odor) of the distributed piriform neurons with aversive or appetitive stimulus can be sufficient to drive an aversive or appetitive behavior (Choi et al., 2011). Thus, patterns of neuronal activity in olfactory cortex are likely to represent odor percepts.

1.1.5 The laminar structure of piriform cortex

The olfactory cortex has simpler laminar architecture than neocortex, with three layers rather than six (Figure 4). In addition, the transcriptional programs that specify olfactory cortex neurons appear to be different from those observed in neocortex. For example, Cux1 is a marker of superficial layers II/III in neocortex, while it delineates deep layers IIIb and III in piriform. In contrast, Fezf2 is a marker of deep layers V and VI in neocortex, while it marks superficial layer Ia cells in piriform. Furthermore, several genes that are expressed in well-segregated neurons in neocortex are co-expressed in subpopulations of piriform cells. For example, Cux1 and Ctip2 are co-expressed in about 30% of piriform layer IIb cells, while they mark distinct, non-overlapping populations of neurons in neocortex layers II/III and V/VI, respectively (Diodato et al., 2016).

Piriform layer I contains dendrites, fiber systems, and GABAergic interneurons. It is subdivided into
a superficial part, layer Ia, which receives dense afferent fibers from the olfactory bulb within the LOT, and a deeper part, layer Ib, which receives recurrent inputs from local excitatory principal neurons in the olfactory cortex as well as from other cortical regions. Layer II/III contains excitatory principal cells with few interneurons (Illig and Haberly, 2003; Suzuki and Bekkers, 2006; Suzuki and Bekkers, 2010a). A high density of associational fibers also makes synaptic contacts within layers II/III. Thus, layer II/III pyramidal cells in piriform cortex receive two distinct classes of glutamatergic synapses: one class conveys primary sensory input, while a different set of associational synapses mediate intra- and intercortical signals. The afferent sensory input from the LOT occurs at synaptic contacts on the distal dendritic tufts of pyramidal cells.

**Figure 4. Piriform cell types and laminar organization.** (a) Major cell types and anatomy of the piriform cortex. Excitatory neurons (semilunar cells (SL), superficial pyramidal neurons (SP) and deep pyramidal neurons (DP)) are colored in green, inhibitory neurons in red. Axons from the LOT are restricted to layer Ia, where they synapse onto pyramidal neurons, semilunar cells and local interneurons such as horizontal cells (HZ) and neurogliaform cells (NG). Interneurons present in layer Ia provide feedforward inhibition to SL/SP cells. Collaterals of SP and SL axons ramify extensively across layers Ib through III. These collaterals excite other SP cells. Feedback inhibition that balances excitation and keeps odor representations sparse is provided mostly by Parvalbumin-expressing neurons (fast spiking multipolar neurons –fMP), Vasoactive intestinal peptide-expressing neurons (bitufted cells) and Somatostatin-expressing neurons (regular spiking multipolar neurons). Dendrites are represented by blue lines, axons by magenta lines.

In vivo patch clamp recordings have shown that piriform neural activity is dominated by intracortical circuits rather than by afferent sensory inputs from the LOT. Excitation of pyramidal neurons is strongly driven by intracortical excitatory connections (Poo and Isaacson, 2011), since pyramidal neurons are interconnected by recurrent excitatory connections that can extend millimeters across the piriform cortex (Franks et al., 2011). A given pyramidal cell could receive 10-fold more intracortical connections than afferent input from the OB. Thus, odor representations in the piriform cortex result from an intracortical balance between recurrent excitation and feedback.
inhibition (Franks et al., 2011a). This circuit organization is thought to provide the neural bases of an autoassociative network for olfactory memory (Haberly and Bower, 1989; Wilson and Sullivan, 2011). In summary, the organization of piriform cortex suggests that it functions to transform incoming sensory inputs into meaningful representation of odor information.

1.1.6 Odor responses in the olfactory cortex

In contrast to the precise spatial odor map of the olfactory bulb, where mitral cells projecting to a single glomerulus represent a single molecular feature of the odorant, in vivo calcium imaging experiments and electrophysiological recordings have found that odor-evoked neural activity is distributed across the piriform cortex without apparent spatial organisation (Duchamp-Viret et al., 1996; Wilson and Yan, 2010; Poo and Isaacson, 2009; Stettler and Axel, 2009; Tantirigama et al., 2017). How are random ensembles of piriform neurons organized in the piriform cortex and how do they encode the identity and intensity of an odor? A substantial fraction of odor-selective piriform neurons exhibit largely concentration-invariant odor responses, and odor identity can accurately be decoded from a subpopulation of piriform neurons (Roland et al., 2017a). Furthermore, using a linear decoder to classify extracellular recordings of single-trial odor responses in piriform, Bolding and Franks observed different piriform coding strategies for odor identity and odor intensity (Bolding and Franks, 2017). The encoding of odor stimuli in the piriform happens in two phases. During the first phase, there is a rapid increase in population spiking, peaking within 50–100 ms after inhalation. During a second phase, response latency is odor concentration-dependent: at low concentrations, the response is slow, but as concentration increases, latency decreases systematically and the two phases of the response become more synchronous. Thus, based on recent optical and electrophysiological recordings, it has been suggested that odor identity is encoded in the spatial patterns of subpopulations of piriform neurons, while temporal features of the odor response may represent odor intensity.

1.1.7 Piriform neural activity and olfactory-driven behaviors: consequences of lesioning the piriform cortex on olfactory behaviors

Based on lesion studies it was suggested that the piriform cortex is necessary for a variety of complex odor-driven behaviors. Odor discrimination or discrimination of a mixture of odors does not appear to be impaired by lesions in the piriform (Zhang et al., 1998; Staubli et al., 1987b; Sacco and Sacchetti, 2010). Piriform lesions do, however, impair olfactory-driven behaviors with higher cognitive demands than odor discrimination alone. For example, piriform lesions result in deficits in an odor delayed non-match-to-sample task that require working memory (Zhang et al., 1998), and in
an impairment in an odor-cued navigation task (Staubli et al., 1987a) that requires working memory and the integration of odor and spatial information. Furthermore, selective lesions of the posterior piriform disrupt the ability to recall remote memories but not recently learned odor-cued fear memories (Sacco and Sacchetti, 2010), suggesting that the posterior piriform might be involved in the long-term storage of odor information. Together, these results suggest that the piriform cortex is involved in tasks that require the integration of olfactory and non-olfactory information.

1.1.8 Experience-dependent plasticity and consequences of artificially activating piriform cortex

It has been hypothesized that piriform circuits are highly plastic according to task demands. By recording cortical activity of the piriform cortex and OB while rats were performing a two-alternative forced choice task it was observed that discrimination of two different mixtures of odors was associated with different stimulus correlation in the piriform ensembles. Learning of a difficult behavioral discrimination task was associated with enhanced stimulus decorrelation (pattern separation) in anterior piriform cortex ensembles (Chapuis and Wilson, 2011) and odor evoked activity in the piriform cortex is decreased following learned aversion (Chen et al., 2011).

Photostimulation of a random ensemble of piriform neurons in the absence of sensory input is sufficient to drive behaviors of different valence: aversive, appetitive and social approach behaviors (Choi et al., 2011).

In humans, using a combination of functional magnetic resonance imaging (fMRI) of the piriform with multivariate analytical techniques it was demonstrated that two initially indistinguishable odor enantiomers become discriminable after an aversive conditioning experience. Thus, the odor discrimination is dependent on associative learning and it is correlated to a change in the spatial representation in the piriform (Li et al., 2008).

Together, these observations suggest that piriform is a highly plastic structure involved in multiple behaviors. However, it remains unknown which piriform neural cell types and pathways are important for driving specific behaviors.

1.2 Experimental approaches to manipulate neural activity

To achieve a deep understanding of neural circuit function it is essential to understand how a distributed neural ensemble of piriform neurons control behavior. Neuroanatomical and neurophysiological studies have provided connectivity maps for brain circuits supporting normal functions. Lesions, electrical stimulation, transient pharmacological manipulations, and genetic tools have generated critical functional insights into how network activity relates to behavior. However,
those tools do not enable for cell type- and pathway- specific activation or silencing of neurons at defined time points.

### 1.2.1 Optogenetics

In a distant field from neuroscience, biologists observed that some microorganisms produce proteins that regulate the flow of electric charge across their membranes in response to visible light. In 1971, Stoeckenius and Osterhelt discovered the bacteriorhodopsins: proteins that act as a single component ion pump that can be activated by photons of green light. The most common opsins used in neuroscience were then identified; halorhodopsins in 1977 and later channelrhodopsins were isolated from the green alga *Chlamydomonas reinhardtii* (Nagel et al., 2002). In 2005, was first tested the introduction of channelrhodopsin in mammalian neurons and their precise response to blue light characterized (Boyden et al., 2005). In the same year, it was demonstrated in Drosophila that it was possible to manipulate behavior by photostimulation of genetically targeted neurons (Lima and Miesenböck, 2005). Nowadays, many optogenetic tools are available to interrogate the functions of genetically defined cell types, with real-time bidirectional control of neuronal activity, and the possibility to manipulate neural circuit elements based on their connectivity.

Optogenetics accomplishes the modulation of neural activity through the expression of light-sensitive ion channels or pumps: when these actuators are expressed in cells of interest, focal light delivery results in neuronal excitation (e.g., channelrhodopsin 2, ChR2) or inhibition (e.g., archaerhodopsin, ArchT, halorhodopsin, NpHR) (Boyden et al., 2005; Fenno et al., 2011). Upon light illumination at different wavelengths such as 480nm, 590nm or 560nm, distinct opsins can be activated. Channelrhodopsin passively transports Na\(^+\), K\(^+\), H\(^+\), Ca\(^{2+}\) down their electrochemical gradients to depolarize neurons. For hyperpolarization, halorhodopsins actively pump Cl\(^-\) into the cell to hyperpolarize neurons, and archaerhodopsins actively pump H\(^+\) out of the cell to hyperpolarize neurons (Hegemman, 2008).

### 1.2.2 Chemogenetics

Chemogenetics is a term that is now used to describe the process by which macromolecules can be engineered to interact with previously unrecognized small molecules. G protein–coupled receptors (GPCRs) and their signal transduction pathways are important for both physiological and pathological processes in neural systems. Many GPCRs coupled to different G proteins have been engineered to regulate receptor functions selectively, and to modulate their signal transduction via drug-like compounds (Roth, 2016).

The first generation of these modified receptors, which includes receptors activated solely by synthetic ligands (RASSLs), therapeutic receptor-effector complexes (TRECs), and neo-receptors,
was created by designed mutagenesis (Pei et al., 2008). However, these engineered GPCRs have potential problems limiting their use in vivo, including for example, high constitutive activity (Conklin et al., 2008; Pei et al., 2008).

In 2008, DREADDs (designer receptors exclusively activated by designer drugs) have been engineered, using a directed molecular evolution strategy in yeast (Armbruster et al., 2007). DREADDs have no constitutive activity, are insensitive to their endogenous ligands, and are exclusively activated by synthetic drug-like compounds. These receptors, commonly referred to as first-generation DREADDs (hM4Di, hM3Dq, Gs-D), evolved from the human muscarinic receptor (Armbruster et al., 2007; Stachniak et al., 2014).

DREADDs no longer respond to their endogenous ligand but, instead, display high affinity for other putatively inert compounds (e.g. clozapine-n-oxide, CNO). Thus, with the DREADD approach, focal or systemic delivery of the DREADD ligand results in neuronal silencing or excitation (Urban and Roth, 2015).

Multiple DREADD actuators are available. The most widely used DREADDs are those based on the human muscarinic receptor (with Gi, Gs, and Gq variants). A DREADD-based option for neuronal silencing (hM4Di), and the equivalent available for neuronal activation (hM3Dq) are both activated by the same ligand, CNO. DREADD-mediated neuronal activation (e.g., through Gq DREADDs) operates through a phospholipase C mechanism. Application of CNO in hM3Dq-expressing mice induces both an increase in presynaptic neurotransmitter release and a modest membrane depolarization (Gomez et al., 2017). Moreover, in slice, activation of Gq-coupled DREADDs induces neurotransmitter release (Buchta et al., 2017). GPCR activation has been linked to pathways mediating cell survival as well as long-term depression and long-term potentiation (Atwood et al., 2014).

1.2.3 Optogenetics versus chemogenetics

A clear benefit of the optogenetic approach is its fast temporal resolution of neuronal control. Even the first generation of optogenetic constructs displayed millisecond-resolution control of neuronal activity (Boyden et al., 2005), with subsequent generations of tools capable of driving neurons at even higher frequencies (Gunaydin et al., 2010; Klapoetke et al., 2014). By contrast, DREADDs do not offer spike-level temporal resolution. Agonist administration results in silencing or activation of neurons for minutes to hours, with the pattern of activation outside of experimental control. If a high degree of temporal control, specific patterns of firing, or rapid switching between activated and inactivated states is required to address a particular question, optogenetics is the method of choice.

DREADD approaches suffer from some of the same limitations as traditional pharmacological approaches. DREADD receptors may desensitize with prolonged activation - GPCR kinase
phosphorylation sites are conserved between muscarinic receptors and DREADDs (Armbruster et al., 2007). While in cases of robust transgene expression, a large pool of receptors is likely to minimize apparent desensitization, in cases where expression is less robust, desensitization may be evident (Roth, 2016). In addition to these pharmacodynamic concerns, DREADD activity depends on the pharmacokinetic properties of the DREADD agonist. Although CNO has been described as pharmacologically inert, recent studies have shown that it can influence amphetamine-induced locomotor activity in rats lacking DREADD receptors (MacLaren et al., 2016), and can partially substitute for clozapine in a drug-discrimination paradigm in both mouse and rat (Porter et al., 2017). Clozapine displays appreciable affinity for native muscarinic receptors, histamine receptors, alpha-adrenergic receptors, and serotonin receptors.

These pharmacokinetic concerns underscore the importance of appropriate controls: it is essential to ensure that the dose of DREADD ligand is without effect on the dependent measure of interest in wild-type (i.e., non–DREADD-expressing) animals. Moreover, development of novel DREADD reagents that avoid desensitization, and/or agonists with desirable pharmacokinetic properties, may address these concerns.

One of the key advantages of both optogenetic and chemogenetic approaches is enhanced spatial control. Because both opsins and DREADDs can be expressed in a cell type–specific manner, targeting small or adjacent structures is achievable with a resolution impossible for conventional drug microinjection. Drug diffusion to adjacent regions, which has been a long-standing concern even in the best-designed microinjection experiments, may be avoided by the genetic targeting of optogenetic or chemogenetic actuators to cells of interest. These approaches have also enabled pathway-specific modulation. By delivering virus to one brain region, and applying light or DREADD agonist to a terminal field, single projection pathways may be modulated.

Most applications of optogenetics and chemogenetics to date have employed relatively focal manipulations. However, targeting multiple components of a distributed network may lead to some challenges. Light distribution in the brain is limited, and thus a relatively small volume of tissue can be activated. In some respects, the enhanced site specificity afforded by limited light distribution (i.e., the region of activated neurons lying underneath the fiber optic) is a strength as it allows for the discrete targeting of adjacent structures. However, because of this limited light distribution, targeting a distributed network would require multiple optic fibers. Importantly, new red-shifted opsins offer superior penetration of brain tissue (Chuong et al., 2014).

Conversely, the chemogenetic approach, which does not require long-term hardware implantation (just viral injection), may be seen as an advantage because it minimizes the complexity of the surgical approach. In addition, one area in which chemogenetics may offer a clear advantage over optogenetics is in scale. Neural silencing of large areas in the mouse brain such as the piriform
cortex, which spans close to two millimeters in length, or brain regions in animals with larger brains than a mouse, presents a challenge for light penetration. Chemogenetics avoids this concern because virus can be delivered to large volumes of tissue and agonist administered systemically. The decision to employ DREADDs or opsins should be tailored to the needs of the scientific question under investigation. If experiments require a high spatiotemporal resolution, optogenetics may be preferable. If experiments require broader spatial coverage or longer-duration activation or silencing, DREADDs may be preferable. Generally, both of these approaches are amenable to activation and silencing, cell type-specific targeting, and pathway-specific manipulations. Both approaches may also be deployed using genetic strategies to isolate populations based on multiple features (INTRSECT) and in an activity-dependent manner (Fos-tagging active cells) (Garner et al., 2012; Liu et al., 2012). However, one must consider for optogenetics the possibility of tissue heating and lesion when stimulating the tissue for long time periods and, since both techniques rely on a viral injection, the variability associated with viral infection of neural tissue. Such inherent variability may be a confounding factor when silencing or activating a certain brain structure in the mouse brain.

For optogenetics, transgenic knock-in mouse lines can overcome limitations linked to an incomplete coverage of neurons within the target region, and the variability in the levels of opsin expression across cells and individual experimental animals. Mouse lines with high-level and Cre-dependent expression of the fluorescently tagged opsins ChR2(H134R)-tdTomato, ChR2(H134R)-EYFP, Arch-EGFP-ER2, or eNpHR3.0-EYFP are now available (Madisen et al., 2012). Together, these techniques have the potential to help us better understand both general network architecture and region- and cell type–specific functions in piriform cortex (Figure 5).

![Diagram](image)

**Figure 5. Main optogenetic and chemogenetic actuators for neural excitation or inhibition.** a) Opsins are membrane-bound proteins that are activated with light, which results in cell activation (depolarization) or inhibition (hyperpolarization). b) DREADDs are a family of designer G-protein-coupled receptors (GPCRs) built specifically for precise control of GPCR signaling pathways for neural excitation or inhibition.

Adapted from Guru et al, 2015
2. EXPERIMENTAL METHODOLOGY
2.1 Mice
Adult (8- to 12-week-old) C57BL6/J male wild-type mice, SST-Cre, PV-Cre, Emx-Cre and Gad2-Cre mice were used in this study. Mice were housed at the animal facility at the CIRB, Collège de France. All experiments were performed according to European and French National institutional animal care guidelines (protocol number B750512/00615.02).

2.2 Immunohistochemistry
Mice were deeply anaesthetized with pentobarbital and transcardially perfused with 20 ml of PBS, followed by 10 ml of 4% paraformaldehyde. Brains were post-fixed for 4 h in 4% paraformaldehyde at 4 °C. Coronal sections (200 µm thick) were prepared using a vibrating-blade microtome (Microm Microtech). Sections were rinsed in PBS and permeabilized in PBS/0.1% Triton X-100 for 1 h, and blocked in PBS/0.1% Triton X-100/2% heat-inactivated horse serum (Sigma) for 1 h. After incubation with primary antibodies at 4 °C overnight, sections were rinsed in PBS/0.1% Triton X-100, three times for 20 min at room temperature, blocked in PBS/0.1% Triton X-100/2% heat-inactivated horse serum for 1 h and incubated with secondary antibodies overnight at 4 °C. The following antibodies were used at the indicated dilutions: rabbit anti-cfos 1:500 (Santa Cruz sc-7270), and chicken anti-GFP 1:1000 (Abcam ab13970), rabbit anti-PV 1:400 (Swant PV25), rabbit anti-dsRed 1:200 (Clontech 632496). Appropriate secondary antibodies (1:1000) conjugated to Cy3 (Jackson Labs) or Alexa 488 (Molecular Probes) were incubated together with Neurotrace counterstain (1:500, Invitrogen). Sections were mounted on SuperFrost Plus (Menzel-Gläser) microscope slides in Fluorescent Vectashield Mounting Medium (Vector Mount). Images were acquired with a Leica SP5 confocal microscope, magnification: 10 or 20x.

2.3 c-Fos stimulation protocol
Animals co injected with CAV2 retrograde virus expressing Cre-recombinase in the region of interest (CAV2-Cre) and conditional AAV vectors carrying channelrhodopsin (ChR2-eYFP) were photostimulated (20Hz, 30sec per minute for 10minutes) an hour before they were perfused (Choe et al., 2015).

2.4 Quantification of c-Fos positive neurons
Immunohistochemistry was performed on piriform sections obtained 0.8–1.2 mm posterior to bregma. The percentage of ChR2+ neurons expressing c-Fos at the center of the injection site was obtained by manual counting. All image processing and quantification was performed in Fiji and Adobe Photoshop CS5. A given field of view was divided into 15 bins, and the fraction of cells in
each bin was calculated as the total number of fos+ cells in each bin divided by the total number of cells in the field of view.

2.5 uDISCO protocol
Mice injected with AAV.DIO.ChR2 and CAV2.Cre were perfused 4 weeks after injection. Brains were then cut into half and uDisco passive clearing protocol was performed (C Pan et al., 2016). All incubation steps were performed in a shaker at 35C. Brains were initially dehydrated in tert-butanol (Sigma-Aldrich) solution at increasing concentrations 30 Vol% (overnight incubation), 50 Vol% (4h incubation), 70 Vol% (4h incubation), 80 Vol% (ON incubation), 90 Vol% (4h incubation), 96 Vol% (4h incubation) and 100 Vol% (4h incubation). Following dehydration, brains were incubated in dichloromethane for delipidation (45min, RT), and finally in BABB (benzyl alcohol + benzyl benzoate 1:2, respectively) with DPE (diphenyl ether/BABB ratio 1:10) and 0.4% Vol vitamin E. Imaging was performed in the ultramicroscope 2 (LaVisionBioTec) in collaboration with François Michel, InMed Imaging Center, Marseille.

2.6 Stereotaxic viral injections and fiber implantation
Mice were anaesthetized intraperitoneally with ketamine/xylazine (100 and 20 mg per kg of body weight, respectively) and prepared for surgery in a stereotactic frame (David Kopf Instruments). For viral injections, a small craniotomy was made above the injection site. For anterograde neural tracing experiments and behavior experiments, 0.7 µl of AAV5.hSyn.hChR2 (H134R)-eYFP.WPRE.hGHpA was stereotaxically injected into the piriform cortex. In the control group, 0.6 µl of AAV1.hSyn.GCamp3-eYFP.WPRE.hGHpA was injected in the piriform cortex. 0.3 µl of canine adenovirus (CAV)-2-Cre was injected into the OB, the CoA, the mPFC or the lEnt. Virus was injected using a glass pipette with a 10–20 µm tip diameter. AAVs were obtained from the U. Penn (Penn Vectors). CAV2-Cre was obtained from the Montpellier Vector Platform (PVM). The following coordinates, based on the Paxinos and Franklin Mouse Brain Atlas were used: piriform cortex: anterior-posterior (AP) -0.60 mm, medio-lateral (ML) 3.95 mm, dorso-ventral (DV) -3.97 mm; OB: AP 0.75 mm and ML -0.75 mm coordinates from the midline rhinal fissure, DV -0.70 mm; medial PFC (including the prelimbic (PrL), infralimbic (IL), and cingulate (Cg) cortex): AP 0.54mm, ML 0.36mm, DV -1.70mm; posteromedial cortical amygdaloid nucleus: AP -2.80, ML 2.76, DV -4.8; lENT: AP -3.90, ML 3.8, DV 2.90.
For behavior experiments, optical fiber implantations were done after viral injection; the skull was cleaned and covered with a layer of Super Bond C and B (Phymep). An optical fiber (200µm, 0.22 NA) housed inside a connectorized implant (SMR, Doric Lenses) was inserted into the brain, with
the fiber tip positioned 200µm above the infection target (piriform cortex). The implants were secured with dental acrylic (Pi-Ku-Plast HP 36, Bredent). Before being moved back to their cages, after the surgery, mice were put in a hot chamber at 37°C until full recovery of their movements.

2.7 Aversive behavior paradigm

2.7.1 Photostimulation protocol

All animals were single-housed and kept on a 12h/12h day/night cycle. Behavioral tests began 13 days post-surgery, and were carried out 6 hr after onset of the day period. Behavioral conditioning took place over 5 days. On the first day, mice were habituated to the fear conditioning box for 40min, on the following day and on the fourth day, the mice were trained to associate the photo stimulation with the foot shock and on the last day mice were tested, 24 hr after the second training session.

The mouse optical fiber implant was connected to a mono fiber patch cord (MFP_200/240/900-0.22_FC-SMC, Doric Lenses) that was connected to a fiber optic rotary joint (FRJ_1x2i_FC-2FC_0.22, Doric Lenses), which receives the light that passes through galvanometric mirrors from the free-space DPSS laser beam (MBL-III-473, CNI lasers). Laser output was maintained at 7-10 mW as measured at the end of the fiber. The conditioning apparatus was a rectangular chamber (9 cm W x 57 cm L x 16 cm H) with a stainless-steel rod floor (custom-made in collaboration with Yves Dupraz from the mechanics workshop of Collège de France). Each half of the conditioning apparatus was connected to an electrically operated switch (custom made in collaboration with Gerard Paresys from the electronic workshop of ENS), which was connected to an aversive stimulator (115 V, 60 Hz, Med Associates) and to a microcontroller board (Mega 2560, Arduino), allowing foot-shock to be applied independently to either side. The testing apparatus was a rectangular chamber (9 cm W x 57 cm L x 16 cm H) with a white PVC floor. Before each training session, laser beam output intensity and shape was adjusted and electrical current in the rod floor was measured (Aversive stimulation current test package, Med Associates). Both training and testing protocols (photo stimulation and foot-shock) were controlled using custom-written Arduino 1.8.3 open software.

Experimental animals were allowed to habituate to the apparatus for 5 min. The conditioning paradigm consisted of 3s of photo stimulation (20 Hz square shaped, 25ms pulses) followed immediately by a 0.5s, 0.65 mA foot-shock. Foot-shock was applied only when the animal was in or near either end of the apparatus, forcing the animal to run to the opposite side. Photo stimulation/shock pairings were spaced 3-4 min apart. Each of the two training sessions consisted of 10 photo stimulation/shock pairings, for a total of 20 pairings. Photo stimulation was applied 7 times over the testing session, every 3-4 min. The sessions were video-recorded.
For experiments in which odorants were involved, an 8-channel olfactometer (Automate Scientific, Berkley, CA) was used to deliver the monomolecular odorants in the conditioning box. The right and left odor port of the fear conditioning box provided the odorants or a continuous air flux at a flow rate of 1L/min in the conditioning box. Compressed air from the wall outlet was controlled using an air pressure regulator (Air Liquide), this air was filtered (disposable filter capsule, Whatman) and delivered to the olfactometer through tygon tubing. The air flux was then split into three streams; one was delivering air to the olfactometer, and the other two streams were providing a continuous air flux to the right odor port and to the left odor port of the conditioning box. Before each of the odor streams, there is an air flux regulator, the air flux is set to 3psi. Valve switching in the olfactometer delivers continuous air flux or odor to the left or right odor port at an approximate flow rate of 1 L/min.

2.7.2 Odor generalization
Behavioral conditioning took place over 5 days. On the first day, mice were habituated for 40 min to the conditioning box, on the following and on the third day (training sessions), they were trained to associate a 5s CS+ odorant (presented 3 times/session) with a 0.5s foot shock. In between CS+ presentations, 2 CS- odorants (not paired with foot shock) were presented 2 times for 5s each during training and testing. Trials were spaced by a three-minute interval. Testing occurred 24 hr after the second training session.

Mice were presented with three monomolecular odorants diluted to 1% in light mineral oil (Fisher Scientific) on the training sessions: Ethyl Acetate (CS+), beta-Citronellol and Eugenol (CS-1, CS-2) (Sigma-Aldrich); on the testing session, mice were presented the same odorants at a different concentration, either 5% or 0.2%.

2.7.3 PV-cell photosuppression
8 weeks old male PV-Cre mice were stereotaxically bilaterally injected with AAV-EF1a-DIO-eArch3.0-EYFP (University of North Carolina, UNC). Injection was followed by bilateral fiber implantation (for more details on the surgery, see photostimulation protocol above) 13 days before the start of the behavior test.

Behavioral conditioning took place over 5 days. On the first day, mice were habituated for 40 min to the conditioning box, on the following and on the third day (training sessions), they were trained to associate a 5s CS+ odorant diluted to 1% in light mineral oil (presented 3 times/session) with a 0.5s foot shock. In between CS+ presentations, 2 CS- odorants (not paired with foot shock), were presented 3 times for 5s. Trials were spaced by a three-minute interval. Testing occurred 24 hr after
On the second training session, the CS+ odorant was presented 2 times for 5s at 5% or 1% (same odor concentration as in training), one time the odorant at 5% or 1% was presented together with bilateral light suppression (561nm, CW, 7-10mW for 5s, MGL-FN-561, CNI laser) and the other time the odorant was presented without bilateral light suppression at 1% or 5%. CS- odorants (1%) were presented two times for 5s each.

### 2.7.4 Chemogenetic inhibition of PV cells

8 weeks old male PV-Cre mice were stereotaxically bilaterally injected with AAV-hSyn-DIO-hM4D (Gi)-mCherry (University of North Carolina, UNC) 13 days before the start of the behavioral tests. Behavioral conditioning took place over 5 days. On the first day, mice were habituated for 40 min to the conditioning box, on the following and on the third day (training sessions), they were trained to associate a 5s CS+ odorant diluted to 1% in light mineral oil (presented 3 times/session) with a 0.5s foot shock and the 2 CS- odorants, presented 3 times for 5s each were not paired with a foot shock. Trials were spaced by a 180s interval. Testing occurred 24 hr after the second training session. On the day of testing, 20 minutes before it's start, mice were injected with CNO (3.2mg/kg of body weight). CS+ odorant was presented 2 times for 5s at 5% or 1% (same odor concentration as in training), CS- odorants (1%) were presented two times for 5s each.

Videos recorded on the testing session of the experiments described above (Photostimulation, Odor generalization, PV-cell photosuppression and chemogenetic inhibition of PV cells) were analyzed using a custom-written Matlab script where we quantified (frame rate 25frames/second): maximum speed before and after stimuli presentation, distance run before and after stimuli presentation and reaction time, after stimuli presentation.

### 2.7.5 Gad2-Cre and Emx-Cre photostimulation protocol

The behavior protocol is the same as the “photostimulation protocol” described above but with some modifications. Gad2-Cre mice were entrained with two different stimulation protocols: 20Hz photostimulation (25ms pulses, 3 sec per trial) or 40Hz photostimulation protocol (12.5ms pulses, 3 sec per trial) of Gad interneurons in the piriform cortex. Emx-Cre mice were entrained with a 20 Hz photo stimulation protocol and the excitatory neurons were infected with a lentiviral vector LV-Ef1α-ChR2:EGFP-IRES-nuclearCherry.

### 2.7.6 Statistics

Statistical analysis was performed in R and GraphPad Prism 7 software.
For protocol 2.7.3, behavioral responses per mouse and per trial for percentage of flight behavior, reaction time and speed were analysed for statistical significance following presentation of CS+1%, CS+1%PS, CS+5%, CS+5%PS, CS-1 and CS-2 using the non-parametric Wilcoxon test.

For protocol 2.7.2 and 2.7.5, behavioral responses in between mice and trials of different behavior conditions for percentage of flight behavior, reaction time and speed were analysed for statistical significance using the non-parametric Mann-Whitney test. In the Results section, p values ≤ 0.05 are represented with *, p value ≤ 0.01 are represented with **, p value ≤ 0.001 are represented with *** and p values ≤ 0.0001 are represented with ****.

2.8 Appetitive operant conditioning
The custom-made set up consisted on a treadmill (550mm, 100mm, 0.7mm; HPC) where the mouse was positioned to perform the task. To help the movement of the mouse, the treadmill was placed above 6 rollers. The mouse had an aluminium head plate implanted (2cm x 0.5cm) that was used to attach the mouse to the set up using two fixating arms. Water was delivered to the mouse through a water port; the water port was connected to a capacitive sensor (AT42QT1010, Sparkfun Electronics). The water was stored in a 50ml tube and it was delivered through a 2-way direct lift solenoid valve (Cole-Parmer). Water reward volume was set to 15µl. We used an Arduino microcard and software to control the behavior task (water and odor presentations, trial duration and randomization).

2.8.1 Operant conditioning protocol
8 weeks old WT mice were implanted with a head plate and allowed to recover for 2 days until showing no signals of pain or distress. On the third day, mice began the water restriction period. Before performing the task mice should weight around 85% of original body weight. This drop is obtained by removing on the first day of deprivation the water access entirely from the animal for 24 hours. Following that period, mice were given (by hand) 0.5ml of water and on the day before the experiment, mice were entrained to associate the context of the experiment (head fixation) with a reward (1-1.5ml of water, to retain weight).

The task is a go/no-go odor discrimination task, mice had to lick after the presentation of an odorant - rewarded stimulus (CS+) and refrain from doing so after a different odorant (CS-). Both odorants were diluted to 1% in mineral oil (Sigma-Aldrich). Mice underwent the go/no-go task in three consecutive phases, the habituation, the first odor pair discrimination and the second odor pair discrimination. The first odor pair presented was Ethyl acetate (Sigma Aldrich) (CS+), and Eugenol (Sigma Aldrich), (CS-), diluted to 1% in mineral oil.
**Habituation phase:** In this phase, mice had to learn to lick after the CS+ odorant. To reach this goal, CS+ stimulus was presented to the mice and they had to touch the water port during a 2-second time window to receive the water reward. Any lick outside of the reward period did not allow mice to get water drops and was counted as a “mistake”. Not licking after a CS+ presentation during the reward period would count as a “miss”. Every successful trial allowed mice to get 15 µl of water and was counted as a “hit”. 10 trials were grouped in a block. Mice had to reach a performance of 90% to proceed to the next phase.

**First odor pair discrimination:** Mice had to learn to discriminate the CS+ from the CS- odorant by licking only in the presence of the CS+ odorant. Each odorant was presented during 2s, the reward period starts 1s after odor presentation and lasts 2.5s. At this stage, ten trials were grouped in a block and separated by a 4 seconds inter-trial interval (ITI). Licking the wrong odorant or not licking after a CS+ presentation led to an increase of the ITI to 8 seconds. Trials per block were randomized and each block contained 5CS+ and 5CS- odorants. Mice performed the task until signals of stress were evident or a decreased performance due to a lack of motivation. To proceed to the next stage, mice had to reach 90% of correct responses in one block followed by two blocks with performance above 80%.

**Second odor pair discrimination:** On this stage, we tested the ability of the mice to generalize the task (odor discrimination) to other odor pairs. We allow reward upon licking for 100 microliters of reward. Following that stage, the mouse has to perform 2/3 blocks of the first odor pair task to make sure the mouse retains the association. Next, the CS+ odor of the new pair is presented during 5/10 trials to make sure the mouse remembers the task. Finally, animal should quickly learn the new odor pair discrimination 50:50 CS+ to CS-.

Data was acquired with Arduino and Cool-term software. The parameters extracted were the performance (percentage of correct licks to CS+ and correct rejections of the CS- / total number of CS+ presentations per block), the total number of licks per block after CS+ or CS- presentation, the number of licks per trial and the reaction time between the odor presentation and the first lick.
3. RESULTS
3.1 Manipulation of odor intensity-independent behaviors in the mouse piriform cortex

3.1.1 Odor intensity-independent behaviors

An animal following a scent trail must be able to reliably recognize the target odor over a large range of concentrations. Such fluctuations in odor concentration pose a challenge to the olfactory system, as odorant – receptor interactions are highly dependent on odorant concentrations. The olfactory system thus has to form a representation of odor identity that is robust to changes in concentration. When odorants bind olfactory receptors in olfactory sensory neurons (OSNs), they initiate a series of intracellular events producing second messenger signals that gate ion channels. In the absence of odor stimuli, basal firing of OSNs is low (0.05 –3 Hz) (Reisert, 2010). Depending on the strength of odorant – receptor interaction, OSNs spike frequency, number of spikes, and latency to first spike can be modulated so that weak signals can be detected and strong signals normalized (Kleene, 1997). Despite such compensatory mechanisms, an increase in odorant concentration is accompanied by robust increases in the number of activated receptors and OSNs, and changes in their firing characteristics (Ma and Shepherd, 2000; Malnic et al., 1999).

In the olfactory bulb (OB), increasing odor concentration results in increased response amplitudes of individual glomeruli and in the activation and suppression of new glomeruli (Smear et al., 2013; Rubin and Katz, 1999). Encoding of odor concentration in the OB is tightly linked to the latency of the response of the output neurons of the OB, mitral and tufted cells (Duchamp-Viret et al., 1999). At higher odor concentration, spike latencies of mitral cell responses are reduced (Cang and Isaacson, 2003; Rinberg et al., 2006; Uchida and Mainen, 2003; Schaef er and Margrie, 2012). Glomerular recruitment is still detectable postsynaptically in patterns of mitral and tufted cell response mapped by GCaMP2 imaging of calcium in dendritic tufts (Fletcher et al., 2009). Thus, the representation of odors in the OB is strongly dependent on odorant concentration.

In the piriform cortex, electrophysiological and in vivo imaging experiments have revealed that piriform odor responses remain sparse and dispersed even when odor-evoked activity in the OB is dense. Therefore, piriform circuits are able to normalize sensory stimuli (Poo and Isaacson, 2011; Roland et al., 2017; Stettler and Axel, 2009). The observation that cortical odor representations are normalized suggests the action of inhibitory neural circuitry within piriform cortex (Wilson and Sullivan, 2011; Chapuis and Wilson, 2011). Since odor-evoked activity in the piriform cortex is mostly due to long range, recurrent excitation from intracortical associational network (Franks et al., 2011; Poo and Isaacson, 2011), it is likely that the balance of excitation and inhibition is achieved by global feedback inhibition, compensating recurrent network excitation. Among the different types of inhibitory neurons present in the piriform cortex, Parvalbumin-expressing (PV+) interneurons and
Somatostatin-expressing (SST+) interneurons are the two most abundant inhibitory neurons and are predominantly positioned in layer III of piriform cortex (Suzuki and Bekkers, 2010). They exhibit contrasting physiological and anatomical properties: PV+ cells are fast-spiking multipolar (fMP) cells and preferentially target cell soma of layer II neurons, whereas SST+ cells are regular spiking multipolar (rMP) interneurons, whose axons preferentially target distant dendrites.

In vivo calcium imaging experiments in the laboratory were done to test if the response properties of these two inhibitory subpopulations of piriform neurons were modulated by odorant concentration (Benjamin Roland and Alexander Fleischmann, unpublished results). A conditional AAV was used to express the fluorescent calcium indicator GCaMP6s (Chen et al., 2013) in SST-Cre or PV-Cre transgenic mouse lines to selectively target SST+ neurons or PV+ neurons. It was found that the fraction of odor-responsive SST+ cells did not scale with the odorant concentration. In contrast, PV+ cell responses were steeply concentration-dependent (Figure 1). The response magnitude of PV+ cell shifted with concentration, while the response magnitudes of SST+ cell did not change (Figure 1a). Furthermore, PV+ cells became more broadly tuned as concentration increased (Figure 1b). The response properties of SST+ cell are consistent with recent extracellular recordings examining the effect of this inhibitory component on piriform odor representations, using extracellular recordings in anesthetized mice (Sturgill and Isaacson, 2015). SST+ cells regulate piriform cortical activity through a purely subtractive operation (i.e., the level of SST+-mediated inhibition does not scale with input strength).

Based on the hypothesis that piriform ensemble activity encodes odor quality independent of odor concentration, and that this function depends on piriform PV cells, I have developed behavioral tests and combined with manipulations of neural activity to test three related questions: 1) Are mice able to generalize a behavioral response across a range of concentrations of the same odorant? 2) Does PV cell photosuppression or chemogenetic silencing in the presence of the odorant at high concentration impair odor concentration-invariant behavior? 3) Are PV cell-dependent mechanisms for odor concentration-invariance maintained across different behavioral paradigms, such as aversive versus appetitive conditioning?
Figure 1. Differential concentration-dependence of piriform PV+ and SST+ interneurons.
Two-photon in vivo imaging experiments reveal distinct odor response properties of piriform PV+ and SST+ interneurons. (a) Cumulative frequency plot of the maximum change in fluorescence (ΔF/F) of responsive trials after odor onset in PV+ (top) and SST+ (bottom) cells. SST+ cells have a higher response magnitude that remains relatively stable across concentration, whereas PV+ cell response magnitudes shift with increasing concentration. (b) Odor tuning of PV+ (top) and SST+ (bottom) cells across increasing concentration. SST+ cells have a lower response probability, largely independent of odor concentration. In contrast, PV+ cells are more responsive and tuning breadth increases with increasing concentration. Error bar: SEM. Benjamin Roland and Alexander Fleischmann, unpublished results.
3.1.1.1 Establishing an odor concentration-invariant behavioral task

It has been speculated that normalization of odor-evoked neural activity in the piriform cortex underlies an animal's ability to identify odors independent of the concentration and olfactory background noise (Bolding and Franks, 2017; Roland et al., 2017; Luo, 2011). Hence, we have established a behavioral test in which an odorant (Ethyl Acetate, conditioned stimulus (CS+), diluted to 1% in light mineral oil) was paired with a foot-shock. After two training sessions, mice readily learned to escape upon exposure to this odorant (CS+), but to remain stationary upon exposure to the CS- odorant, an odorant not paired with a foot shock during training (beta-Citronellol and Eugenol, conditioned stimulus (CS-1, CS-2), diluted to 1% in light mineral oil). During testing, we then varied the concentration of the CS+ odorant, using a five-fold increase and decrease, corresponding to 5% and 0.2% odorant concentration.

We found that mice escaped from the CS+ in 92% of trials when the CS+ was presented at 1% concentration, the same concentration as during training (average response across three trials per mouse, n (number of mice) = 4). Mice escaped in 72% of trials when the CS+ was presented at 0.2% (n = 6), and in 80% of trials when the CS+ was presented at 5% (n = 5) (Figure 2a). For all 3 concentrations of CS+, the average response was significantly higher than the behavioral response following the presentation of the CS- (average response: 27% of trials, n = 5), showing that mice generalized the behavior across odorant concentration but not odorant identity.

To further characterize this behavioral response, we calculated the speed and reaction time after CS+ presentation. Mice responded to CS+ presentation at 1% with a short response time (1.5 s), but the response was delayed when the odorant was presented both at a higher or lower concentrations (1.75 s for the odorant presented at 5%, 2.2 s for the odorant presented at 0.2%). Furthermore, the average speed following CS+ presentation at 1% was 40.7 cm/s, but was reduced when the concentration of the CS+ was changed during testing. The speed following CS+ presentation at 0.2% was 31.3 cm/s, the speed following CS+ presentation at 5% was 28.7 cm/s (Figure 2b, c).

Taken together, these results suggest that mice generalized an aversive behavioral response across a 25-fold range of different odorant dilutions. The fraction of correct behavioral responses remained unchanged (Figure 2a). Interestingly, however, we observed that changing the concentration of the conditioned odorant stimulus during testing caused a slight delay in the behavioral response, and a decrease in the average response speed.
Figure 2. Odor concentration-independent fear conditioning.

a) Percentage of escape behavior after CS+ presentation at three different concentrations, and following CS-1 and CS-2 presentation. (b) Speed per trial after CS+ presentation at three different concentrations. (c) Reaction times per trial after CS+ presentation at three different concentrations. Error bars: SEM.

3.1.2 PV cells as a candidate cell population to mediate concentration-invariance in piriform

3.1.2.1 PV cell photosuppression

Exposing mice to an odorant that was paired with foot shock leads to a reliable, experience dependent escape behavior. Importantly, mice were able to detect and identify the conditioned odor stimulus when presented at a range of different concentrations. We next tested the conditioned behavioral responses of mice while modulating PV cell activity using optogenetics. If PV cells are important for preserving the identity of an odor across a range of concentrations, given that their activity increases with increasing odor concentrations, we hypothesized that the modulation of PV cell activity will selectively impair the generalization of the learned behavioral response to varying concentrations of the conditioned odor.

We took advantage of transgenic mice expressing the Cre recombinase specifically in PV cells (PV-Cre) (Hippenmeyer et al., 2005) to selectively express conditional AAV expressing the light-driven proton pump Archaerhodopsin (Arch), isolated from the archaeabacteria Halorubrum genus (Han et al., 2011). The previously established odor concentration-independent fear conditioning protocol was modified as follows: during training, we presented the conditioned odorant (CS+) paired with foot
shock (3 times per training session), as well as 2 odorants that were not paired with foot shock (CS-1 and CS-2, two times each per training session). All odorants were presented at a dilution of 1% in light mineral oil. During testing, the CS+ odorant was presented once at a dilution of 1% or 5% each, and once at a dilution of 1% or 5% each but together with bilateral photosuppression of piriform PV cells (laser stimulation at a wavelength of 561nm, continuous wave mode, 7-10mW for 5s). CS-1 and CS-2 odorants at 1% concentration were presented two times each.

Consistent with our previous results, we found no significant differences in the percentage of flight behavior when the CS+ was presented at 1% or 5%. In both conditions, mice escaped from the CS+ in 69.5% or 61.2% of the trials respectively (average response per mouse, n = 6). Furthermore, light-induced PV+ cell silencing did not significantly change the behavioral response. Similar results were obtained when pairing the CS+ at 1% with photosuppression of PV cell (66.7%), and when pairing the CS+ at 5% with photosuppression of PV cell (66.7%). Mice did not escape from CS-1 and CS-2 odorants (35.5% and 36.2%, average response per mouse, n = 6), confirming that mice discriminated between the CS+ and CS- (Figure 3b). Thus, mice were able to generalize the aversive behavior across two odor concentrations, and photosuppression of PV cells did not dramatically alter the behavioral response.

In addition, we analyzed the behavioral response by measuring speed and reaction time following CS+ presentation on testing. The average speed following CS+ presentation at the same concentration as in training (1%) was 44.3 cm/s and reaction time 2.17s. Mice responded faster when the CS+ was presented at 5% concentration but the speed was decreased (reaction time 0.67s, speed 23.5 cm/s). Similar increase in the response time and decrease in speed was observed when pairing CS+ at 1% with photosuppression of PV cells (reaction time 0.67s, speed 36.1 cm/s). Both reaction time and speed following pairing of CS+ at 5% with photosuppression of PV cells were slightly increased (reaction time 2.6s, speed 53.8 cm/s). Thus, no consistent effects of photosuppression could be correlated with the behavior response.

We noticed that escape behavior for all experimental conditions was highly variable among mice (Figure 3d). For example, mouse 6 escaped from the CS+ at 1% in 67% of trials, and in 100% of trials when the CS+ was paired with photosuppression. When the CS+ was presented at 5%, mouse 6 escaped in 67% of trials, but failed to escape (0% escape behavior) when the CS+ was paired with photosuppression. Mouse 6 thus exhibited a selective loss of concentration-invariant escape behavior upon selective silencing of piriform PV+ cells, consistent with our initial model.

One possible explanation for the observed variability in the behavioral response of individual mice could be the specificity and number of infected PV+ neurons in piriform cortex. We have confirmed with previous experiments that in PV-Cre transgenic mice, infection with conditional AAVs carrying different reporter genes resulted in the selective targeting of PV-immunoreactive
neurons. Furthermore, after completing mice behavioral analyses, we performed immunohistochemistry to test the variability of PV cell infection at the injection site. We could not identify any variables, such as the size of the infected area or the density of labeled PV+ neurons, which would explain the variability of the observed behavioral response (Figure 3a,d). Figure 3c illustrate an example of labeled PV+ piriform neurons, in which anti-Parvalbumin immunoreactivity (in red) and Arch-EYFP immunoreactivity (in green) are co-localized in a large fraction of piriform neurons.

Taken together, we found that despite the strong modulation of piriform PV+ cell activity by odor concentration, photosuppression of PV-cells was not sufficient to impair the generalization of the aversive behavior across two different odor concentrations. The following technical limitations could explain the lack of observed behavioral phenotypes and the variability in the behavior response among mice.

1) We do not have experimental evidence to support the assumption that in our experiments, piriform PV+ cells were effectively silenced. In vivo electrophysiological recordings of neural activity of piriform PV+, Archaerhodopsin-expressing cells were done by our collaborators (Kevin Bolding and Kevin Franks, Duke University), with the prediction that upon PV cell photosuppression, additional pyramidal cells would be disinhibited and overall piriform activity would be increased. This prediction could not be verified, because in vivo photosuppression of PV cells in a controlled manner could not be achieved: attempts with moderate laser power (10mW) did not elicit any detectable change in piriform spiking patterns, while attempts with high laser power (50mW) caused epileptic seizures in the mouse.

2) Our behavioral paradigm, fear conditioning, has limitations that might explain the variability observed in the behavioral outcome. An aversive behavior induces stress, which, together with the stress generated form the surgery and fiber implantation, might impair the behavioral response. In addition, the small number of training and testing sessions limit the behavioral read-out (less than 10 test trials). Using an alternative behavior assay, such as the appetitive operant conditioning, we could test the behavior response driven by the data obtained during testing, consisting of hundreds of licks (higher statistical power).

3) We only tested a small range of odorant concentrations. It is possible that the range in odor concentration was not large enough to require a significant modulation in PV cell activity to support a concentration-invariant behavioral response. Furthermore, while odor delivery is controlled by the olfactometer, mice are freely moving in the behavior box, therefore the encountered odor concentration likely varies according to the proximity of the mouse to the odor port.
**Figure 3. Photosuppression of piriform PV cells in an odor concentration-independent fear-conditioning paradigm.**

(a) Histological section of a PV-Cre transgenic mouse expressing Arch in the piriform cortex (Arch-EYFP shown in green), anti-PV immunoreactivity is shown in red, nuclear counterstain in blue. (b) Percentage of escape behavior after CS+ presentation at 1% and 5% concentration (CS+1%, CS+5%), and after CS+ presentation at 1% and 5% concentration with simultaneous photosuppression of PV+ cells (CS+1%+PS, CS+5%PS). The percentage of escape behavior after the presentation of CS-1 and CS-2 odorant is shown in gray. Average response per mouse, n (number of mice) = 6, error bars: SEM. (c) Example of labeled PV+ piriform neurons, in which anti-Parvalbumin immunoreactivity (in red) and Arch-EYFP immunoreactivity (in green) are co-localized in a large fraction of piriform neurons. (d) Percentage of escape behavior of individual mice following stimulus presentation, as in Figure 3b. Each number (1-6) represents a different mouse and the average response per mouse to a conditioned stimulus.

### 3.1.2.2 Chemogenetic inhibition of piriform PV cells

Inhibition of neurons using optogenetics provides tight temporal control and reversible inhibition, however, inhibition is constrained to the area where the light is spread in the tissue. Alternatively, suppressing neural activity using chemogenetics can provide inhibition in larger areas and does not cause tissue damage resulting from the implantation of optical fibers (Gomez et al., 2017). Therefore, we have performed a pilot experiment to test, as described for optogenetic manipulations above, whether the modulation of PV+ cell activity selectively impairs an odor concentration-invariant behavioral response. Here, we used chemogenetics to selectively perturb PV+ cell activity.
hM4D(Gi)-mCherry is a modified form of the human M4 muscarinic (hM4) receptor. It can be activated by the inert clozapine metabolite clozapine-N-oxide (CNO), which engages the Gi signaling pathway. Gi signaling in neurons opens potassium channels, resulting in an influx of potassium ions that decreases the resting membrane potential of neurons and their capacity to depolarize. Thus, neurons expressing hM4Di treated with CNO have decreased firing rates.

PV-Cre mice were bilaterally injected with conditional hM4D (Gi)-mCherry, and after recovery from surgery, mice were trained to associate a CS+ odorant diluted to 1% with foot shock. The presentation of CS-1 and CS-2 odorants was not paired with foot shock. On testing day, mice were injected with CNO (3.2mg/kg of body weight) 20 minutes before the testing session. During testing, the CS+ was presented at 5% and 1%, CS-1 and CS-2 were presented at 1%.

We did not find striking differences in the percentage of escape behavior when the CS+ was presented at 1% or 5%. Mice escaped from the CS+ in 75% or 50% of the trials, respectively. The percentage of flight behavior in response to the CS-1 and CS-2 odorants was lower, 33.5% (average response per mouse, n = 2) (Figure 4b). However, as for optogenetic manipulation of PV+ cells, variability in the behavioral response of the two mice to the different stimuli limited the interpretation of the observed behavioral responses. Expression of hM4Di in piriform PV+ cells was verified using immunohistochemical analysis (Figure 4a). We could not identify any variables, such as the size of the infected area or the density of labeled PV+ neurons, which would explain the variability of the observed behavioral response.

Figure 4. Chemogenetic inhibition of piriform PV cells in an odor concentration-independent fear-conditioning paradigm.

a) Histological section of a PV-Cre transgenic mouse expressing hM4di in PV cells in the piriform cortex. hM4di-mCherry is shown in red, nuclear counterstain in blue. (b) Percentage of escape behavior after chemogenetic inhibition of PV cells. The CS+ was presented at two different concentrations (CS+1%, CS+5%). In grey, percentage of escape behavior after the presentation of CS-1 and CS-2; average response per mouse, n = 2, error bars: SEM.
Taken together, chemogenetic inhibition of PV cells caused a moderate decrease in the response to the CS+ odorant when it was presented at higher concentration. However, due to the observed variability in behavior, and the small number of mice used in this experiment we cannot conclude that the inhibition of PV cell activity was responsible for the impairment in the odor concentration-independent behavior.

### 3.1.3 Establishing an alternative behavioral task

The generalization of a behavior across a range of concentrations is not specific to classical aversive conditioning (Wojcik and Sirotin, 2014; Homma et al., 2009). Therefore, to expand the repertoire of behavioral tests, I have next set up an appetitive go/no go operant conditioning task. During the first phase of training, water-restricted mice have to touch the water port and lick from it to receive the water reward. During the second phase, mice are entrained to lick from the water port after the presentation of a CS+ odorant. Finally, during the third training phase, mice have to touch the water port and lick after the presentation of an odorant that will be the rewarded stimulus (CS+), and refrain from doing so after the presentation of a different odorant (CS-). Custom-written software allowed us to quantify the behavioral response of the mouse according to the following parameters: the performance per block (licking in the presence of the rewarded odorant (hit) and not licking to the non-rewarded odorant (correct rejections), divided by the total number of trials in a block, 10), the total number of licks per block after CS+ or CS- presentation, the number of licks per trial, and the reaction time between the odor presentation and the first lick.

An example of the read-out of the behavioral performance of a mouse is shown in Figure 5a. A water-restricted mouse was entrained to discriminate between two odorants, the rewarded odorant (CS+: ethyl acetate at 1% concentration) and the non-rewarded odorant (CS-: eugenol at 1% concentration). We considered that mice had correctly learned the odor discrimination task when they reached a performance criterion of 90% in one block, followed by a performance of 80% in the two following blocks (Figure 5b).
Figure 5. Odor discrimination in a go/no-go operant conditioning task.

a) The mouse is performing a go/no-go odor discrimination task in the operant conditioning setup. (b) Odor discrimination between the rewarded odor and the non-rewarded odor. The mouse reached a performance of correct responses of 83% after 900 trials (block 9). Indicated on the y-axis is the percentage of correct responses per block, i.e. licks in the presence of the rewarded odorant (hit) and no licks in the presence of the non-rewarded odorant (correct rejection), divided by the total number of trials. In grey: percentage of correct responses per block; in green: percentage of correct licks to the rewarded odorant (CS+), and in red: percentage of correct rejections to the non-rewarded odorant (CS-).

Unfortunately, due to time constrains, I was not able to use this task for testing concentration invariance of odor discrimination. Importantly, however, this behavioral test will complement results obtained from fear conditioning experiments and provide several advantages for future experiments. First, the behavior paradigm is more flexible. The duration of the training and testing sessions can be adapted to individual mice, and testing can be extended for longer periods of time when performing complex discrimination tasks. Furthermore, data obtained during testing consist of hundreds of licks, as opposed to less than 10 test trials obtained during aversive conditioning. Therefore, the power of statistical analyses is substantially increased. Finally, testing can be done using rare ‘catch trials’. For example, the behavioral response to odors at varying concentrations, and in the presence or absence of perturbations of neural activity, can be tested without training the mice to adapt to varying experimental conditions.

3.1.4 Summary

Taken together, the experiments described above have provided new insights into experimental approaches that can be employed to probe neural mechanism of sensory processing in the piriform cortex that underlie the transformation of sensory input into behavioral output.

First, we show that mice are able to generalize an odor-driven behavioral response across a range of different odor concentrations, without losing the ability to identify the odor. We have established an odor fear conditioning task to test for odor identity generalization across different odor intensities.
Mice were able to identify and escape from the conditioned stimulus, and generalize this behavior response to when the odorant was presented at higher or lower concentrations.

Second, PV+ cell activity in piriform cortex is dependent on odorant concentration (Benjamin Roland and Alexander Fleischmann, unpublished data). Using optogenetics and chemogenetics we aimed at determining the role of piriform PV+ cells in the fear-conditioning assay. Overall, silencing of PV+ cell activity did not significantly change the behavioral response of mice to odorants when presented at different concentration. Thus, PV+ cells may not be essential for an odor concentration-independent fear learning response. However, the interpretation of our data is limited by the variability of the behavioral responses we observed. For example, silencing of piriform PV+ cells abolished odor concentration generalization of the escape behavior in two mice. Testing PV+ cell activity in other odor concentration-independent behavioral paradigms, and combining optogenetics with in vivo electrophysiological recordings to quantify the effect of neural silencing will provide more consistent data and overcome the current technical limitations.
3.2 Dissection of cortical olfactory circuit functions in mice: cell type-specific functions in the mouse piriform cortex

In this section, we aim to understand the organization and function of neural circuits in the mouse piriform cortex. The neural circuit mechanisms operating in the piriform cortex have been suggested to serve as an association cortex integrating the input from the olfactory bulb to form coherent odor objects (Haberly and Bower, 1989; Wilson and Sullivan, 2011).

Moreover, piriform neural circuits undergo experience-dependent synaptic plasticity, and activation of random ensembles of piriform neurons is sufficient to drive a learned behavior in the absence of sensory input (Choi et al., 2011; Chapuis and Wilson, 2011). Thus, piriform neural circuits have been implicated in odor perception, and olfactory learning and memory. Piriform efferent pathways project to several higher brain centers implicated in behavioral output, including the anterior olfactory nucleus (AON), the medial prefrontal cortex (mPFC), the olfactory tubercle (OT), the cortical amygdala (CoA), the lateral entorhinal cortex (lEnt) and the agranular insular cortex (AI) (Diodato et al., 2016; Chen et al., 2013; Luskin and Price, 1983). Potential functional differences in the ability of these different piriform projection neurons to relate sensory input to the control of behavior remain unknown.

We hypothesized that different subsets of neurons within the ensemble of piriform neurons may differ in their ability to drive a behavioral output. We here devised an experimental approach, based on the selective activation of distinct piriform neural cell types, to test our hypothesis.

We expressed the light-activated cation channel channelrhodopsin (ChR2, Boyden et al., 2005) in piriform neurons, using intersectional viral gene transfer. We then tested if photostimulation of ChR2-expressing neurons could serve as a conditioned stimulus to entrain learned aversion, when paired with foot shock as the unconditioned stimulus. We initially tested if photostimulation of excitatory and inhibitory piriform neural subpopulations was sufficient to drive a learned behavioral response. Second, we tested if different subpopulations of piriform projection neurons, defined by their target selectivity (OB, mPFC, CoA or lEnt), showed functional differences in driving a fear response. Our data reveal robust differences in the ability of different piriform subnetworks to drive learned aversion.

3.2.1 Piriform inhibitory neurons fail to support learned aversion

Initially, to set up the behavioral paradigm, we targeted ChR2 expression to piriform principle cells, defined by their expression of the transcription factor Emx1, by injecting conditional AAV-ChR2 in the piriform cortex of Emx1-Cre transgenic mice (Gorski et al., 2002). This approach had previously been shown to support aversive conditioning (Choi et al., 2011). Mice were trained to associate photostimulation of ChR2-expressing EMX+ cells as the conditioned stimulus (CS+) with foot
shock as the unconditioned stimulus (US). During testing, the CS+ was presented in the absence of the US, and mouse behavior was analyzed from video recordings.

We found that the behavioral readout was variable across different mice: some mice escaped from the CS+ (as described in Choi et al., 2011), while others failed to escape. Mice failing to escape often exhibited weak seizure-like episodes, characterized by immobility, extensive grooming, contraction/extension of the limbs along with arching of the back for \( \sim 10 \) seconds (data not shown). This observation is not unanticipated, given that piriform cortex is the most susceptible brain region for epileptic seizures, which results from an imbalance between neural excitation and inhibition ('area tempestas', Piredda and Gala, 1985; Morimoto et al., 2004).

We hypothesized that these seizure-like episodes, induced by photostimulation of excitatory neurons in piriform cortex, could be caused by the over-excitation of the piriform neurons. One possible explanation for this effect, which was not reported in previous studies, is the use of AAV instead of lentiviral vectors, which results in denser labeling of neurons at the injection site and higher levels of ChR2 expression per infected neuron (data not shown) (Kantor et al., 2014). We therefore next used a conditional Lentivirus expressing ChR2:EEFP-IRES-nuclear-mCherry (Figure 6a). We found that photostimulation of Emx1+ neurons was indeed sufficient to drive an aversive response in 60% of trials. In a pilot experiment, we next attempted to correlate the number of infected neurons at the injection site with their ability to drive behavioral response. Behavioral responses were observed if the number of infected cells was superior to 200 (Figure 6b). Importantly, 200-500 excitatory neurons were sufficient to drive an aversive behavior without eliciting seizure-like behavior. Thus, conditioned escape behavior provides a behavioral readout for exploring potential functional differences between different ensembles of piriform neurons to drive behavior.
Figure 6. Photostimulation of Emx1+ neurons in the piriform cortex is sufficient to drive learned aversion.

a) Histological section of an Emx1-Cre transgenic mouse expressing ChR2:YFP-nuclear-mCherry in piriform cortex. Nuclei are labeled in red, neural processes in green, Neurotrace counterstain is shown in blue. Damage from the optical fiber is visible above the injection site. (b) Percentage of escape behavior in response to the photostimulation of ChR2+ neurons, relative to the number of infected cells per mouse.

Random ensembles or piriform neurons, as well as ensembles of piriform excitatory neurons can serve as a conditioned stimulus to drive learned aversion. An important general question is whether any ensemble of piriform neurons can be entrained to drive this behavioral response. One possibility is that independent of neural cell type and connectivity, photostimulation of any ensemble of neurons in piriform cortex can be linked with behavioral output through aversive conditioning. Alternatively, the ability of neural ensembles to drive a behavioral response may be constraint by cellular and/or network properties.

We therefore next asked if piriform inhibitory neurons can be entrained to elicit learned aversion. Piriform inhibitory neurons play important functions in odor processing, for example by synchronizing odor-evoked activity across multiple olfactory areas (Isaacson and Scanziani, 2011; Stokes and Isaacson, 2010). We asked if this subpopulation of piriform neurons was sufficient to drive an aversive response. We expressed ChR2 in Gad2-expressing inhibitory neurons in the piriform cortex, using stereotaxic injection of conditional AAV-ChR2:YFP in Gad2-Cre transgenic mice (Makinae et al., 2000) (Figure 7a). We trained mice to associate photostimulation of piriform interneurons with foot shock, and then tested if inhibitory neurons in the piriform were sufficient to drive a behavior.

We initially photostimulated inhibitory neurons at 20Hz, using the same protocol as for photostimulation of Emx1+ piriform neurons. We observed that upon testing, mice failed to efficiently respond to photoactivation. Photoactivation resulted in an average escape behavior in only 12% of trials (7 trials per mouse, n = 8). In 14% of trials, mice exhibited signs of stress, such as
tail shaking or stationary behavior. Comparable results were obtained in a group of control mice, in which AAV encoding the calcium indicator GCaMP3 was injected instead of ChR2. Photostimulation of the control group (7 trials per mouse, n = 2) resulted in an average escape behavior of 7%, while in 29% of trials, mice exhibited signs of stress (Figure 7b).

One possible explanation for the observed lack of behavioral response is that interneurons require photostimulation at different frequencies, and that 20Hz photostimulation is not sufficient to drive synchronized activity of these neurons. For example, inhibition of a sub-type of interneurons, parvalbumin-expressing interneurons, suppresses gamma oscillations in vivo (König et al., 1996; Womelsdorf et al., 2007), and photostimulation of PV+ interneurons is sufficient to generate emergent 40Hz-frequency rhythmicity in PV+ interneurons in prefrontal cortex and inhibit spikes in pyramidal cells (Sohal et al., 2009). Enhanced GABAergic inhibition in the OB was observed in electrophysiological recordings of mitral cells following photostimulation of inhibitory neurons at 40Hz but not at 10Hz (Alonso et al., 2012).

We therefore performed the experiment in a separate cohort of mice, but we stimulated Gad2+ neurons at a frequency of 40Hz. We observed that photostimulation of ChR2+ inhibitory neurons at 40Hz resulted in an average escape behavior of 27% (7 trials per mouse, n = 5). Mice presented signs of stress in 14% of trials. Results obtained in a control group expressing GCaMP3 instead of ChR2 were similar. 40Hz photostimulation of the control group (7 trials per mouse, n = 4) resulted in an average escape behavior of 13% and signs of stress were detectable in 7% of trials (Figure 7c). Taken together, these results clearly show that under comparable experimental conditions, excitatory and inhibitory ensembles of neurons differ in their ability to drive learned aversion.

Figure 7. Photostimulation of inhibitory neurons in the piriform cortex fails to drive learned aversion. a) Histological section of a Gad2-Cre transgenic mouse expressing ChR2:EYFP in inhibitory neurons in the piriform cortex. Damage from the optical fiber is visible above the injection site. (b) Percentage of aversive responses to photostimulation at 20Hz or 40Hz (c). Each dot in the graphic represents the average response of each mouse to photostimulation. The behavioral response was classified as ‘run’ (escape behavior) or as ‘stress response’ (tail shaking or immobility). Photostimulation of piriform interneurons was ineffective in driving learned aversive behaviors.
3.2.2 Distinct piriform output pathways differ in their ability to drive learned aversion

The experiments described above begin to identify differences in the ability of different piriform neural cell types in supporting conditioned aversion. We next asked whether functional differences in piriform ensembles could also be determined amongst distinct subnetworks of piriform projection neurons. This work is currently being finalized for submission for publication and here presented as an independent manuscript draft.
Olfactory cortex output pathways exhibit robust differences in their ability to drive learned aversion

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Abstract

The olfactory (piriform) cortex contains a large diversity of neural cell types, defined by their molecular identity, morphology, functional properties and projection target specificity. How these distinct neural cell types contribute to the control of olfactory-driven behaviors remains unknown. Here, we use a behavioral task in which optogenetic activation of subpopulations of piriform neurons is associated with an unconditioned aversive stimulus. We find that piriform neurons projecting to the olfactory bulb or the medial prefrontal cortex efficiently drive a learned escape behavior. In contrast, neurons projecting to the cortical amygdala or the lateral entorhinal cortex failed to support a behavioral response. Our results suggest that different piriform neural subnetworks exhibit robust differences in their ability to relate neural activity to behavioral output.
Introduction

The detection of odorants is accomplished by odorant receptors, expressed on the dendrites of olfactory sensory neurons in the main olfactory epithelium. In mice, olfactory sensory neuron express one of 1,000 odorant receptor genes present in the genome (Zhang and Firestein, 2002; Niimura, 2012), and project to one of two spatially stereotyped glomeruli in the olfactory bulb (OB) (Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994). The output neurons of the olfactory bulb, the mitral and tufted cells, then send projections through the lateral olfactory tract to several higher olfactory centers in the brain, including the piriform cortex, the anterior olfactory nucleus, the olfactory tubercle (OT), the cortical amygdala (CoA), and the lateral entorhinal cortex (lENT) (Haberly and Price, 1978; Ghosh et al., 2011; Sosulski et al., 2011).

The piriform cortex is the largest cortical area receiving direct inputs from OB mitral and tufted cells, and mitral and tufted cells projections to piriform are widespread and diffuse, lacking apparent topographical organization (Ghosh et al., 2011; Igarashi et al., 2012; Sosulski et al., 2011). Single piriform neurons receive convergent inputs from mitral and tufted cells belonging to multiple glomeruli, allowing for the integration of the segregated patterns of odor-evoked glomerular activity (Davison and Ehlers, 2011; Franks et al., 2011). Furthermore, piriform networks are shaped by olfactory learning and experience, and optogenetic activation of piriform neural ensembles is sufficient to drive learned behaviors (Chapuis and Wilson, 2011; Choi et al., 2011). Together, these data suggest that the piriform cortex plays important functions in odor perception, and olfactory learning and memory.

Odor information encoded by piriform ensembles must then be transmitted to downstream target areas to support multimodal sensory integration and motor control. Piriform cortex neurons send feedback projections to the OB as well as efferent projections to several cortical and sub-cortical targets, including the medial prefrontal cortex (mPFC), the OT, the CoA, and the lENT (Chen et al., 2014; Diodato et al., 2016; Haberly and Price, 1978; Johnson et al., 2000; Mazo et al., 2017). Recent experiments have begun to shed light onto the organization of piriform output pathways. For
example, piriform neurons projecting to different subdivisions of the orbitofrontal cortex exhibit distinct spatial topography along the antero-posterior axis of piriform cortex (Chen et al., 2014). Furthermore, it has recently been shown that distinct subpopulations of piriform projection neurons segregate into distinct piriform layers: neurons projecting to the OB and the mPFC are enriched in deep piriform layers IIb and III, while neurons projecting to the CoA and the IENT are predominantly present in the superficial piriform layer IIa. These different classes of piriform projection neurons can also be distinguished based on their morphology and the expression of distinct molecular markers (Diodato et al., 2016). However, the relevance of this organization of distinct piriform output pathways for olfactory-driven behaviors remains unknown.

The functional characterization of distinct piriform output pathways, through manipulation of neural activity, is complicated by the fact that OB mitral and tufted cells project to multiple higher olfactory centers in cortex, which, in turn, are strongly interconnected (Franks et al., 2011; Illig and Haberly, 2003; Johnson et al., 2000; Luskin and Price, 1983; Mazo et al., 2017). Such parallel neural pathways may interfere with perturbations in the activity of subpopulations of piriform neurons, potentially confounding the interpretations of behavioral phenotypes. We therefore devised an alternative experimental strategy to probe the functional properties of distinct piriform subpopulations, which is based on the direct optogenetic activation of piriform neurons and allows us to test the sufficiency of distinct piriform subpopulations to drive a behavioral response. We find that subpopulations of projection neurons in the deep piriform layers IIb and III, selectively targeted based on their connections with the OB or the mPFC, reliably drive a learned escape behavior, upon association of light-induced neural activity with an unconditioned aversive stimulus. In contrast, neurons in superficial piriform layer IIa, targeted based on their connections with the CoA or the IENT failed to support this learned escape behavior under our experimental conditions. Our data provide evidence for differences in the ability of piriform subnetworks to link neural activity with behavioral output.
Results

OB- and lENT-projecting piriform neurons have distinct output targets in the anterior telencephalon

To confirm and extend the characterization of the projection targets of distinct classes of piriform neurons we used an intersectional viral tracing approach. We injected the retrogradely transported Canine Adeno Virus 2 expressing Cre recombinase (CAV2-Cre) (Junyent and Kremer, 2015; Schwarz et al., 2015) into the OB and the lENT of adult mice, and we infected piriform cortex neurons with conditional Adeno-Associated Virus expressing channelrhodopsin (AAV-ChR2-EYFP). Cre-mediated recombination in piriform neurons results in the expression of ChR2-EYFP in distinct subpopulations of piriform projection neurons. ChR2-EYFP is robustly expressed on cell somata and axon terminals, and thus provides an anterograde neural tracer to identify brain regions innervated by piriform fibers. As previously reported, we observed that OB- and lENT-projecting piriform neurons can be distinguished based on their laminar positioning and morphology (Diodato et al., 2016, Figure 2, and data not shown). We then analyzed EYFP fluorescence on axonal processes in coronal sections through the brain. As expected, in mice in which CAV2-Cre was injected into the OB, we detected dense labeling of EYFP-positive axons in the granule cell layer of the OB (Figure 1e). In addition, we observed EYFP-positive axons in the mPFC, the OT, and the insular cortex (IC) (Figure 1f). These data suggest that OB-projecting neurons send axon collaterals to several other target areas in the anterior telencephalon. In contrast, targeting lENT-projecting neurons resulted in only minimal labeling of the OB, the mPFC, the OT, and the IC (Figure 1h, i). Labeling could be observed in superficial fiber tracts in the anterior telencephalon, however, the precise projection targets of these fibers could not be resolved in this analysis. We also observed differences in the more posterior projection targets of piriform neurons targeted by CAV2-Cre injections into the OB and the lENT (Figure 1g, j). However, the potential spread of virus into adjacent areas including the CoA and the lENT precluded a more detailed analysis. Taken together, these experiments demonstrate that distinct piriform projection neurons can be targeted using
intersectional viral-genetic tools, and that OB- and lENT-projecting piriform neurons have largely non-overlapping target areas.
Photostimulation of OB- and CoA-projecting piriform neurons activates distinct subnetworks of neurons

We next photoactivated distinct ChR2-expressing subpopulations of piriform projection neurons and monitored light-induced neural activity using immunohistochemical detection of the transcription factor c-Fos. Stimulation through implanted optical fibers in piriform cortex resulted in robust induction of c-Fos immunoreactivity (Figure 2a, c). While the numbers of c-Fos-positive neurons were highly variable (number of Fos neurons following photostimulation of OB-projecting neurons, average per mouse: 6356, 4433, 114, and 161 and number of Fos neurons following photostimulation of CoA projecting neurons, average per mouse: 665, 926, 1003, 480), likely due to the variability of the numbers of infected neurons and the efficiency of their activation by light, the laminar distribution of c-Fos-positive neurons was consistently different between the two targeted subpopulations. When photostimulating OB-projecting neurons, c-Fos-positive cells were predominantly observed in the deep piriform layers IIb and III (Figure 2b). In contrast, when stimulating CoA-projecting neurons, c-Fos expression was robustly enriched in superficial piriform layer IIa cells (Figure 2d).

It is important to note that light-induced c-Fos expression was not confined to ChR2-expressing piriform neurons. c-Fos-positive, ChR2-negative neurons were observed within and outside of the targeted sublayer of piriform cortex, i.e. layers IIb and III for OB-projecting neurons, and layer IIa for CoA-projecting neurons. The spread of c-Fos immunoreactivity beyond the neurons directly responsive to photostimulation is consistent with the activation of piriform neurons through extensive recurrent excitatory connections (Franks et al., 2011; Poo and Isaacson, 2011). Nevertheless, despite such intra-cortical propagation of light-induced neural activity, photoactivation of OB- and CoA-projecting neurons resulted in the activation of distinct ensembles of piriform subnetworks.
Different classes of piriform projection neurons differ in their ability to drive learned aversion

We next adapted a previously established behavioral paradigm, in which photoactivation of piriform neurons paired with foot shock drives a learned escape behavior (Choi et al., 2011). To test whether the activity of distinct subpopulations of piriform neurons was sufficient to elicit conditioned aversion, we selectively expressed ChR2 in OB- and CoA-projecting neurons. An additional cohort of mice, in which we expressed the calcium indicator GCaMP3 instead of ChR2 in piriform neurons, was used to control for the specificity of light-induced neural activity as the conditioned stimulus (CS). Training was performed in a rectangular box, in which mice could move freely. Foot shock as the unconditioned stimulus (US) was applied to the side of the box where the mouse was located at the time of photostimulation, allowing the mouse to escape from the aversive stimulus by running towards the opposite side of the box. The CS-US presentation was randomly applied to either side, depending on the location of the mouse. After two training sessions, mice were tested in a box with identical dimensions, to determine whether photoactivation of neurons alone was sufficient to elicit learned aversion.

Escape behavior was quantified using three parameters. We measured the maximum speed of mice and the distance traveled, during a 10 second (s) time window before and after the beginning of photostimulation. Furthermore, we determined the time between the beginning of photostimulation and a behavioral response (“reaction time”) during the same time period. For each mouse, data were averaged across 7 individual trials. Similar results were obtained when analyzing individual trials independently (Supplementary Figure 1). We found that photostimulation of OB-projecting neurons reliably elicited a robust behavioral response. Mice exhibited an increase in the maximum speed after photostimulation, and increase in the distance traveled, and a short reaction time (maximum speed before vs. after photostimulation: 19.5 vs. 30.6 cm/s, p = 0.0098; distance run before before vs. after photostimulation: 35.7 vs. 44.4 cm, p = 0.2324; reaction time: 4.2 s; Figure 3c-e). In contrast, photoactivation of CoA-projecting neurons failed to produce a behavioral response (maximum
speed before vs. after photostimulation: 18.3 vs. 17.8 cm/s, \( p = 0.5887 \); distance run before vs. after photostimulation: 31.2 vs. 28.9 cm, \( p = 0.4961 \); reaction time: 6.7 s; Figure 3c-e). A similar lack of behavioral response was evident in control mice, in which GCaMP3 instead of ChR2 was expressed in piriform neurons (maximum speed before vs. after photostimulation: 10.53 vs. 8.7 cm/s, \( p = 0.4403 \); distance run before vs. after photostimulation: 16.3 vs. 15.6 cm, \( p > 0.99 \), reaction time: 8.5 s; Figure 3c-e).

To further extend our analysis we next targeted mPFC- and lENT-projecting piriform neurons. mPFC-projecting neurons substantially overlap with OB-projecting neurons, while lENT-projecting neurons largely overlap with CoA-projecting neurons (Diodato et al., 2016). Based on the observations described above, we predicted that mPFC-projecting neurons, similar to OB-projecting neurons, may support a conditioned behavioral response, while lENT-projecting neurons, similar to CoA-projecting neurons, may fail to do so. We found that photoactivation of mPFC-projecting neurons was indeed sufficient to elicit learned aversion, reflected in an increase in the maximum speed and distance traveled, and a short reaction time (maximum speed before vs. after photostimulation: 12.76 vs. 21.75 cm/s, \( p = 0.0625 \); distance run before vs. after photostimulation: 24.5 vs. 32.3 cm, \( p = 0.313 \), reaction time: 5.5 s, Figure 3c-e). In contrast, photoactivation of lENT-projecting neurons failed to produce a reliable behavioral response (maximum speed before vs. after photostimulation: 10.68 vs. 9.87 cm/s, \( p = 0.5887 \); distance run before vs. after photostimulation: 20.2 vs. 15.4 cm, \( p = 0.3095 \), reaction time: 8.5 s, Figure 3c-e). Finally, to compare between different cohorts of mice, which can exhibit differences in baseline motor activity, we calculated the escape ratio, defined by the maximum speed after photostimulation divided by the maximum speed before photostimulation (Figure 3f). This analysis further illustrates that OB- and mPFC-projecting subpopulations of piriform neurons are sufficient to drive a learned escape behavior, while CoA- and lENT-projecting neurons fail to do so. Taken together, these data identify functional differences in the ability of distinct piriform subnetworks to relate neural activity to behavioral output.
Behavioral output does not correlate with the number of active neurons

Differences in the ability of distinct subpopulations of piriform neurons to drive learned aversion may reflect differences in the numbers of photoactivated neurons, rather than functional differences between different piriform subnetworks. To test this possibility, we trained an additional cohort of mice, and we determined the numbers of c-Fos-positive neurons directly after behavioral testing. Consistent with our results described above, we observed higher escape ratios in mice in which OB- and mPFC-projecting neurons had been targeted, compared to two mice in which CoA-projecting neurons had been targeted (one mouse with OB-targeted neurons: escape ratio 1.49, one mouse with mPFC-targeted neurons: escape ratio 2.14, two mice with CoA-targeted neurons: escape ratios 1.25 and 1.11). However, we found fewer c-Fos-expressing neurons in the OB- and mPFC-targeted mouse compared to the two CoA-targeted mice (mouse with OB-targeted neurons: 373 c-Fos-positive piriform cells, mouse with mPFC-targeted neurons: 241 c-Fos-positive cells; mice with CoA-targeted neurons: 599 and 412 c-Fos-positive cells). Thus, a higher number of photoactivated, c-Fos-positive neurons does not correlate with an increase in learned aversion, suggesting that functional differences in the different neural subnetworks underlie the observed differences in the behavioral responses.
Discussion

We established a viral-genetic approach to target distinct subpopulations of piriform projection neurons, and we tested their ability to relate neural activity to behavioral output. We found that neurons in the deep piriform layers IIb and III were capable of eliciting robust escape behavior upon pairing of light-induced neural activity with foot shock as the unconditioned stimulus. In contrast, targeting superficial piriform layer IIa cells failed to produce a behavioral response under these experimental conditions. Our data thus identify functional differences amongst different piriform output pathways.

We previously showed that OB- and mPFC-projecting piriform neurons are enriched in piriform layers IIb and III, exhibit the morphological characteristics of superficial and deep pyramidal cells, and express molecular markers that delineate their projection target specificity (Diodato et al., 2016). In contrast, the majority of CoA- and lENT-projecting neurons are piriform semilunar cells and are located in the superficial piriform layer IIa. Previous work further suggests that piriform projection neurons can extend axon collaterals to multiple target regions (Diodato et al., 2016; Mazo et al., 2017). Neural tracing experiments using the retrogradely transported CAV2 virus provide an opportunity to label the entire axonal projections of neurons, based on a single selected output target area (Schwarz et al., 2015). We found that OB-projecting neurons elaborate axons to multiple additional target areas, including the mPFC, IC, and the OT. In contrast, axons emanating from lENT-projecting neurons only minimally innervated these brain regions. An important limitation of this experimental approach is the likely spread of conditional AAV-ChR2-EYFP virus into areas adjacent to piriform cortex. While viral infection was well confined within piriform layers and across its dorso-ventral axis, viral spread appeared to be less confined along the antero-posterior axis. We observed a few infected neural cell bodies in regions posterior to piriform cortex, such as in the amygdala and entorhinal cortex (data not shown). Therefore, our analysis of axonal projection patterns is potentially confounded by labeled axons from nearby regions, in particular in more posterior brain regions. Future experiments using sparse and focal expression of a neural tracer,
combined with the tracing of individual axons from identified neurons in cleared brain preparations should provide improved resolution to resolve these questions.

Photostimulation of ChR2-expressing piriform neurons resulted in c-Fos immunoreactivity in ChR2-positive and ChR2-negative neurons, both within and beyond the targeted piriform sub-layers (Figure 2). Such propagation of light-induced neural activity is likely to reflect the activity of excitatory connections within and across piriform layers (Franks et al., 2011; Suzuki and Bekkers, 2011). However, despite extensive intracortical connectivity, photostimulation of distinct ChR2-expressing subpopulation of piriform neurons resulted in the activation of piriform ensembles with distinct laminar distribution. It will be interesting to explore patterns of light-induced neural activity beyond the piriform cortex. The analysis of c-Fos expression patterns in direct piriform target areas as well as in more distant neural processing centers may provide insights into how neural activity is propagated through neural circuits in the brain to elicit motor output.

Photoactivation of OB- and mPFC-projecting piriform neurons effectively drove learned aversion. Two training sessions, each consisting of 10 pairings of photoactivation with foot shock, were sufficient to elicit a robust escape behavior when photoactivation was presented in the absence of foot shock during testing. In contrast, photoactivation of CoA- and lENT-projecting neurons did not produce a behavioral response under the same experimental conditions. A trivial explanation for this observation could be that viral injections into the CoA and the lENT did not target a sufficiently large population of piriform neurons. We consider this explanation unlikely, based on the following two observations. First, the number of light-induced c-Fos-positive neurons was highly variable when targeting OB-projecting neurons (Figures 2). Despite such variability, we found that OB-targeted neurons reliably elicited a robust escape behavior. Second, we tested, albeit in a small cohort of mice, whether the number of c-Fos-positive neurons correlated with escape behavior. We found that fewer than 400 c-Fos-positive neurons in OB- and mPFC-targeted mice were sufficient to elicit conditioned aversion, while more than 400 c-Fos-positive neurons in CoA-targeted mice failed to elicit the behavioral response. A more plausible explanation of the observed
differences is that distinct piriform subpopulations require a different photostimulation regimen to entrain a learned behavior. For example, photostimulation of CoA- or lENT-projecting neurons at different frequencies and/or laser power may support the association of foot shock with a behavioral response. Such a model would suggest that functional differences between the piriform subnetworks, such as differences in neural excitability and network plasticity underlie the observed differences in behavioral output.

An alternative possibility consistent with our observations is that distinct subnetworks of piriform neurons are specialized to control different behavioral responses. Fear conditioning in mice, for example, can result in escape behavior or freezing, depending on experimental constraints (Fadok et al., 2017). It will therefore be interesting to test if in conditions in which mice cannot escape from the unconditioned stimulus, CoA- and lENT-projecting neurons can support a conditioned freezing response. Finally, different subpopulations of piriform neurons may be dedicated to support behaviors of different valence, such as aversive or appetitive conditioning, and experience-dependent social behaviors (Choi et al., 2011). The experimental approach we have developed provides an opportunity to probe the sufficiency of different piriform output pathways to support different learned behaviors.
Methods

Mice
Adult (8- to 12-week-old) C57BL/6/J male wild-type mice were used in this study. Mice were housed at the animal facility at the CIRB, Collège de France. All experiments were performed according to European and French National institutional animal care guidelines (protocol number B750512/00615.02).

Immunohistochemistry
Mice were deeply anaesthetized with pentobarbital and transcardially perfused with 20 ml of PBS, followed by 10 ml of 4% paraformaldehyde. Brains were post-fixed for 4 h in 4% paraformaldehyde at 4 °C. Coronal sections (200 µm thick) were prepared using a vibrating-blade microtome (Microm Microtech). Sections were rinsed in PBS and permeabilized in PBS/0.1% Triton X-100 for 1 h, and blocked in PBS/0.1% Triton X-100/2% heat-inactivated horse serum (Sigma) for 1 h. After incubation with primary antibodies at 4 °C overnight, sections were rinsed in PBS/0.1% Triton X-100, three times for 20 min at room temperature, blocked in PBS/0.1% Triton X-100/2% heat-inactivated horse serum for 1 h and incubated with secondary antibodies overnight at 4 °C. The following antibodies were used at the indicated dilutions: rabbit anti-cfos 1:500 (Santa Cruz sc-7270), and chicken anti-GFP 1:1000 (Abcam ab13970). Appropriate secondary antibodies (1:1000) conjugated to Cy3 (Jackson Labs) or Alexa 488 (Molecular Probes) were incubated together with Neurotrace counterstain (1:500, Invitrogen). Sections were mounted on SuperFrost Plus (Menzel-Gläser) microscope slides in Fluorescent Vectashield Mounting Medium (Vector). Images were acquired with a Leica SP5 confocal microscope, with 10x or 20x objectives.

c-Fos stimulation protocol
Animals injected with CAV2 virus expressing Cre-recombinase in the region of interest (CAV2-Cre), and conditional AAV vectors carrying channelrhodopsin (ChR2-eYFP) were photostimulated (20Hz, 30sec per minute for 10minutes) and processed for immunohistochemical analysis one hour later.

Quantification of c-Fos positive neurons
Immunohistochemistry was performed on piriform sections obtained 0.8–1.2 mm posterior to bregma. The percentage of neurons expressing c-Fos at the center of the injection site (3 histological sections) was obtained by manual counting. All image processing and quantification
was performed in Fiji and Adobe Photoshop CS5. A given field of view was divided into 15 bins, and the fraction of cells in each bin was calculated as the total number of c-Fos+ cells in each bin divided by the total number of cells in the field of view.

**Stereotaxic viral injections and fiber implantation**
Mice were anaesthetised intraperitoneally with ketamine/xylazine (100 and 20 mg per kg of body weight, respectively) and prepared for surgery in a stereotactic frame (David Kopf Instruments). For viral injections, a small craniotomy was made above the injection site. For anterograde neural tracing experiments and behavior experiments, 0.7 µl of AAV5.hSyn.hChR2 (H134R)-eYFP.WPRE.hGHpA was stereotaxically injected into the piriform cortex. In the control group, 0.6 µl of AAV1.hSyn.GCamp3-eYFP.WPRE.hGHpA was injected in the piriform cortex. 0.3 µl of canine adenovirus (CAV)-2-Cre was injected into the OB, the CoA, the mPFC or the lEnt. Virus was injected using a glass pipette with a 10–20 µm tip diameter. AAVs were obtained from the University of Pennsylvania (Penn Vectors). CAV2-Cre was obtained from the Montpellier Vector Platform (PVM). The following coordinates, based on the Paxinos and Franklin Mouse Brain Atlas were used: piriform cortex: anterior-posterior (AP) -0.60 mm, medio-lateral (ML) 3.95 mm, dorso-ventral (DV) -3.97 mm; OB: AP 0.75 mm and ML -0.75 mm coordinates from the midline rhinal fissure, DV -0.70 mm; medial PFC (including the prelimbic (PrL), infralimbic (IL), and cingulate (Cg) cortex): AP 0.54mm, ML 0.36mm, DV -1.70mm; posteromedial cortical amygdaloid nucleus: AP -2.80, ML 2.76, DV -4.8; IENT: AP -3.90, ML 3.8, DV 2.90.

For behavior experiments, optical fiber implantations were performed directly after viral injection; the skull was cleaned and covered with a layer of Super Bond C and B (Phymep). An optical fiber (200µm, 0.22 NA) housed inside a connectorized implant (SMR, Doric Lenses) was inserted into the brain, with the fiber tip positioned 200µm above the infection target (piriform cortex). The implants were secured with dental acrylic (Pi-Ku-Plast HP 36, Bredent). Before being moved back to their cages, after the surgery, mice were put in a warm chamber at 37°C until full recovery.

**Aversive behavior paradigm**
Photostimulation protocol
All animals were single-housed and kept on a 12h/12h day/night cycle. Behavioral tests began 13 days post-surgery, and were carried out 6 hr after onset of the day period. Behavioral conditioning took place over 5 days. On the first day, mice were habituated to the fear conditioning box for 40 min, on the following day and on the fourth day, the mice were trained to associate the photostimulation with the foot shock and on the last day mice were tested, 24 hr after the second training session.
The optical fiber implant was connected to a mono fiber patch cord (MFP_200/240/900-0.22_FC-SMC, Doric Lenses) that was connected to a fiberoptic rotary joint (FRJ_1x2i_FC-2FC_0.22, Doric Lenses), which receives the light that passes through galvanometric mirrors from the free-space DPSS laser beam (MBL-III-473, CNI lasers). Laser output was maintained at 7-10 mW as measured at the end of the fiber. The conditioning apparatus was a rectangular chamber (9 cm W x 57 cm L x 16 cm H) with a stainless-steel rod floor. Each half of the conditioning apparatus was connected to an electrically operated switch, which was connected to an aversive stimulator (115 V, 60 Hz, Med Associates) and to a microcontroller board (Mega 2560, Arduino), allowing foot-shock to be applied independently to either side. The testing apparatus was a rectangular chamber (9 cm W x 57 cm L x 16 cm H) with a white PVC floor. Before each training session, laser beam output intensity and shape was adjusted and electrical current in the rod floor was measured with an aversive stimulation current test package (Med Associates). Photostimulation and foot shock were controlled using Arduino 1.8.3 open software.

Experimental animals were allowed to habituate to the apparatus for 5 min. The conditioning paradigm consisted of 3s of photostimulation (20 Hz square-wave shape, 25ms pulses) followed immediately by a 0.5s, 0.65 mA foot shock. Foot shock was applied only when the animal was in or near either end of the apparatus, forcing the animal to run to the opposite side. Photo stimulation/shock pairings were spaced 3-4 min apart. Each of the two training sessions consisted of 10 photo simulation/foot shock pairings, for a total of 20 pairings. Photo stimulation was applied 7 times over the testing session, every 3-4 min. All sessions were video-recorded.

Videos recorded during the testing session were analyzed during the 10 seconds time period before and after stimuli presentation using a custom-written Matlab script to quantify: maximum speed before and after stimuli presentation, distance run before and after stimuli presentation and reaction time after stimuli presentation.

Statistics
Statistical analysis was performed in R and GraphPad Prism 7 software. Behavioral responses per mouse and per trial for speed and distance were compared before and after CS+ presentation and analysed for statistical significance using the non-parametric Wilcoxon test. Behavioral responses in between mice and trials of different behavior conditions for reaction time and flight ratio were analysed for statistical significance using the non-parametric Mann-Whitney test. In Figure 3 and Supplementary Figure 1, p values ≤ 0.05 are represented with *, p value ≤ 0.01 are represented with **, p value ≤ 0.001 are represented with *** and p values ≤ 0.0001 are represented with ****.
References


Figure 1. Target areas of OB- and IEnt-projecting piriform neurons. Schematic representations of the injection sites of conditional AAV-ChR2-EYFP in the piriform, and CAV2-Cre virus in the OB (a) and in the IEnt (c), below is represented the piriform injection site (b,d). Coronal sections of the target areas of OB-projecting piriform neurons (e-g) and of IEnt-projecting piriform neurons (h-j) to the olfactory bulb (OB), medial prefrontal cortex (mPFC), insular cortex (IC), olfactory tubercle (OT) and the lateral entorhinal cortex (IEnt). EYFP labeling in green, neurotrace counterstain in blue. (k-m) In dark grey, are represented the main target regions of piriform neurons; below the reference AP coordinates of each coronal section. n (number of mice) = 4, 4 sections per mouse.
Figure 2. Photoactivation of distinct subpopulation of piriform projection neurons. Immunohistochemical analysis of coronal sections of piriform cortex after photostimulation of subpopulations of ChR2-expressing neurons. AAV-ChR2-EYFP-expressing neurons are in green, c-Fos immunoreactivity in red, neurotrace (NT) in blue. (a) Subpopulations of neurons targeted via their projections to the olfactory bulb (OB). (c) Subpopulations of neurons targeted via their projections to the cortical amygdala (CoA). (b,d) Laminar distribution of c-Fos-expressing neurons upon photostimulation of OB- and CoA-targeted subpopulations. c-Fos-expressing neurons are enriched in the superficial layer IIa in CoA-targeted subpopulations, while c-Fos expression is observed in deeper piriform neurons in OB-targeted mice. n (number of mice) = 4.
Figure 3. OB- and mPFC-projecting, but not CoA- and IENT-projecting piriform neurons are sufficient to drive learned aversion. (a) Fear conditioning assay set up and optogenetics in freely moving mice. (b) Schematic representation of the injection sites of AAV-flex-ChR2 in the Piriform and CAV2-Cre in the different target areas: OB, mPFC, CoA and IEnt. (c) Maximum speed in cm/s, measured during 10 s windows before and after photostimulation, of mice in which distinct subpopulations of piriform neurons were entrained to drive a learned escape behavior. OB-projecting neurons: n = 10, CoA-projecting neurons: n = 9, mPFC-projecting neurons: n = 5, IENT-projecting neurons: n = 6, and AAV-GCamp3 expressing neurons (control group), n = 10. (d) Distance traveled before and after photostimulation. (e) Reaction time after photostimulation. (f) Flight ratio, defined as the maximum speed after photostimulation, divided by the maximum speed before photostimulation. Each dot represents data obtained from individual mice, averaged across seven trials. Bars represent the mean +/- SEM.
Supplementary Figure 1. OB- and mPFC-projecting, but not CoA- and lENT-projecting piriform neurons are sufficient to drive learned aversion. 
a) Maximum speed per trial in cm/s, measured during 10 s windows before and after photostimulation, of mice in which distinct subpopulations of piriform neurons were entrained to drive a learned escape behavior. OB-projecting neurons: n = 66, CoA-projecting neurons: n = 62, mPFC-projecting neurons: n = 32, lENT-projecting neurons: n = 42, and AAV-GCamp3 expressing neurons (control group), n = 69. 
(b) Distance traveled per trial before and after photostimulation. 
(c) Reaction time per trial after photostimulation. 
(d) Flight ratio, defined as the maximum speed after photostimulation, divided by the maximum speed before photostimulation per trial. Each dot represents individual trials. Bars represent the mean +/- SEM.
3.2.3 Summary

Taken together, our experiments have contributed to functionally characterize different subpopulations of neurons in the piriform cortex. We have demonstrated, using optogenetic tools and a fear conditioning behavioral assay, the sufficiency of excitatory neurons and the insufficiency of inhibitory neurons to drive an aversive behavior. Additionally, distinct subpopulations of piriform neurons that segregate into distinct layers of the piriform cortex are functionally distinct. OB- and mPFC-projecting piriform neurons are sufficient to drive a behaviour, but CoA- and lEnt-projecting piriform neurons are not capable of driving an aversive behavior. Thus, the ability to drive learned aversion depends of neural activity patterns in the piriform cortex. Characterizing light-induced neural activity in direct piriform target areas as well as more distant neural circuit components will shed light on how neural activity is coupled to motor output.
4. DISCUSSION
4.1 Odor concentration invariance

It was our initial goal to understand how mice could generalize olfactory perception and behavior across a range of different odor concentrations, and what neural circuit mechanisms could be responsible for this odor concentration-invariance. As an experimental approach we decided to perturb the activity of piriform PV cells during an olfactory fear-conditioning task using opto- and chemogenetics.

Silencing PV-cell in an odor concentration-independent conditioning task did not consistently impair the behavioral response across a range of concentrations of the conditioned odor stimulus. However, we have observed high variability of the behavioral responses, which limits the interpretation of our results. Using an alternative behavior assay, such as the appetitive operant conditioning I set up, we could test the behavioral response using data obtained during testing sessions over extended periods of time and consisting of hundreds of trials, thus providing higher statistical power. Furthermore, we could more precisely control the concentration and duration of the odor presentation, as the mouse would be head-fixed and exposed to the odor stimulus in a highly reproducible manner. Finally, the range of concentrations we tested in our behavior paradigm (0.2%, 1% and 5% dilutions of pure odorant in mineral oil) may not have been large enough to require a significant modulation in PV cell activity to support a concentration-invariant behavioral response. Extending the concentration range of odors would likely increase the requirements for the olfactory system to efficiently normalize signal intensity.

By silencing PV cells in an odor concentration-independent behavior we have tested the necessity of piriform PV cells for odor concentration invariance. Overall activity in piriform cortex does not scale with increasing odor concentrations (Bolding and Franks, 2017) but PV cell activity is increased with increasing concentrations of an odor (see Results, Figure 1). We therefore hypothesized that by silencing PV cell in the presence of increasing concentrations of an odor, odor-evoked neural activity would increase with odor concentration, thus perturbing concentration-invariant piriform odor representations. However, in vivo imaging experiments in anesthetized mice, performed in parallel with the behavioral experiments described in this thesis, failed to show robust concentration-dependent changes in piriform odor responses (B. Roland and A. Fleischmann, unpublished).

What are plausible explanations for the observed lack of phenotypes? First, it could be argued that piriform PV cells simply do not have a function in mediating signal normalization in the piriform cortex. This would suggest that other piriform neural circuit components play important functions in mediating concentration-invariance. Alternatively, opto- and chemogenetic manipulations of piriform PV cell activity could elicit compensatory changes in network function, such that other neural cells types could take over. Different classes of piriform interneurons may contribute to the
stabilization of cortical odor representations. For example, SST or VIP interneurons might be recruited to compensate for the disinhibition of excitatory neurons and thus maintain the representation of odors sparse and dispersed. Interestingly, however, in the absence of SST interneuron activity in the piriform cortex, pyramidal neurons and PV interneurons are disinhibited. This was demonstrated by extracellular recordings in the piriform of SST-Cre mice exposed to odors, following photo-suppression of SST neurons (Sturgill and Isaacson, 2015). Thus, SST cells regulate sensory response via subtractive inhibition, independent of odor stimulus intensity.

In the primary visual cortex of mice, the interplay of PV and SST cell activity has been implicated in signal normalization and orientation tuning of visual cortex principle cells. While initial reports on the precise functions of PV and SST cells in visual cortex have been contradictory, further studies showed that differences in the experimental design, such as in the duration of laser stimulation during optogenetic manipulations, can account for the observed differences. Furthermore, compensatory changes in the recruitment of PV cells provide a potential explanation for the observed effects of SST cell silencing on principal cell activity. Importantly, no behavioral correlate for PV and SST cell function in mouse visual cortex has yet been reported (Pfeffer et al., 2013; El-Boustani and Sur, 2014; Atallah et al., 2012; Lee et al., 2014).

4.1.1 Future directions
An alternative experimental approach to assess the function of neural circuit components is to artificially activate neurons, independently from the natural stimulus. By decoupling neural activity from stimulus control one can inject noise into the system and assess the behavioral consequences of such perturbations. We have shown that mice can detect and identify an odorant across a range of different concentrations, and respond with a learned escape behavior to the odor that was previously paired with an unconditioned aversive stimulus. If piriform PV cell activity is required for odor concentration-invariance, artificially activating piriform PV cells through opto- or chemogenetic activation independent from the odor stimulus may be expected to interfere with, or delay the acquisition of this task.
4.2 Piriform efferent pathways and odor fear learning

Piriform cortex is an associative cortex, which is thought to link sensory input with context and experience. It is connected to multiple brain areas involved in learning and memory. Here, we have demonstrated, using viral-genetic and optogenetic tools to manipulate the activity of specific piriform neural cell types, that different piriform output pathways differ in their ability to drive learned aversion. First, we showed that excitatory, but not inhibitory neurons are sufficient to drive a learned aversion. Second, we found that piriform neurons projecting to the OB or mPFC, but not piriform neurons projecting to the CoA or lEnt can support efficient olfactory fear conditioning. Potential explanations for the observed functional differences in piriform subpopulations of neurons may be explained by differences in target specificity and/or intrinsic properties. For example, OB-projecting piriform neurons are enriched in layers IIb and III and have the morphological characteristics of pyramidal neurons. CoA-projecting piriform neurons are located in layer IIa and have the morphology of semilunar cells. Semilunar cells and pyramidal neurons differ in local connectivity: while pyramidal neurons form functional connections with other neurons in the piriform cortex (Franks et al., 2011), SL cells form little or no recurrent connections with local excitatory neurons (Suzuki and Bekkers, 2011; Suzuki and Bekkers, 2006; Choy et al., 2017). These findings are consistent with our observations that OB- and CoA-projecting piriform neurons activate distinct subnetworks of neurons. OB- projecting piriform neurons activate predominantly deep cells in piriform layers IIb and III, while CoA-projecting piriform neurons activate superficial piriform layer IIa cells.

What cellular or network characteristics are required to drive learned aversion? Is network function encoded in the cell type, its connectivity, or in the plasticity driven by the activation of the distinct subsets of neurons? A possibility consistent with our observations is that distinct subsets of piriform neurons are specialized to control different behavioral responses. Thus, it will be interesting to test if complementary to the learned aversion, in a behavioral task of different valence, such as appetitive conditioning, the same output pathways are sufficient to drive behavior.

Furthermore, we observed that some mice following photostimulation of CoA-projecting piriform neurons, but not of OB-projecting piriform neurons, displayed freezing behavior. Our behavioral experiments are designed to quantify escape behavior, but do not allow for more detailed analysis of freezing behavior. It is surprising that CoA- projecting piriform neurons are not sufficient to drive an escape behavior even though the CoA is involved in multiple aversive behaviors (Root et al., 2014). Thus, additional behavioral tests, combined with more detailed analysis of mouse behavior, could yield interesting additional information about the coupling of neural activity with different behavioral outputs. Recent 3D imaging tools combined with computational models allow the
identification of behavior modules expressed during a variety of experiments and the characterization of mouse behavior at a sub-second spatiotemporal scale when stimuli are presented (Wiltschko et al., 2015).

4.2.1 Future directions

1) Test the necessity of piriform output pathways to drive learned aversion

We have demonstrated the sufficiency of OB and mPFC output pathways of the piriform cortex to drive learned aversion. Are these piriform output pathways also necessary to drive the behavioral response? The Kremer lab (Institute of Molecular Genetics, Montpellier) has developed new viral tools to test the necessity of specific output pathways to drive a behavior.

We will inject in WT mice a retrogradely transported CAV2 virus expressing the Cre recombinase under the doxycycline-inducible promoter (CAV.rtTACre) in the output target of the piriform we want to silence: OB and mPFC. In the piriform, we will co-inject: a lentivirus expressing Flp recombinase (LV.Flp) and an AAV expressing ChR2 in the presence of Flp recombinase and absence of Cre recombinase (AAV-hSyn CreOFF/FlpON hChR2(H134R)-EYFP) (Fenno et al., 2014). This viral strategy will allow for the conditional deletion of subpopulations of ChR2-expressing neurons projecting to the OB and mPFC. We will entrain random ensembles of piriform neurons to drive learned aversion, and after training delete OB- and mPFC-projecting neurons from the entrained ensemble. We hypothesize that mice will fail to exhibit the learned behavioral response, which would provide evidence for the necessity of the targeted neural pathways to relate neural activity to a learned behavioral response.

2) Viral genetic tools to silence and activate distinct neural networks in the mouse

The use of intron recombinase sites enabling combinatorial targeting (INTRSECT) and the combination of chemogenetics and optogenetics can be used to manipulate local or distal microcircuits engaged in driving a behavior.

The INTRSECT approach (Fenno et al., 2014) allows for the selection of neurons based on multiple features through the use of multiple recombinase enzymes and recognition sites (i.e., both Cre and Flp recombinases). For example, INTRSECT allows for the selection of neurons of a certain cell type that project to a region of interest, and it allows for the selection of neurons in one area that project to one target region but avoid another.

In addition to this approach, it is possible to employ both DREADD and optogenetic tools in parallel. Finally, optogenetic actuators have been developed that selectively respond to blue, yellow, green, orange, and red light (Guru et al., 2015). These variants can similarly allow for multiplexing within a single mouse, although the selectivity with which individual actuators are activated depends
on the experimental conditions, opsin pairs, and intensity of light delivery. Some opsin pairs offer minimal spectral overlap. Chrimson and Chronos for example, are activated by red and blue light, respectively, and have been effectively multiplexed in vivo enabling within-subject targeting of multiple cell types for excitation (Klapoetke et al., 2014). Similarly, Chrimson could be multiplexed with ChR2 as there is minimal overlap in the activation spectra. For multiplexed optogenetic excitation and inhibition, ChR2 can be multiplexed with eNpHR3.0 with minimal spectral overlap (Zhang et al., 2007).

3) Development of tools for tracing the entire axonal tree of piriform output pathways
We found that OB-projecting neurons co-project to multiple additional target areas, including the mPFC, IC, and the OT. In contrast, lENT-projecting neurons only minimally innervated these brain regions (see article in preparation).

Those findings were obtained by confocal imaging of histological brain sections. By sectioning the brain, we lose structural information, and the reconstruction of the neural output pathways is compromised. Thus, characterization of the axonal projections is best performed in intact tissue. We have used uDisco to image the entire half brain of OB-projecting and lEnt-projecting piriform neurons (Figure 1) (Pan et al., 2016). Clearing of the two half brains was obtained following uDISCO protocol, and we were able to image the entire cleared brains with good endogenous signal preservation (without performing immunohistochemistry). Even though it is possible to distinguish the OB and lEnt-projection patterns in Figure 1, the injection site is not specific to the piriform. Furthermore, we are improving the protocol to reduce the background fluorescence.
Figure 1. Distinct subpopulations of piriform neurons exhibit distinct projection patterns. Images of horizontal optical sections of two half brains after the uDISCO transparisation protocol. On the left panel, the piriform - OB pathway was targeted (co-injection of conditional AAV.ChR2 in the piriform and CAV2-Cre in the OB). On the right panel, the piriform - IEnt pathway was targeted (co-injection of conditional AAV.ChR2 in the piriform and CAV2-Cre in the IEnt). Projections to the OB, AON, LOT and IEnt are distinct. Heat map representation for the signal intensity, low signal is represented in blue and strong signal in yellow.
5. BIBLIOGRAPHY
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