Target-specific control of olfactory bulb periglomerular cells by GABAergic and cholinergic basal forebrain inputs

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Abstract (165 words)

The olfactory bulb (OB), the first relay for odor processing in the brain, receives dense GABAergic and cholinergic long-range projections from basal forebrain (BF) nuclei that provide information about the internal state and behavioral context of the animal. However, the targets, impact and dynamic of these afferents are still unclear. I studied how BF synaptic inputs modulate activity in diverse subtypes of periglomerular (PG) interneurons using optogenetic stimulation and loose cell-attached or whole-cell patch-clamp recording in OB slices from adult mice. GABAergic BF inputs potently blocked PG cells firing except in a minority of calretinin-expressing cells in which GABA release elicited spiking. Parallel cholinergic projections excited a previously overlooked PG cell subtype via synaptic activation of M1 muscarinic receptors. Low frequency stimulation of the cholinergic axons drove persistent firing in these PG cells thereby increasing tonic inhibition in principal neurons. Taken together, these findings suggest that modality-specific BF inputs can orchestrate synaptic inhibition in OB glomeruli using multiple, potentially independent, inhibitory or excitatory target-specific pathways.

Introduction

Basal forebrain (BF) nuclei innervate many regions of the brain including the entire neocortex, hippocampus, amygdala, thalamus and hypothalamus with diffuse long-range projections releasing GABA, ACh and, more rarely, glutamate. These projections provide cues about the behavioral context and internal state of the animal. They modulate multiple synaptic, cellular and network processes at a variety of temporal and spatial scales thereby regulating sensory perception, metabolic functions such as food intake, brain states and important cognitive functions including attention, arousal, memory or learning (Ballinger et al. 2016; Picciotto, Higley, and Mineur 2012).

The olfactory bulb (OB), the first region that processes olfactory information in the brain, receives massive cholinergic and GABAergic BF projections that principally originate in the nucleus of the horizontal limb of the diagonal band of Broca (HDB) and in the magnocellular preoptic nucleus (MCPO)(Zaborszky et al. 1986). Cholinergic signaling within the OB modulates olfactory learning and memory (Devore, de Almeida, and Linster 2014; Devore, Manella, and Linster 2012; Ravel, Elaagouby, and Gervais 1994; Ross, Bendahmane, and Fletcher 2019), odor discrimination (Chan et al. 2017; Chaudhury, Escanilla, and Linster 2009; Doty, Bagla, and Kim 1999; Li and Cleland 2013; Mandairon et al. 2006; Smith et al. 2015), odor habituation (Ogg et al. 2018) and social interactions (Suyama, Egger, and Lukas 2021). How ACh modulates these behavioral demands is less clear. Cholinergic axons innervate all layers of the OB and preferentially form synapses on inhibitory interneurons (Hamamoto et al. 2017; Kasa et al. 1995). Yet, a sparse subclass of deep short axon cells in the internal plexiform layer is the only synaptic target of BF cholinergic axons known to date (Case et al. 2017). Deep short axon cells are few compared to granule cells and PG cells, the two prevalent classes of GABAergic interneurons in the OB. Moreover, cholinergic synapses are particularly abundant in the glomerular layer (Hamamoto et al. 2017) where muscarinic receptors exert a strong control on intraglomerular inhibition (Liu et al. 2015) suggesting that PG cells are another likely target of cholinergic axons.

BF GABAergic afferents innervate all layers of the OB at least as densely as cholinergic axons but only few studies have examined their function in odor processing (Bohm, Brunert, and Rothermel 2020; Nunez-Parra et al. 2013). This is a difficult question because BF GABAergic afferents innervate many types of interneurons including granule and PG cells

which inhibit principal neurons (Hanson, Swanson, and Arenkiel 2020; Nunez-Parra et al. 2013; Sanz Diez, Najac, and De Saint Jan 2019; Villar, Hu, and Araneda 2021) as well as deep short-axon cells which inhibit granule and PG cells (Case et al. 2017; Sanz Diez, Najac, and De Saint Jan 2019). Thus, depending on their target, BF GABAergic inputs may inhibit or disinhibit principal neurons. Moreover, BF GABAergic inputs have target-specific release properties suggesting they arise from distinct populations of BF GABAergic neurons (Sanz Diez, Najac, and De Saint Jan 2019). Finally, the effect on principal neurons may be hard to predict as GABA is excitatory in some PG cells (Parsa, D'Souza, and Vijayaraghavan 2015) and can be co-released along with ACh from cholinergic terminals. Co-transmission of the two transmitters occurs onto deep short axon cells (Case et al. 2017) but it remains unclear whether co-release is systematic in the OB as it is in the hippocampus (Takacs et al. 2018) or target-specific as in the cortex (Desikan et al. 2018; Saunders, Granger, and Sabatini 2015).

In such complex context, an important step towards understanding the influence and function of the BF inputs in the OB is to investigate the connections, temporal dynamics and functional impact of each BF pathway. Recent papers have examined BF GABAergic inputs onto granule cells and their influence on adult-born granule cells survival (Hanson, Swanson, and Arenkiel 2020), on the firing of principal neurons and on low field potential rhythmic activity (Villar, Hu, and Araneda 2021). Here, I examined how synaptic release of GABA and ACh from BF fibers modulates the activity of the various PG cells. PG cells are small axonless GABAergic interneurons with dendritic projections in a single glomerulus. Collectively, they regulate glutamate release from olfactory sensory neurons (OSNs) terminals (Murphy, Darcy, and Isaacson 2005; Shao et al. 2009) as well as spike timing (Geramita and Urban 2017; Najac et al. 2015) and respiration-coupled theta rhythms in output neurons (Fukunaga et al. 2014; Villar, Hu, and Araneda 2021). PG cells are classified in two broad classes with largely similar morphologies but distinct excitatory inputs: type 1 PG cells receive direct excitatory inputs from OSNs whereas type 2 PG cells are not connected to OSNs and receive glutamatergic inputs from the dendrites of mitral and tufted cells (Shao et al., 2009). In our previous study we have shown that BF GABAergic inputs are an additional criterion that differentiate PG cell subclasses (Sanz Diez, Najac, and De Saint Jan 2019). This is summarized in Fig.1. First, BF GABAergic neurons contact type 2 PG cells but not type 1 PG cells (Fig.1A). Second, BF GABAergic IPSCs have target-specific time courses in three classes of type 2 PG

cells (Fig.1B) that also differ on the basis of their olfactory nerve (ON)-evoked excitatory response, firing properties and molecular markers (Fig. 1C). For clarity, I hereafter introduce a new nomenclature and call these three classes type 2.1, type 2.2 and type 2.3 PG cells. Type 2.1 cells correspond to the most abundant calretinin (CR)-expressing PG cells. They conserve intrinsic properties of immature neurons i.e. they have a remarkably high membrane resistance ($> 1 \text{ G}\Omega$, usually higher than in other PG cells), they do not fire or fire at most a single and often small action potential, they receive little excitatory inputs and their output is uncertain (Benito et al. 2018; Fogli Iseppe, Pignatelli, and Belluzzi 2016). Type 2.2 cells are labeled in the Kv3.1-eYFP mouse and includes calbindin (CB)-positive as well as CB-negative cells. They receive short bursts of excitatory inputs from mitral and tufted cells and, in turn, indiscriminately release GABA onto these principal neurons (Najac et al. 2015). Finally, type 2.3 PG cells are a previously ignored subclass of regularly firing PG cells with no known chemical marker, remarkably slow BF IPSCs and distinctive prolonged ON-evoked plurisynaptic excitatory responses (Sanz Diez, Najac, and De Saint Jan 2019). Their output connections have not been determined.

Figure 1. BF GABAergic inputs define three subtypes of PG Cells

A, Diagram of the glomerular microcircuit. PG cells are small GABAergic interneurons (red) surrounding each glomerulus. OSNs-innervated type 1 PG cells do not receive BF GABAergic inputs whereas type 2 PG cells do. Three subtypes of type 2 PG cells have been identified in Sanz Diez et al. (2019) based on their synaptic, molecular and intrinsic properties. See introduction for details. **B**, Representative optogenetically-evoked BF GABAergic inputs in the different subtypes of type 2 PG cells. Amplitudes are normalized for comparison. Data from Sanz Diez et al. 2019. **C**, Summary table of the properties that distinguish different PG cell subtypes. Cases are left empty when the properties have not been determined, are diverse or uncertain.

I used optogenetic stimulations in OB slices to induce synaptic release of ACh and/or GABA from BF fibers and patch-clamp recording to examine PG cells responses. The results demonstrate that PG cell subtypes are differentially controlled by BF afferents and reveal that the previously overlooked type 2.3 subtype is a central player in mediating BF muscarinic modulation of glomerular inhibition.

RESULTS

Synaptic release of ACh activates a subset of PG cells

ChR2 fused with eYFP was first targeted to BF cholinergic neurons by injecting a viral construct into the HDB/MCPO of *Chat*^{Cre} mice (Fig.2A). Choline acetyltransferase (ChAT) immuno-detection on brain sections from these mice (hereafter called ChAT mice) confirmed the expression of ChR2 in neurons expressing endogenous ChAT (Fig.2B). ChR2 expression, as indicated by eYFP labeling in the HDB/MCPO, was almost exclusively found in cholinergic neurons (89% of the cells positive for eYFP were also ChAT+, 440 double+ cells from a total of 495 eYFP+ cells in coronal slices from 4 mice) with an infection rate of 34% (440 double+ cells from a total of 1305 ChAT+ cells). Consistent with previous studies (Hamamoto et al. 2017; Kasa et al. 1995; Rothermel et al. 2014; Smith et al. 2015), eYFP-labeled cholinergic axons arising from BF cholinergic neurons densely innervated the OB and were abundant in the glomerular layer (Fig.2C and Fig.2-figure supplement 1).

Next, I used loose cell-attached (LCA) recording in acute OB slices from these mice to monitor spiking activity in randomly chosen PG cells while BF cholinergic axons were periodically stimulated every 2 seconds (0.5 Hz) using a single brief (1 ms) flash of blue light (Fig.2D). This photostimulation induced a significant increase in baseline spike frequency in 7% of the PG cells tested (n=23/350). No other kind of response was observed. It is noteworthy, however, that more than half of the cells tested were silent or fired only rarely. A cholinergic input may simply be unnoticeable in these cells using LCA spike recording. Responsive PG cells had a low baseline spiking activity in control condition (range 0-6 Hz, mean 1.2 ± 1.6 Hz, n=14) and switched to a higher frequency firing mode (mean frequency 5.3 ± 2.7 Hz, n=14) as soon as low frequency photostimulations started. This sustained spiking regime was maintained throughout the trials with a stimulation (>1 min)(Fig.2E-F).

Photo-evoked responses persisted in the presence of NBQX (10 μ M), D-AP5 (50 μ M) and mecamylamine (50 μ M)(n=5, Fig.2E-F), which inhibit AMPA, NMDA and nicotinic ACh receptors, respectively. This cocktail of antagonist blocks a possible direct nicotinic excitation

of PG cells (Castillo et al. 1999) as well as a putative indirect glutamatergic excitation following the nicotinic activation of mitral and tufted cells (Liu et al. 2015).

Figure 2: BF cholinergic neurons activate a subset of PG cells

A, Schematic of the virus injection in *Chat^{Cre}* mice (top) and coronal section (bottom), at about Bregma -0.1 mm, 20 days after injection. **B**, ChR2-eYFP expression (green) in ChAT-expressing cholinergic neurons (magenta) in the BF. Scale bar 50 μ m. Right panels, zoom on the boxed region. **C**, ChR2-eYFP-expressing axons in the OB. Scale bar 100 μ m. DAPI staining (blue) delimits layers (ONL: olfactory nerve layer; GL: glomerular layer; EPL: external plexiform layer; GCL: granule cell layer). Higher resolution image in Fig.2-figure supplement 1. **D**, Experimental design for recording BF-evoked responses in OB slices. **E**, Top, two representative 2 s-long LCA recording episodes (scale bar 200 pA) and raster plot of spiking activity in control condition and when BF axons were photostimulated every 2 s (blue arrow, 1 ms flash, 490 nm). Photostimulation started at episode 31 (1 flash/episode, blue line). Bottom, raster plot for the same cell, same experiment in the presence of blockers. **F**, Average firing rate per episode (2 s each) in ACSF (green) or in the presence of blockers (violet). Low frequency photostimulation (0.5 Hz) started at episode 31. **G**, representative recording (scale bar 50 pA), raster plot and PSTH (20 consecutive trials, bin 200 ms) of an excitatory response evoked in ACSF by a single photostimulation of the BF cholinergic axons at blue arrow and dotted line. Pale areas within the PSTH indicate the two periods that were compared in I. The non-

selective mAChR antagonist scopolamine (10 μ M) blocked the evoked excitation (bottom, scale bar 50 pA). **H**, Average firing rate per bin and per 15 s-long episode for 21 cells recorded in ACSF. Each grey line corresponds to a cell; the black line indicates the ensemble average. Photostimulation at blue arrow. **I**, Firing rate before (pre) and after (post) photostimulation of BF axons in ACSF (green), in the presence of NBQX, D-AP5 and mecamylamine (violet) or in the presence of the mAChR antagonist atropine (n=4) or scopolamine (n=4)(red). Each line indicates a cell; blue circles are the means. Paired t-test or Wilcoxon signed-rank sum test (for atro/scopo).

I then used a longer (15-20s) interval between each flash to examine the time-course of the excitatory response. A single flash evoked a reliable and long-lasting increase in firing rate (Fig.2G). Spike rate increased on average 3 fold compared to baseline activity (from 2.2 ± 2.1 Hz before the flash to 6.6 ± 3.3 Hz after the flash, n=21, Fig.2I), peaked about 1 s after the flash and returned to baseline frequency after about 5-10 s (Fig.2H). Evoked excitation persisted in the presence of NBQX, D-AP5 and mecamylamine (spike frequency increased from 2.1 ± 2.8 Hz before the flash to 8.0 ± 3.5 Hz after the flash, n=11, Fig.2I) and was totally blocked by atropine (10 μ M, n=4) or scopolamine (10 μ M, n=4), two non-selective antagonists of metabotropic muscarinic ACh receptors (mAChRs)(Fig.2G, 2I). Thus, transient temporally and spatially precise synaptic release of ACh from BF cholinergic axons strongly excites a subset of PG cells via the activation of mAChRs. The principal focus of this study is on this previously unknown cholinergic pathway.

PG cells excited by a muscarinic input also receive an inhibitory GABAergic input from separate BF neurons

Nearly all cholinergic neurons in the BF express the molecular machinery to both synthesize and package GABA into synaptic vesicles (Saunders, Granger, and Sabatini 2015). Synaptic co-transmission of GABA from cholinergic axons would be expected to block or reduce spiking if GABA is inhibitory. If GABA is excitatory, co-release of GABA would be expected to trigger spikes even in the presence of AChR antagonists. None of these possible GABAergic responses was seen in ChAT mice suggesting that GABA is not co-transmitted together with ACh. However, PG cells may still receive a GABAergic input from separate BF neurons. To test this possibility, a virus encoding ChR2-eYFP was injected into the HDB/MCPO of *dlx5/6*^{Cre} mice, a transgenic line that expresses the Cre recombinase in neurons originating from the embryonic ganglionic eminence during development, which includes GABAergic as well as cholinergic neurons in the forebrain (Monory et al. 2006)(Fig.3A). Accordingly, we showed in our previous study that recombination induces the

expression of ChR2-eYFP in several populations of GABAergic neurons as well as in ChAT-expressing cholinergic neurons in the BF (Sanz Diez, Najac, and De Saint Jan 2019).

Figure 3: In *dlx5/6* mice, a single photostimulation of BF axons evokes a biphasic inhibition-excitation response in PG cells with a muscarinic excitation

A, Schematic of the virus injection in the HDB/MCPO of *dlx5/6*^{Cre} mice. **B**, ChR2-eYFP expression in BF axons (yellow) in a sagittal section of the OB. DAPI staining (blue) delimits the different layers (GL: glomerular layer; EPL: external plexiform layer; MCL: mitral cell layer; GCL: granule cell layer). Scale bar 100 μm. Higher resolution image in Fig.3-figure supplement 1. **C**, Representative spiking response, raster plot and cumulative PSTH (10 consecutive sweeps, 200 ms/bin) of a typical biphasic inhibition-excitation response evoked by a single photostimulation of BF fibers and recorded over 15 s in a PG cell from a *dlx5/6* mouse. **D**, Average spiking frequency per bin (200 ms) and per episode. Each grey line corresponds to a cell. The black line is the ensemble average. Photostimulation at blue arrow. Only one cell in the dataset responded with a long-lasting excitation that was not preceded by an inhibitory component (Fig.3-figure supplement 3). **E**, The non-selective mAChR antagonist atropine (10 μM) blocked BF-evoked excitation. **F**, Firing rate before (pre) and after (post) photostimulation of BF axons in ACSF (green), in the presence of NBQX, D-AP5 and mecamylamine (violet) or in the presence of the muscarinic receptor antagonist atropine (n=7) or scopolamine (n=2) (red). Each line indicates a cell. Blue circles indicate means. Paired t-test.

Axonal projections of Chr2-eYFP-expressing neurons densely innervated the OB in *dlx5/6* mice, especially in the glomerular layer and in the granule cell layer (Fig.3B and Fig.3-figure supplement 1). This projection pattern is consistent with earlier reports in *GAD2-cre* mice (Bohm et al., 2020; Villar et al., 2021) or *vGAT-cre* mice (Hanson et al., 2020), two other lines often used to label BF GABAergic neurons. In OB slices from *dlx5/6* mice, 0.5 Hz photostimulations affected post stimulus spiking activity in 30% of the cells tested (116/383). Responses were heterogeneous but three clearly distinct types of responses were evoked (Fig.3-figure supplement 2): 12% of the cells tested (45/383) responded with a transient inhibition of spiking immediately after the stimulation, as expected for a classical inhibitory input (see also Fig.8). 7% of the cells responded with a brief excitation (29/383 cells), i.e. the photostimulation induced a single spike, sometimes two (see also Fig.9). Finally, 11% of the cells (42/383 cells) responded with a transient inhibition of spiking after each flash and with a robust and long-lasting increase in baseline firing rate as soon as low frequency photostimulations started. This last group of cells with dual responses likely correspond to those receiving a muscarinic excitation. Consistent with this, a single flash evoked a robust and long-lasting increase in firing rate in these cells (Fig.3C), similar as in *ChAT* mice. This excitation persisted in the presence of NBQX, D-AP5 and mecamylamine and was blocked by atropine ($n=7$) or scopolamine ($n=2$)(Fig.3C-F). However, contrasting with the exclusive excitatory muscarinic responses in *ChAT* mice, spiking was also transiently blocked after the photostimulation. PG cells with these dual responses had a low and irregular firing activity in control condition (range 0-4 Hz, mean 1.95 ± 1.6 Hz, $n=13$) and switched to a sustained higher frequency firing mode upon the 0.5 Hz low frequency photostimulations in ACSF (mean 8.6 ± 3 Hz, $n=13$, Fig.4 A-C). This sustained spiking regime was also induced when low frequency stimulations were done in the presence of NBQX, D-AP5 and mecamylamine ($n=10$, Fig.4 D-F). When delivered every 2s, each flash was followed by a period of spiking inhibition (Fig.4 A-E). Photo-evoked spiking inhibition lasted 391 ± 142 ms ($n=21$), persisted in the presence of NBQX, D-AP5 and mecamylamine ($n=10$, Fig.4 D-F) and was blocked by the GABA_A receptor antagonist gabazine (5 μ M)(Fig.4 G-H). Gabazine did not prevent the ACh-mediated increase of basal firing evoked by the 0.5 Hz photostimulations ($n=8$, Fig.4 G-I). Only one cell with the muscarinic excitatory response lacked the GABAergic component in *dlx5/6* mice (Fig.3-figure supplement 3). Although rare, this single case confirms that the cholinergic neurons stimulated in *dlx5/6* mice do not co-transmit ACh and GABA. All

together, the results are consistent with previous investigations demonstrating that although BF cholinergic neurons express molecular markers of GABAergic neurons, only a fraction of them co-releases GABA and ACh (Saunders, Granger, and Sabatini 2015). They further indicate that a subset of PG cells receives both ACh and GABA inputs from separate BF axons. GABA release inhibits spiking through GABA_A receptors whereas ACh release activates mAChRs and produces a long-lasting excitation.

Figure 4: In *dlx5/6* mice, a monosynaptic GABAergic input inhibits spiking in cells with a muscarinic response

A, Two representative traces (scale bar 100 pA) showing spiking activity in control condition and while BF axons were photostimulated with a single flash per episode every 2 s (blue arrow). Middle, corresponding raster plot. Photostimulations started at episode 31 (blue line). Bottom, cumulative PSTH (bin size 20 ms) in the same cell for the trials with a photostimulation (blue arrow). **B**, average firing rate per bin (20 ms) and per episode for 17 cells ($\pm$ sem, grey bars) while BF axons were photostimulated every 2 s with a single flash (at blue arrow). **C**, average firing frequency per episode (2 s each) for 13 cells recorded in ACSF in control condition (no light, episodes 1-30) and during photostimulation of the BF

267 *afferents once per episode (31-60). D-F, Same as in A-C in the presence of NBQX (10 μ M), D-AP5 (50 μ M), and*
268 *mecamylamine (MECA, 50 μ M). Traces, raster plot and PSTH in D are from the same cell as in A. G-I, Same as in A-C when*
269 *gabazine (GBZ, 5 μ M) was added to the cocktail of blockers. Traces, raster plot and PSTH in G are from the same cell as in*
270 *A and D.*

272 **Endogenous ACh release elicits a slow muscarinic EPSC in type 2.3 PG cell**

273 In our previous report, we did not detect any BF-evoked muscarinic EPSC in whole-cell
274 recordings from various PG cell subtypes in dlx5/6 mice (Sanz Diez, Najac, and De Saint Jan
275 2019). To understand the mechanism underlying photo-evoked muscarinic excitation, I re-
276 examined this question using a slightly different K-gluconate-based internal solution
277 supplemented with phosphocreatine to improve ATP supply. PG cells receiving a muscarinic
278 input were first identified using LCA spike recording and then whole-cell (WC) patched with a
279 pipette filled with the new internal solution (n=5 cells in ChAT mice and n=12 in dlx5/6 mice).
280 In dlx5/6 mice, a single photostimulation evoked a biphasic response initiated by an outward
281 IPSC followed by a slow rising inward current in 7/12 cells (Fig.5A). The slow EPSC was
282 undetectable in 5 other cells. In ChAT mice, BF axons photostimulation induced a slow
283 inward EPSC in 5/5 cells. Consistent with the LCA experiments, the slow inward current was
284 not preceded by an outward IPSC, further confirming that cholinergic axons do not release
285 GABA on these PG cells (Fig.5D). In both transgenic lines, photo-evoked muscarinic EPSCs
286 recorded at negative holding potentials (from -30 to -60 mV) were small and often at the
287 limit of detection (max 10 pA, mean 5 ± 3 pA, data obtained in ChAT and dlx5/6 mice pooled
288 together). In current-clamp, at membrane potential close to the resting potential, photo-
289 evoked EPSPs did not exceed 5 mV (mean 3.1 ± 1.1 mV, 2 cells in ChAT mice and 3 cells in
290 dlx5/6 mice)(Fig.5A and 5D). Unfortunately, muscarinic EPSC/EPSP ran down in few minutes,
291 precluding any further characterization.

Figure 5: BF cholinergic inputs produce a slow muscarinic EPSC in type 2.3 PG cells

A, Biphasic GABAergic-muscarinic voltage-clamp (VC) and current-clamp (CC) whole-cell responses to a single photostimulation (blue arrow) of BF axons in a cell from a *dlx5/6* mouse. Responses recorded in the presence of NBQX, D-AP5 and mecamylamine. The current is an average of 8 consecutive sweeps, the voltage response is a single trace eliciting 4 spikes (truncated for display). **B**, Morphology of a biocytin-filled PG cell in which photostimulation of BF axons (eYFP-positive, green) produced a dual GABA-ACh response in a *dlx5/6* mouse. DAPI staining (blue) shows the outline of the glomerulus. Scale bar 20 μ m. Inset: voltage responses of this cell to depolarizing and hyperpolarising current steps (20 pA, 500 ms). Scale bar 20 mV. **C**, Photo-evoked GABAergic IPSC recorded in the same PG cell as in **A** at a holding potential of 0 mV. Average of 10 sweeps. The decay was best fitted with two exponentials (magenta) with a weighted decay time constant of 153 ms. Inset: distribution histogram of the decay time constants of photo-evoked GABAergic IPSCs in PG cells with a mixed GABA/ACh response in *dlx5/6* mice. **D**, Photo-evoked muscarinic EPSC (VC) and EPSP (CC) recorded in ACSF in a PG cell from a *ChAT* mouse. Average of 6 consecutive sweeps for the EPSC, single trace for the EPSP. **E**, Morphology of a biocytin-filled cell that responded to the photostimulation of BF cholinergic axons with a muscarinic excitation in a *ChAT* mouse. Scale bar 20 μ m. Blue: DAPI; green: eYFP positive BF axons. Inset: membrane voltage responses of this cell to the injection of current steps (-20/+35 pA, 500 ms). Scale bar 20 mV. **F**, Long-lasting barrage of EPSCs evoked by an electrical stimulation of the olfactory nerves (black arrow, 0.1 ms/100 μ A) in the cell shown in **E**. Inset: zoom on the earliest phase of the response (6 consecutive traces, truncated for display). Responses had onset latencies >2 ms. The distribution histogram shows the duration of the ON-evoked response elicited in 10 cells receiving a muscarinic excitation (7 cells from *dlx5/6* mice and 3 cells from *ChAT* mice).

Intrinsic, synaptic and morphological properties of the ACh-responsive PG cells were also examined. In addition to the 17 WC-recorded cells from *ChAT* and *dlx5/6* mice, the dataset included WC recordings from previous experiments in *dlx5/6* mice in which a photo-evoked muscarinic excitation was noticed in the cell-attached configuration but not detected in the WC mode (n=9). The morphology of each cell was assessed at the end of the recording by visual inspection of the dye present in the internal solution. Moreover, six cells filled with biocytin were successfully recovered for post-hoc morphological reconstruction. All had the typical morphology of PG cells. Their soma was small, ovoid or round, with no apparent

axon. Thin dendrites projected within a single glomerulus (Fig.5B and 5E). On average, their electrical membrane resistance was $1061 \pm 524 \text{ M}\Omega$. They all fired regularly at up to 100 Hz with overshooting action potentials in response to depolarizing current steps (Fig.5B and 5E). Another striking hallmark of these PG cells was their prolonged response to a single electrical stimulation of the olfactory nerves. ON-evoked response consisted of a barrage of fast EPSCs lasting several hundreds of ms (average $1760 \pm 927 \text{ ms}$, $n=10$)(Fig.5F). Evoked responses had an onset latency $>2\text{ms}$ (average $3.14 \pm 0.66 \text{ ms}$) as typically seen in plurisynaptic ON-evoked responses of type 2 PG cells (Sanz Diez, Najac, and De Saint Jan 2019; Najac et al. 2015). Finally, in *dlx5/6* mice, photo-evoked BF IPSCs (amplitude range 10-186 pA, mean $62 \pm 40 \text{ pA}$) were particularly slow (average decay time constant $162 \pm 55 \text{ ms}$, range 69-258 ms, $n=16$)(Fig.5C). Together, these intrinsic and synaptic properties unambiguously identify type 2.3 PG cells as the principal and perhaps unique synaptic target of BF cholinergic axons among PG cells.

To confirm that type 2.3 PG cells are the only PG cell target of BF cholinergic neurons, I examined whole-cell responses evoked by a single flash in other types of PG cells in ChAT mice ($n=54$). Photo-evoked responses were recorded in voltage-clamp at 0 mV and at negative holding potential in order to detect possible monosynaptic GABAergic, nicotinic or muscarinic responses. Membrane properties and ON-evoked responses were examined when possible to identify each cell type. Photostimulation of the BF fibers did not evoke any response in type 1 PG cells ($n=4$), type 2.1 PG cells ($n=21$) and type 2.2 PG cells ($n=17$). 12 additional PG cells that could not be firmly classified did not respond either. Only one PG cell, that could not be unambiguously classified, responded with a fast IPSC, a rare event that possibly reflected unspecific expression of ChR2 in BF GABAergic neurons. Together, these results indicate that BF cholinergic axons innervating the glomerular layer of the OB have a unique and selective synaptic target among PG cells and do not release GABA.

M1 mAChR mediates the muscarinic excitation

Next, I determined what muscarinic receptor mediates the cholinergic response in type 2.3 PG cells and what downstream mechanism produces the excitation. There are five types of mAChRs and three of those (M1, M3 and M5) are coupled to excitatory $G_{q/11}$ proteins that activate $\text{PLC}\beta$, causing hydrolysis of PIP2 into DAG and IP3. Activation of these receptors most often depolarizes neurons. M1 receptors are widely expressed in the OB (Le Jeune et

al. 1995) and their blockage impairs olfactory-evoked fear learning (Ross, Bendahmane, and Fletcher 2019) making them a likely candidate. To test this hypothesis, I examined the effects of pirenzepine (1-2 μ M), a selective antagonist of M1 receptors, on BF-induced muscarinic excitation in both ChAT (n=3 cells) and dlx5/6 mice (n=7). Experiments were done using LCA recording in the presence of glutamate and nicotinic receptor antagonists. Pirenzepine fully blocked the excitation evoked by light stimulation of the BF fibers in 6 cells and reduced its strength in 4 cells (Fig.6A and 6B). On average, post stimulation spike frequency was reduced 4-fold by pirenzepine (control: 10 ± 3.4 Hz; pirenzepine: 2.6 ± 3.3 Hz, $p < 0.0001$, data from ChAT and dlx5/6 mice pooled together, Fig.6B). These results support the idea that M1 receptors mediate the muscarinic excitation of type 2.3 PG cells.

Could M1 receptors serve as a molecular marker for type 2.3 PG cells? To address this question, I examined the distribution of an mRNA transcript of Chrm1, the gene encoding M1 mAChRs, using RNAscope fluorescence in situ hybridization (ISH) in OB sections. Hybridization signals were found around DAPI-stained nuclei in all layers of the OB (Figure 6, figure supplement 1A and 1B). This widespread distribution is consistent with an earlier autoradiography binding study (Le Jeune et al. 1995) and with more recent ISH data in the Allen brain atlas (<https://mouse.brain-map.org/experiment/show/73907497>) (Lein et al. 2007) or immunohistochemical data in the human protein atlas (<https://www.proteinatlas.org/ENSG00000168539-CHRM1/brain>) (Sjostedt et al. 2020). The signal was particularly strong in granule cells (Fig.6, figure supplement 1B- D), consistent with previous functional data demonstrating that M1 mAChRs potentiate the excitability of granule cells (Pressler, Inoue, and Strowbridge 2007). However, all the granule cells recorded in this study in ChAT mice in the LCA mode were silent and remained silent during the low frequency photostimulations of the cholinergic fibers (n=17, not shown). M1 could still be a useful molecular marker of type 2.3 PG cells if it is strongly and consistently expressed in these cells and have minimal expression in other cell types in the glomerular layer. However, most cells around glomeruli expressed the mRNA transcript of Chrm1. Cells with no signal were more abundant in the glomerular layer than in the granule cell layer but still represented a small fraction of the cells (Fig.6, figure supplement 1C and 1D). Moreover, ISH combined with immunodetection of CR or tyrosine hydroxylase (TH), two commonly used markers of non-overlapping juxtglomerular cell types, revealed that the Chrm1 transcript was expressed in 85 % of the CR-positive (n=372 cells counted) and in 94% of the TH-positive

cells (n=181 cells) (Fig.6, figure supplement 1E and 1F). These results thus suggest that multiple cell types in the glomerular layer express M1 receptors.

M1 receptors classically suppress the M current, a slow voltage-activated potassium current mediated by KCNQ2/3 potassium channels and active at resting membrane potential (Brown and Passmore 2009; Suh and Hille 2008). KCNQ2 subunits are strongly expressed in PG cells (Cooper et al. 2001). The fast run-down of the BF-evoked muscarinic EPSC in whole-cell recordings as well as its strong dependence on intracellular ATP are also consistent with this downstream mechanism. Cell dialysis is indeed often deleterious for M-currents because M-channels opening depends on PIP2 binding, a process that is itself highly dependent on intracellular ATP supply for PIP2 phosphorylation (Suh and Hille 2008; Zhang et al. 2003). To investigate whether closure of the M current causes the muscarinic depolarization of type 2.3 PG cells, I applied the selective M-channel antagonist XE-991 (10 μ M) in the presence of NBQX, D-AP5 and mecamylamine. XE-991 had two noticeable effects in type 2.3 PG cells recorded in LCA. First, it increased baseline spike frequency in all the cells tested (n=14, 8 in dlx5/6 mice, 6 in ChAT mice, Fig.6C-E) suggesting that M-channels are indeed open at rest in type 2.3 PG cells and hyperpolarize their membrane potential. Second, although XE-991 did not fully block photo-evoked muscarinic excitation in most cells (Fig.6C and in Fig.6F), it reduced the strength of BF-induced excitation (Fig. 6D and 6E). A persistent photo-evoked excitation is expected if the muscarinic EPSP is partially blocked while the membrane potential is more depolarized compared to control condition. Overall, these data support the hypothesis that activation of M1 mAChRs depolarizes type 2.3 PG cells by blocking a potassium M current.

Figure 6: M1 mAChRs mediate BF-evoked excitation by closing M channels

A, Photo-evoked LCA responses and cumulative PSTHs (over 22 consecutive sweeps, 200 ms/bin) recorded in a PG cell from a *dlx5/6* mouse in control condition (left) and in the presence of the M1 mAChR antagonist pirenzepine (2 μ M). Scale bar for traces 50 pA. *B*, Summary graphs. Firing rate before (pre) and after (post) photostimulation of BF fibers in control condition (left, paired t-test) and in the presence of pirenzepine (PIR, 1 or 2 μ M, paired Wilcoxon signed-rank sum test). Right graph shows that pirenzepine decreased BF-evoked excitation in every cell tested (paired t-test). Partial washout was obtained in 5 cells. Cells were recorded in ChAT mice ($n=3$, black lines) and in *dlx5/6* mice ($n=7$, grey lines). *C*, Photo-evoked LCA responses and cumulative PSTHs recorded in a PG cell from a *dlx5/6* mouse showing the effects of the M-channel blocker XE-991 (10 μ M) on spiking frequency. BF fibers were photostimulated with a single flash (blue arrow and dotted line). *D*, XE-991 increased baseline spiking rate (blue line, measured during a 15 s time period preceding the flash) and decreased post stimulus spike frequency (grey line). Average from 14 cells (8 in *dlx5/6* mice, 6 in ChAT mice). Each episode was 30 s long. *E*, summary graph showing the two effects of XE-991 on each cell. Paired Wilcoxon signed-rank sum tests. *F*, Firing rate before (pre) and after (post) photostimulation of BF fibers in control condition (left, paired Wilcoxon signed-rank sum test) and in the presence of XE-991 (t-test). Experiments were all done in the presence of NBQX (10 μ M), D-AP5 (50 μ M) and mecamylamine (50 μ M). Means are the blue circles.

Muscarinic excitation of type 2.3 PG cells leads to an increase of inhibitory synaptic inputs in principal neurons

PG cells are classically viewed as a source of inhibitory GABAergic synaptic currents (IPSCs) for mitral and tufted cells, the two OB output channels that project to distinct cortical

areas. However, the targets and output properties of type 2.3 PG cells have never been specifically investigated. Thus, to evaluate the impact of the muscarinic excitation of type 2.3 PG cells, I recorded IPSCs in mitral cells and in superficial or middle tufted cells (s/mTC) in the presence of NBQX, D-AP5 and mecamylamine (Fig.7A). I also examined IPSCs in external tufted cells (eTCs)(Fig. 7A). Although it is still unclear whether eTCs have axonal projections outside the OB like other tufted cells, they play a major role in processing incoming OSNs information by coordinating rhythmic activity within each glomerulus and by providing feedforward excitation to various types of neurons including PG, mitral and tufted cells (De Saint Jan et al. 2009; Hayar et al. 2004; Najac et al. 2011; Najac et al. 2015).

Photostimulation of the cholinergic axons significantly increased IPSCs frequency in eTCs (n=9) and in s/mTCs (n=10) (Fig.7) whereas no response was found in mitral cells (n=17). However, three of the recorded mitral cells had severed apical dendrites and most of the intact cells (10/14) projected in glomeruli located deep within the slice, where light stimulation may be less efficient. Baseline IPSCs frequency greatly varied across cells and was on average smaller in s/mTC (7.0 ± 5.1 Hz) compared to eTC (15.7 ± 10 Hz, $p=0.0285$, t-test). Yet, IPSCs increased in similar proportion and with comparable time course in both cell types after photostimulation (Fig.7-figure supplement 1). Data were thus pooled in Fig.7D and 7E. Thus, low frequency photostimulation led to a rapid and persistent increase in IPSCs frequency in 8/10 eTC and in 10/23 s/mTC. This response was blocked (n=8) or reduced (n=3) when the experiment was repeated in the presence of 2 μ M pirenzepine (Fig.7C and 7E). Pirenzepine had little effect in 4/15 cells. A single photostimulation also transiently increased IPSCs frequency in eTC (n=9) and s/mTC (n=8)(Fig.7B and 7D). Addition of pirenzepine attenuated this response in 10/11 cells. Together, these results suggest that type 2.3 PG cells release GABA onto tufted cells following their excitation by muscarinic activation. This increased in inhibitory synaptic inputs could lead to profound changes on the activity and output of the OB network.

Figure 7: BF muscarinic inputs lead to an increase of inhibitory synaptic inputs in tufted cells

A, Morphologies of two biocytin-filled tufted cells recorded in different slices from ChAT mice. The localization of the soma and the presence or not of lateral dendrites in the external plexiform layer (arrowheads) distinguish a sTC (left) and an eTC (right). DAPI staining (blue) shows the outline of glomeruli. ChR2-eYFP-expressing cholinergic fibers are visible in green. Scale bars 50 μ m. **B**, Increase of inhibition evoked by a single photostimulation (blue arrow) of the cholinergic axons in a sTC (top). Pirenzepine (2 μ M) blocked this response (bottom). 5 traces are superimposed in each condition. PSTHs show the cumulative number of IPSCs/bin (200 ms) across 15 consecutive trials. **C**, Four consecutive traces of spontaneous IPSCs recorded in an eTC in control condition (top) and during low frequency photostimulation of the cholinergic BF fibers (1 flash every 2 s at blue arrow, middle). Pirenzepine (2 μ M) blocked the increase of IPSC frequency evoked by the photostimulations (bottom). **D**, Average IPSC frequency per 200 ms bin and per episode for 17 tufted cells (8 s/mTC and 9 eTC). BF axons were photostimulated once (blue arrow). Pirenzepine was tested on 11/17 cells and reduced photo-evoked increase of IPSCs. **E**, average IPSC frequency per episode (2 s) for 18 cells (10 s/mTC and 8 eTC). Photostimulation of the cholinergic fibers at 0.5 Hz rapidly and persistently increased IPSC frequency. Pirenzepine was tested in 15/18 cells. Experiments were done in ChAT mice in the presence of NBQX, D-AP5 and mecamylamine. Individual data points are shown in Figure 7- Figure supplement 1.

BF GABAergic inputs inhibit type 2.2 PG cells

Beside type 2.3 PG cells described above, a second group of cells in dlx5/6 mice responded to the optogenetic stimulation of the BF axons with a transient block of spiking immediately after the photostimulation (n=45/383, 12% of the cells tested)(Fig.8). However, unlike type 2.3 PG cells, they did not show any evidence of parallel cholinergic excitation. Baseline firing activity did not rapidly increase when BF fibers were photostimulated at 0.5 Hz and a single flash did not elicit a long-lasting mAChR-evoked increase in firing (Fig.8-figure supplement 1). In these cells, basal firing frequency was, on average, higher than in type 2.3

PG cells (mean 7.8 ± 5.6 Hz, $n=21$, $p=0.00058$, Mann-Whitney Rank Sum Test). However, the nature of this activity (single spike vs. burst of spikes) and its frequency (range 2-21 Hz) varied greatly across cells (Fig.8-figure supplement 2). Spontaneous spiking was also diversely affected by NBQX, D-AP5 and mecamylamine, being totally blocked in some cells ($n=3$) and not affected in others ($n=9$). In the latter, BF-induced inhibition persisted in the presence of the blockers ($n=9$) and was blocked by gabazine (Fig.8B, $n=7$) suggesting that it was caused by a direct GABAergic BF input. The duration of the BF-induced inhibition also varied across cells. In about half of the cells, the BF input induced a shorter (<200 ms) inhibition than in type 2.3 PG cells whereas inhibition was longer and similar as in type 2.3 PG cells in others (Fig.8C). However, it is noteworthy that cells with a prolonged inhibition often fired at low rate or fired irregularly with bursts of spikes (Fig.8-figure supplement 2). In these cells, the duration of BF-induced inhibition, as measured as the mean delay between the flash and the first spike after the flash, varied across sweeps making this estimate imprecise (Fig.8-figure supplement 2A).

Cells with BF-induced spiking inhibition and no evidence for cholinergic excitation in the cell-attached configuration were subsequently characterized in the whole-cell mode ($n=7$, Fig.8D). Their electrical membrane resistance was 1119 ± 789 mOhm. BF axons photostimulation evoked large IPSCs (amplitude range 69-464 pA, mean 170 ± 144 pA) with an average decay time constant of 37 ± 9 ms, that was significantly shorter than in type 2.3 PG cells ($p<0.0001$, Mann-Whitney Rank Sum Test). Three cells in which ON-evoked responses were recorded responded with a short burst of EPSCs (duration <150 ms). Onset latencies (2.45 ± 0.23 ms) were consistent with plurisynaptic responses. Firing properties were more heterogeneous. Injection of a depolarizing current step evoked a burst of action potentials followed by a plateau ($n=3$ cells, as in the example shown in Fig.8D) or sustained firing of action potentials ($n=3$, not shown). In 5/6 cells, injection of an hyperpolarizing step caused a voltage sag suggesting the activation of an I_h current. All together, these properties match well with those of type 2.2 PG cells (Najac et al. 2015). This suggests that type 2.2 PG cells only receive an inhibitory GABAergic input from the BF.

Figure 8: type 2.2 PG cells are inhibited by the BF GABAergic input

A, 10 superimposed LCA traces and the corresponding PSTH for 44 consecutive trials (bin 20 ms) in a cell from a *dlx5/6* mouse. Photostimulation of the BF fibers (blue arrow) transiently blocked spiking. Bottom, average firing frequency per bin (20 ms) and per episode for 28 cells that were inhibited by the BF input, without evidence of parallel cholinergic excitation (Figure 8-figure supplement 1). **B**, BF-induced spiking inhibition persisted in the presence of NBQX, AP5 and mecamylamine but was blocked by gabazine. The two PSTHs are from the same cell as in **A** (bin 20 ms). Bottom, average firing rate per bin (20 ms) and per episode for 7 cells in control conditions (grey line, 6 cells in the presence of blockers, 1 cell in ACSF) and when GBZ (5 μ M) was added (blue). **C**, Duration of the post-stimulus spiking inhibition in cells with a BF-induced inhibitory response (blue) vs. in cells with a biphasic inhibition-muscarinic excitation response (grey). See also Figure 8-figure supplement 2 for caveats in these measurements. **D**, Whole-cell characterization of a cell with an inhibitory response. Top, BF impact on firing (cell-attached recording, 38 episodes are superimposed) and BF-evoked IPSCs (whole-cell recording, 15 superimposed episodes). The histogram compares the decay time constants of photo-evoked GABAergic IPSCs in 7 PG cells that were only inhibited (blue bars) vs. in cells with a mixed GABA/ACh response (grey bars). Bottom left, ON-evoked EPSCs (left, 4 superimposed traces). Onset latencies >2 ms (inset) are consistent with a plurisynaptic response. Bottom right, current-clamp voltage responses to current steps.

GABA is excitatory in a minority of CR-expressing type 2.1 PG cells

Finally, photo-evoked BF inputs elicited a single spike (Fig.9A) or, more rarely, a doublet (Fig.9C) in a small number of cells in *dlx5/6* mice ($n=29/383$, 7% of the cells tested). These excitatory responses were not seen in ChAT mice, persisted in the presence of NBQX, D-AP5 and mecamylamine ($n=6$) and were blocked by gabazine ($n=4$, Fig.9B and 9D) suggesting that they were caused by a direct excitatory GABAergic input. It would not be surprising if GABA was depolarizing in CR-expressing PG cells which retain many properties of newborn immature neurons (Benito et al. 2018). Consistent with this hypothesis, most of the cells with an excitatory response (26/29) had no or little (<1 Hz) spontaneous firing activity, similar as CR+ PG cells (Benito et al. 2018; Fogli Iseppe, Pignatelli, and Belluzzi 2016). However, photo-evoked action potentials occurred within a short delay after the flash, as expected for synaptically-driven spikes, in only 15/29 cells (average spike timing 13.4 ± 9.9 ms, Fig.9A and 9E). In the other cells, the average delay was longer (329 ± 191 ms) and more variable ($n=14$, Fig.9C and 9E) suggesting that distinct mechanisms drive the two types of response. Strikingly, the photo-evoked BF synaptic input evoked a small but detectable gabazine-sensitive capacitive current that was inward in 14/15 cells responding with early spikes (Fig.9A-B, 9E) whereas it was outward in all the cells responding with delayed spikes (Fig.9C-D, 9E). Thus, a likely explanation of the data is that a depolarizing GABAergic input directly triggered early spikes whereas delayed spikes could be induced by rebound depolarization following an hyperpolarizing GABAergic IPSP, as often seen in CR+ PG cells (Benito et al. 2018). Eight cells responding with early action potentials in the cell-attached mode (average timing 20 ± 19 ms) were subsequently characterized in the WC configuration. All of them had properties consistent with those of CR+ PG cells, i.e. a large input resistance (6 ± 3.1 G Ω), a characteristic voltage response to depolarizing current steps and a large and fast photo-evoked BF IPSC (amplitude range 133-1300 pA, mean 370 ± 139 pA; weighted decay time constant 7.7 ± 3.6 ms)(Fig.9G).

The BF input induced a detectable capacitive current, but no spike, in 70 additional cells, all with no or little spontaneous activity (these cells were classified as non-responsive in Fig.3 figure supplement 2). BF-evoked capacitive current were inward in 54% of these cells ($n=38/70$) and outward in the other cells ($n=32$, Fig.9F). Eight of these cells were characterized with whole-cell recording and all displayed the typical intrinsic and synaptic properties of CR+ PG cells including a high membrane resistance (3 ± 1.2 G Ω) and large and

fast BF IPSCs (amplitude: 284 ± 107 pA; decay 12.2 ± 2.9 ms). All together, this analysis suggests that the BF GABAergic input is depolarizing in as much as half of the CR-expressing type 2.1 PG cells that may have an elevated chloride reversal potential, more depolarized than the membrane potential. However, this input drives spiking in only a minority of them.

Figure 9: BF-evoked GABAergic excitation in a fraction of type 2.1 PG cells

A, example of a BF-evoked excitatory response in a PG cell. One representative LCA recording episode (duration 2 s) is shown on top. Photostimulation of the BF fibers (blue arrow) induced an inward current followed by a single spike (inset). Each tick is a spike in the raster plot of these cell responses (stimulation: episodes 31-61). Bottom, corresponding PSTH. Note the temporal precision of the evoked spikes. Bin size 50 ms. **B**, Same cell as in **A**. The evoked inward current and the evoked spike both persisted in NBQX, D-AP5 and mecamylamine but were blocked by gabazine. At least four traces are superimposed in each condition. **C**, Another example of a PG cell responding to the photostimulation with action potentials. Photostimulation (blue arrow, episodes 31-65 in the raster plot) induced an outward current (inset) and, in some trials, a delayed doublet of spikes. Bottom, corresponding cumulative PSTH (bin size 50 ms). **D**, Same cell as in **C**. Gabazine blocked both the evoked outward current and the evoked spikes. **E**, Distribution histogram of the average spike timing in each cell responding with spikes. Cells in which the stimulation evoked an inward capacitive current are in violet, cells in which the stimulation evoked an outward current are in green. **F**, Total number of cells responding with an inward (violet) or an outward (green) capacitive current followed or not with evoked-spikes. **G**, Whole-cell characterization of a cell excited by the BF input. Left, BF-evoked spike response (LCA recording, top, 20 consecutive episodes are superimposed) and BF-evoked IPSC (whole-cell recording, 20 consecutive responses). Right: current-clamp voltage responses to current steps in the same cell. The distribution histogram compares the decay time constants of photo-evoked GABAergic IPSCs in the 7 PG cells that were excited (violet bars) vs. in type 2.2 cells with an inhibitory response (blue) and in type 2.3 cells with a mixed GABA/ACh response (grey bars).

Discussion

This study shows that OB-projecting BF neurons have diverse impacts on PG cells. GABAergic inputs potently block the discharge of type 2.2 and type 2.3 PG cells with a target-specific time course. In contrast, GABA release is excitatory and eventually triggers action potentials in a fraction of type 2.1 PG cells. Data also reveal that BF cholinergic fibers strongly and exclusively excite type 2.3 PG cells. Thus, intraglomerular inhibition of principal neurons mediated by PG cells can be modulated in various ways by multiple BF pathways that potentially regulate olfactory processing in a context and behavior-specific manner.

A previously ignored cholinergic pathway

The main finding of this study is that endogenous phasic ACh release from BF cholinergic neurons selectively evokes a remarkably strong and reliable muscarinic excitation in a previously overlooked PG cell subtype, which are referred to as type 2.3 PG cells. This novel cholinergic pathway concerns a small population of neurons lacking a molecular marker and evokes small muscarinic EPSP/EPSCs that rapidly runs down in whole-cell recording, explaining why it has been missed until now. Here, I provide pharmacological evidence suggesting that the slow cholinergic response is mediated by M1 mAChRs that suppress a M current. This downstream mechanism classically washes out quickly and future experiments using perforated patch-clamp experiments will be necessary to confirm this hypothesis.

This pathway adds to other mAChR-dependent mechanisms capable of increasing tonic inhibition in mitral and tufted cells. Activation of M1 receptors increases the excitability of granule cells, the most abundant interneurons in the OB, by potentiating current-evoked afterdepolarization (Pressler, Inoue, and Strowbridge 2007). mAChR activation also directly enhances transmitter release at reciprocal dendrodendritic synapses between mitral and granule cells (Castillo et al. 1999; Ghatpande and Gelperin 2009) or between juxtaglomerular interneurons and mitral/tufted cells (Liu et al. 2015). However, there is yet no evidence that endogenous ACh can recruit these previously described pathways. In the previous studies, ACh or cholinergic agonists were exogenously applied on slices. This results in prolonged and uniform activation of synaptic and extrasynaptic ACh receptors and induces multiple concomitant effects that may not necessarily be evoked by physiological release of ACh, even in case of strong afferent activity in cholinergic neurons leading to diffuse volume transmission (Unal, Pare, and Zaborszky 2015).

Physiological implications

Like other sensory systems, olfactory perception is context-dependent. This modulation already takes place in OB circuits, where odor-evoked neural responses depend on reward (Doucette and Restrepo 2008) or on the difficulty of the task (Koldaeva, Schaefer, and Fukunaga 2019) and are shaped by learning and experience (Martin et al. 2004; Chu, Li, and Komiyama 2017; Ross and Fletcher 2018). Whether BF cholinergic innervation of the OB plays a role in context-dependent neuromodulation is suspected, but has never been proven.

In the cortex, transient increased cholinergic signaling signals a transition to a behaviorally important context and adjust neural output to improve task performances (Gritton et al. 2016; Kuchibhotla et al. 2017; Pinto et al. 2013). In pavlovian learning paradigms, BF cholinergic neurons respond with brief and temporally precise burst of activity to reward or aversive stimuli and to conditioning stimuli, including olfactory cues (Guo, Robert, and Polley 2019; Hangya et al. 2015; Hanson, Brandel-Ankrapp, and Arenkiel 2021; Crouse et al. 2020). This results in fast and precise transients of ACh in target sensory cortical areas (Guo, Robert, and Polley 2019) or in the basolateral amygdala (Crouse et al. 2020). The physiological dynamics of ACh within the OB are unknown but it is tempting to speculate that similar ACh transients are evoked in the OB during olfactory guided aversive or appetitive learning, a behavior that critically depends on M1 mAChRs in the OB (Ross, Bendahmane, and Fletcher 2019).

I showed that a single stimulation of the cholinergic BF axons, which evokes transient, temporally and spatially precise release of ACh, triggers a long-lasting discharge in type 2.3 PG cells. This target-specific muscarinic response likely involves synaptic or perisynaptic mAChRs and provides support for phasic, spatially restricted cholinergic transmission as opposed to spatially diffuse volume transmission (Sarter and Lustig, 2020). The same stimulus repeated every two seconds, a low frequency stimulation that is insufficiently strong to induce massive diffusion of ACh in the extracellular space, rapidly transforms type 2.3 PG cells that usually fire at low rate into tonically active neurons that fire at high frequency. This, in turn, leads to a rapid and persistent increase of synaptic inhibitory currents in principal neurons, thus potentially driving OB circuits in a different state of

activity. This target-specific muscarinic transmission may have widespread circuit implications because single cholinergic axons frequently ramify and innervate multiple glomeruli in different OB areas (Hamamoto et al. 2017). This could ultimately reduce the firing rate in output neurons but could also shape the temporal structure of mitral and tufted cells output at diverse time scales. Inhibitory inputs regulate spike timing and synchrony in mitral and tufted cells (Najac et al. 2015; Schoppa 2006; Shao et al. 2012) and inhibition of eTCs might modulate slow glomerulus-specific coordinated activity (De Saint Jan et al. 2009; Hayar et al. 2004; Najac et al. 2011). Based on previous studies on ACh functions, increased inhibition driven by the muscarinic excitation of type 2.3 PG cells may improve olfactory perception of behaviorally important odorants. Interestingly, BF GABAergic fibers innervating type 2.3 PG cells potentially block their activity and could act as a powerful brake to reverse the cholinergic effects. Future *in vivo* experiments will be necessary to explore these possibilities.

In vivo, optogenetic stimulation of the cholinergic axons in the OB of *Chat*^{Cre} mice increases mitral and tufted cells spontaneous and odor-evoked firing (Bohm, Brunert, and Rothermel 2020; Rothermel et al. 2014). This result seems at odds with the expected implications of the new muscarinic pathway described in this study. However, photostimulation was strong and sustained in these *in vivo* studies (light continuously on for 10 s). Although it is difficult to compare stimulations *in vivo* and in slices, a prolonged photostimulus could recruit additional cholinergic pathways that need volume transmission to be activated and that have opposite impacts compared with those of type 2.3 PG cells. For instance, TH-expressing dopaminergic/GABAergic juxtglomerular neurons express the M2 mAChR (Crespo et al. 2000; Hamamoto et al. 2017). Activation of these receptors inhibits tonically active TH-expressing cells (Pignatelli and Belluzzi 2008) that provide an inhibitory drive to mitral and tufted cells (Liu, Puche, and Shipley 2016; Whitesell et al. 2013; Zhou et al. 2020). Thus, understanding the physiological impact of the muscarinic excitation of type 2.3 PG cells on the OB output and network activity will require targeted stimulations that selectively engage this pathway *in vivo* as well as specific approaches to determine the *in vivo* activity of the cholinergic afferents.

Multiple cell-type specific pathways for BF control of glomerular inhibition

Results of this study also provide new insights into PG cell diversity. Immunohistochemical studies have already demonstrated that the few classical markers commonly used to label PG cells do not label all of them (Panzanelli et al. 2007; Parrish-Aungst et al. 2007; Whitman and Greer 2007). Yet, most functional studies only distinguish type 1 and type 2 PG cells and ignore type 2's diversity. CR-expressing type 2.1 PG cells are by far the most abundant, representing 40 to 50% of the entire PG cell population. They are predominately generated postnatally and persist in an immature stage in terms of connectivity and membrane properties (Benito et al. 2018). The present data suggest that BF GABAergic inputs are excitatory in a fraction of them, likely the most immature, whereas GABA is inhibitory in the other PG cells. The functional impact of this excitation is unclear because it concerns a minority of PG cells and there is no evidence that immature CR+ PG cells form output synapses. However, this result may explain why GABA appears predominantly excitatory in calcium imaging of unidentified PG cells (Parsa, D'Souza, and Vijayaraghavan 2015). Type 2.3 PG cells constitute about 20% of the whole PG cell population and are approximately as numerous as type 2.2 PG cells (Sanz Diez, Najac, and De Saint Jan 2019). These regular spiking interneurons have pluri-synaptic long-lasting ON-evoked responses and receive remarkably slow BF IPSCs that readily distinguish them from type 2.1 and type 2.2 PG cells. As shown here, their muscarinic input is another selective feature. Although their input and output connections are not firmly established, type 2.3 PG cells are presumably activated by the glutamate released from mitral and tufted cell dendrites and most likely release GABA unselectively onto mitral and tufted cells. Consistent with this idea, a previous study showed that mAChR activation within glomeruli increases IPSCs equally well in mitral and tufted cells (Liu et al. 2015). The present data confirm that type 2.3 PG cells inhibit various classes of tufted cells but I found no evidence that they also inhibit mitral cells. However, this negative result has to be interpreted with caution as it is challenging in slices from adult mice to find mitral cells projecting in surface glomeruli, a technical requirement for optimal LED stimulation of the cholinergic afferents.

The functional implications of PG cells diversity are not known. Cholinergic and GABAergic inputs from the BF may provide physiological tools to manipulate each PG cell subtype selectively in future studies exploring this question. Like elsewhere in the brain, BF GABAergic neurons are highly diverse and each cell population makes cell type-specific long-range connections (Do et al. 2016) and plays specific functions. For instance, somatostatin

(SOM)- and parvalbumin (PV)-expressing sub-populations have distinct impacts on arousal control (Anacleit et al. 2018; Xu et al. 2015) or on food intake (Zhu et al. 2017). Distinct classes of BF GABAergic neurons may thus modulate distinct classes of PG cells. Similarly, muscarinic excitation of type 2.3 PG cells may involve a specific population of BF cholinergic neurons. There are at least two distinct types of BF cholinergic neurons that differ in their firing modes and synchronization properties and that are differently engaged during behaviors (Laszlovszky et al. 2020). This specificity could also rely on connectivity. For instance, BF cholinergic neurons modulating distinct areas are driven by specific combinations of synaptic inputs (Do et al. 2016; Gielow and Zaborszky 2017; Zaborszky et al. 2015; Zheng et al. 2018). The recent discovery of a genetically-defined sub-population of cholinergic neurons that selectively innervates a specific subgroup of deep short axon cells in the OB (Case et al. 2017) also supports the hypothesis of cell-specific innervation of OB interneurons by specific subsets of cholinergic neurons. Hence, each of the BF neuromodulatory pathways innervating PG cells might be independently recruited during specific tasks or internal states.

Materials and Methods

Animals and ethical approval. All experiments procedures were approved by the French Ministry and by the local ethic committee for animal experimentation (CREMEAS; agreement number/reference protocol: APAFIS#5250-2016042115058488v3 and v7). Mice were housed in the animal facility (Chronobiotron, UMS3415, CNRS, University of Strasbourg) with ad libidum access to food and water in accordance with the European convention 2010/63/EU on the protection of animals used for scientific purposes. Adult heterozygous *dlx5/6*^{Cre} mice (n=36, 34 females - 2 males, C57BL6/J background)(Jackson Laboratory stock no : 008199)(Monory et al. 2006) and *Chat*^{Cre} mice (n=44 of either sex, CD1 background)(Jackson Laboratory stock no: 006410)(Rossi et al. 2011) were used in this study.

Stereotaxic viral injection. 3-8 weeks old mice were anesthetized with intraperitoneal injection of Zoletil 50 (TILETAMINE/ZOLAZEPAM, 60-70 mg/kg) and Rompun 2% (xylazine, 18-20 mg/Kg) and placed in a stereotaxic apparatus. Metacam (meloxicam, 2 mg/Kg, SC injection) and Lurocaine + Bupivacaine (2 mg/Kg both, SC, local) were administered prior

incision. Mice were craniotomized and a volume of 300-400 nl of AAV9.EF1a.DIO.hChR2(H134R).eYFP.WPRE.hGH was stereotactically injected in the left hemisphere at 0/+0.2 mm AP, 1.4/1.6 mm ML and 5.4 /5.6 mm DV from bregma. Viruses were purchased from the University of Pennsylvania Viral Vector Core (RRID: Addgene # 20298; virus titer 1.8×10^{13} vg/mL) and from the Canadian Neurophotonics Platform Viral Vector Core Facility (RRID:SCR_016477, virus titer 9×10^{12} GC/mL). After surgery, Antisedan (atipamezol)(2.5%) was injected IP and mice were rehydrated with 0.5 ml of NaCl 0.9% and placed under a heating lamp. Mice recovered during 2-4 weeks after injection before anatomical or physiological experiments.

Slice preparation. Mice were killed by cervical dislocation and the OB rapidly removed in ice-cold oxygenated (95% O₂-5% CO₂) cutting solution containing (in mM): 83 NaCl, 26.2 NaHCO₃, 1 NaH₂PO₄, 2.5 KCl, 3.3 MgSO₄, 0.5 CaCl₂, 70 sucrose and 22 D-glucose (pH 7.3, osmolarity 300 mOsm/l). Horizontal OB slices (300 µm-thick) were cut using a Microm HM 650V vibratome (Microm, Germany) in the same solution; incubated for 30-40 minutes at 34°C; stored at room temperature (RT) in a regular artificial cerebrospinal fluid (ACSF) until use. ACSF contained (in mM): 125 NaCl, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂ and 25 D-glucose and was continuously bubbled with 95% O₂-5% CO₂.

Electrophysiological recordings. Slices were transferred to a recording chamber and perfused with ACSF at 32-34°C under an upright microscope (SliceScope, Scientifica, Uckfield, UK) with differential interference contrast (DIC) and fluorescence optics. Spontaneous action potential firing activity was monitored using loose cell-attached (LCA) recording (15-100 MΩ seal resistance). LCA recordings were made in the voltage-clamp mode of the amplifier (multiclamp 700B, Molecular Devices, Sunnyvale, CA) with no current injected through the pipette. In these conditions, large and fast membrane potential changes such as action potentials are detected as capacitive currents flowing across the patch capacitance (Barbour and Isope 2000). A regular patch pipette filled with ACSF was used on several successively recorded cells. Whole-cell PG cell recordings were made with glass pipettes (4-7 MΩ) filled with a regular K-gluconate-based internal solution containing (in mM): 135 K-gluconate, 2 MgCl₂, 0.025 CaCl₂, 1 EGTA, 4 Na-ATP, 0.5 Na-GTP, 10 Hepes (pH 7.3, 280 mOsm, 15 mV junction potential). The intracellular solution used to record the muscarinic EPSC was adapted from (Lawrence et al. 2006) and contained (in mM): 110 K-gluconate, 4 MgCl₂, 0.1

777 EGTA, 4 Na₂-ATP, 0.5 Na₂-GTP, 10 Hepes, 10 Phosphocreatine (pH 7.3, 250 mOsm/L, 15 mV
778 junction potential). Whole-cell voltage-clamp recording from mitral and tufted cells were
779 made with an internal solution containing (in mM): 120 Cs-MeSO₃, 20 tetraethylammonium-
780 Cl, 5 4-aminopyridine, 2 MgCl₂, 0.025 CaCl₂, 1 EGTA, 4 Na-ATP, 0.5 Na-GTP, and 10 HEPES,
781 (pH 7.3, 280 Osm/L, 10 mV junction potential). Atto 594 (10 μM, Sigma) was systematically
782 added to the internal solution in order to visualize the cell morphology during the recording.
783 Optical stimulation of the BF axons was done using a blue LED (490 nm, pE 100, CoolLED Ltd.,
784 Andover, UK) directed through the 40X objective of the microscope at 50-100% of its
785 maximum power (5 mW at the objective output) and driven by the Axograph X acquisition
786 software (Axograph Scientific). Olfactory nerves projecting inside a given glomerulus were
787 electrically stimulated using a theta pipette filled with ACSF. The electrical stimulus (100 μs)
788 was delivered using a Digitimer DS3 (Digitimer, Welwyn Garden City, UK). Recordings were
789 low-passed filtered at 2-4 kHz and digitized at 20 kHz using the AxoGraph X software. In
790 whole-cell voltage-clamp recordings, access resistance was not compensated. Voltages
791 indicated in the paper were corrected for the junction potential.

792 Cell selection. PG cells were selected based on the small size of their cell body and their
793 position within the first rings of cells surrounding the glomerulus. Although they have a
794 larger cell body, it cannot be excluded that TH-expressing cells or eTC have been erroneously
795 included in the LCA recording dataset. However, these cells usually have a remarkable
796 spontaneous activity patterns (eTC are rhythmically bursting, TH+ cells have a highly regular
797 rhythmic discharge) and cells with this kind of activity constituted a minority of the dataset.
798 eTC, superficial or middle tufted cells (collectively called s/mTC) and mitral cells were
799 identified based on the localization of their soma and the presence or not of lateral
800 dendrites in the external plexiform layer, as seen during whole-cell recording by visual
801 inspection of the dye-filled cell morphology. In addition, some cells were filled with biocytin
802 for post-hoc anatomical reconstruction. Thus, eTC were selected based on their large pear-
803 shaped soma within the glomerular layer, a short and thick apical dendrite extensively
804 ramifying into a single glomerulus and the lack of lateral dendrites. In addition, they often,
805 but not always, spontaneously fired short burst of action potentials in the cell-attached
806 configuration even in the presence of NBQX, d-AP5 and mecamylamine. Superficial tufted
807 cells were found at the border between the glomerular layer and the external plexiform

layer. Compared to eTC, their soma was located further from the glomerulus into which they projected and they had long lateral dendrites extending into the external plexiform layer. Middle tufted cells and mitral cells had large cell bodies located in the external plexiform layer and in the mitral cell layer, respectively, a thick apical dendrite projecting into a single glomerulus and long lateral dendrites in the external plexiform layer.

Morphological reconstruction. Neurobiotin or biocytin (Vector Laboratories INC., Burlingame, CA) was added to the intracellular solution (1mg/ml). The patch pipette was slowly retracted after the recording to avoid damaging the cell body. The slice was then fixed in 4% paraformaldehyde overnight, washed 3 times in PBS and incubated in a permeabilizing solution containing Alexa Fluor 555 conjugated streptavidin (1 µg/ml; Thermo Fischer Scientific, Waltham, MA) overnight. After 3 rinses with PBS, sections were mounted in Vectashield Hardset with DAPI (Vector laboratories, Inc., Burlingame, CA). Labeled cells were imaged with a confocal microscope (Leica TCS SP5 II).

Immunohistochemistry: ChAT mice expressing ChR2-eYFP in BF neurons were deeply anesthetized with Zolazepam tiletamine/Xylazine and transcardially perfused with PBS at room temperature followed by 4% paraformaldehyde (PFA, 4°C). Brains were removed, postfixed 3-6 hrs in 4% PFA at 4°C, rinsed in PBS and incubated in PBS until cut on a vibratome (VT 1000S, Leica). 50 µm-thick coronal sections were collected and stored in PBS. For ChAT staining, sections were incubated overnight at 4°C with a goat anti-ChAT (1:1000; Millipore Cat# AB144P, RRID:AB_2079751) in Tris-Triton buffer containing 2% donkey serum and 0.2% Triton X100. After three washes in Tris-Triton, sections were incubated 1 hour at room temperature with Alexa fluor 647 conjugated donkey anti-goat (1:500; Thermo Fisher Scientific Cat# A-21447, RRID:AB_2535864). After three washes, sections were mounted in Prolong Diamond Antifade Mountant (ThermoFisher) or Vectashield Hardset with DAPI. Images were taken using a Leica TCS SP5 II confocal microscope or a Zeiss Axio Imager M2 for mosaic images. Immunostained and EYFP-expressing cells were manually counted using the cell counter plug-in on Fiji software.

Fluorescence in situ hybridization (FISH) combined with immunohistochemistry was done using the RNAscope® Multiplex Fluorescent Reagent Kit V2 (Advanced Cell Diagnostics Cat. #323100) according to the manufacturer's protocols. Briefly, whole brains from 6 weeks old

838 transcardially perfused mice (n=3, M and F wild-type littermates from the *dlx5/6*^{Cre} colony)
839 were extracted and immediately placed in 4% PFA, post-fixed 6 hrs in 4% PFA at 4°C and
840 cryoprotected with successive incubations in 10%, 20% and 30% sucrose solution. OBs were
841 then embedded in Tissue-Tek® OCT, frozen on dry ice and stored at -80°C until sliced with a
842 cryostat (Leica CM3050 S) into 10-15 µm coronal sections, adhered to SuperFrost Plus slides
843 (VWR) and immediately refrozen at -80°C.

844 Unless otherwise stated, the probe and all reagents were provided in the RNAScope
845 Multiplex Fluorescent Reagent Kit v2. On day 1, samples were washed in PBS to remove OCT,
846 incubated 30 min at 60°C, post-fixed in 4% PFA for 15 minutes at 4°C and dehydrated with
847 50% (x1), 70% (x1), and 100% (x2) ethanol washes for 5 minutes. Slides were air-dried and a
848 barrier drawn around the tissue section with an Immedge hydrophobic barrier pen (Vector
849 Laboratories, Burlingame, CA). Endogenous peroxidase activity was blocked using hydrogen
850 peroxide for 10 min at RT. Sections then underwent antigen retrieval by submersion into
851 boiling (~98°C-102°C) co-detection target retrieval solution for 5 min and were rinsed in
852 distilled water (5 times) and then in PBS-tween (PBS-T, 1 time). Sections were incubated
853 overnight at 4°C with primary antibodies (rabbit anti-CR, 1:1000; Swant Cat# 7697,
854 RRID:AB_2721226 or rabbit anti-TH, 1:500, Millipore Cat# AB152, RRID:AB_390204) diluted
855 in the co-detection antibody diluent provided by the manufacturer.

856 On day 2, slices were washed in PBS-T 3 times for 2 min, post-fixed in PFA 4% for 30 min at
857 RT, washed 4 times in PBS-T and treated with protease reagent for 30 min at 40 °C. After
858 rinsing twice in distilled water, sections were incubated with the RNAScope probe for Chrm1
859 (RNAScope® Probe- Mm-Chrm1, target region 851 – 1994; catalog #495291-C1) for 2 h at
860 40°C for the hybridization step. Sections were then washed twice in wash buffer at RT, then
861 incubated in Multiplex v2 AMP 1 (40°C for 30 min), AMP 2 (40°C for 30 min) and AMP3 (40°C
862 for 15 min) for the amplification steps. After rinsing twice in wash buffer, sections were
863 incubated in HRP-C1 for 15 min at 40°C, washed twice in wash buffer and incubated 30 min
864 at 40°C with the Opal 570 fluorophore (AKOYA BIOSCIENCES, catalog #FP1488001KT) to mark
865 Chrm1. Sections were washed and subsequently incubated in HRP blocker for 15 min at
866 40°C. I then further incubated the sections in a secondary antibody (Alexa fluor 488 goat
867 anti-rabbit; 1:500; Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165) diluted in co-
868 detection antibody diluent for 45 min at RT to visualize CR or TH. The slides were then

869 washed in PBS-T, incubated with DAPI for 30 s at RT and covered with ProLong gold antifade
870 mounting medium (Thermo Fisher Scientific. RRID:SCR_015961).

871 Confocal images were acquired using a Leica TCS SP5 II confocal microscope through a 20X
872 air objective and a 63X oil-immersion objective. Images were analyzed using the Fiji
873 software. Hybridization signal was converted into a binary mask by setting a single
874 threshold. Hybridization dots were counted using the analyze particle function on Fiji
875 Software and DAPI-stained cells were manually counted using the cell counter plug-in.
876 Normalized fluorescence intensity profiles were estimated within a 650X50 μm area
877 spanning from the nerve layer to the granule cell layer and centered on the mitral cell layer.

878 Drugs. Chemicals used to prepare cutting, recording and internal solutions were acquired
879 from MilliporeSigma, Carl Roth and Fisher Scientific. 6-nitro-7-sulfamoylbenzo[f]quinoxaline-
880 2,3-dione (NBQX), D-2-Amino-5-phosphonopentanoic acid (D-AP5), 2-(3-carboxypropyl)-3-
881 amino-6-(4 methoxyphenyl)pyridazinium bromide (gabazine), mecamylamine hydrochloride
882 and atropine were purchased from Abcam Biochemicals. Scopolamine hydrobromide,
883 Pirenzepine and XE-991 were purchased from Tocris Bioscience.

884 Electrophysiological analysis. Action potential capacitive currents were automatically
885 detected by the Axograph X software using an amplitude threshold. The timing of each spike
886 was used to construct PSTHs (peri stimulus time histograms) representing the total number
887 of action potentials per time period (bin of 20 ms for 2 s-long episodes, of 200 ms for 15 s or
888 longer episodes) across several consecutive sweeps (>30 for 2 s-long episodes, >10 for 15 s-
889 long episodes). For cell classification, a post-stimulus spiking inhibition was a statistically
890 significant (paired t-test or paired Wilcoxon signed-rank sum test) decrease in spike rate
891 within a 100 ms or 200 ms-long time period immediately after the flash compared to the
892 same period preceding the flash across at least 30 consecutive trials. The duration of spiking
893 inhibition was calculated as the average duration between the flash and the first spike fired
894 after the flash. A muscarinic excitation was a statistically significant increase in spike
895 probability within a 1 s period starting 1 s after the flash compared to the 1s period
896 immediately preceding the flash across at least 10 consecutive trials.

897 Photo-evoked IPSC amplitudes were measured as the peak of an average response
898 computed from multiple sweeps. The decay of photo-evoked IPSCs was most often best

fitted with a double exponential with time $t=0$ at the peak of the current. Time constant values indicated in the text are weighted decay time constants calculated using the following equation: $\tau_w = (\tau_1 A_1 + \tau_2 A_2) / (A_1 + A_2)$ where τ_1 and τ_2 are the fast and slow decay time constants and A_1 and A_2 are the equivalent amplitude weighting factors.

EPSCs and IPSCs were automatically detected by the Axograph X software using a sliding template function. The onset of ON-evoked EPSCs was measured at 5% of the first peak of the response. The latency of an ON-evoked EPSC was defined as the time interval between the beginning of the stimulation artifact and the onset of the first EPSC. To estimate the duration of an ON-evoked plurisynaptic excitatory response, PSTHs representing the cumulative number of EPSCs per 20 ms bin across several consecutive sweeps were constructed. I then determined the time needed after stimulation for the EPSC frequency to come back to baseline frequency + 2 SD during at least five consecutive bins. Baseline frequency was calculated over the 25 bins (i.e. 500 ms) preceding the stimulation.

Data are presented as mean $\pm$ SD in the text and as mean $\pm$ SEM in graphs for display purpose. Data points from experiments were tested for normality using a Shapiro–Wilk test. Experiments with a normal distribution were tested for statistical significance with a paired Student’s t-test. Experiments with skewed distributions were tested for statistical significance using a paired Wilcoxon signed-rank sum test. For experiments comparing data points from different cells, statistical significance was determined using an unpaired t-test (normal distribution) or a Mann–Whitney test (non normal distribution).

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932

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934

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Figure supplements

Figure 2-figure supplement 1: Higher resolution image of Fig.2C showing the distribution in the OB of ChAT mice of eYFP-expressing axons from BF cholinergic neurons. The two channels used to make this image are shown on top.

Figure 3. Figure supplement 1 : Higher resolution image of Fig.3B showing the distribution in the OB of a *dlx5/6* mouse of eYFP-expressing axons from BF cholinergic and GABAergic neurons. The two channels used to make this image are shown on top.

Figure 3. Figure supplement 2: BF inputs have various impacts on PG cells activity in *dlx5/6* mice

A, Example of an inhibitory response. In this cell, photostimulation of the BF fibers (blue arrow, 1 ms flash, 490 nm) transiently inhibited spiking. Top, a representative 2 s-long LCA recording episode (scale bar 20 pA). Middle, cumulative PSTH (bin size 20 ms) in the same cell for 30 consecutive episodes with stimulation. Bottom, raster plot showing spiking activity in control condition (episodes 0-30) and while BF axons were photostimulated once per episode with a single flash (episodes 31-60, blue line). **B**, Example of an excitatory response where BF fibers photostimulation (episode 27-55) induced an inward current and a spike (red star in the inset). Scale bar 30 pA. Corresponding PSTH and raster plot, same as in A. **C**, example of a dual inhibition-excitation response where BF axons photostimulation inhibited spiking after the flash but also induced a muscarinic increase in baseline firing rate (see raster plot). Scale bar 200 pA. PSTH and raster plot, same as in A and C. **D**, Probability of occurrence of the various types of LCA responses induced on PG cells firing by a single optogenetic stimulation of the BF axons.

*Figure 3-figure supplement 3: the unique example, in a *dlx5/6* mouse, of a cell responding with a long-lasting excitation that was not accompanied by a transient inhibitory phase immediately after the flash*

Figure 6-figure supplement 1: RNAscope imaging of *Chrm1* mRNA expression in the OB

A, Widespread expression of *Chrm1* mRNA in an OB section. Single mRNA molecules of *Chrm1* appear as punctate green dots, DAPI staining in white. The yellow rectangle indicates the area where fluorescence was quantified in **B**. **B**, Normalized fluorescence intensity profile averaged across six OB sections of similar size. Quantification of fluorescence intensity was done within a 50 μm X 650 μm area centered on the mitral cell layer. The black trace is the average; the grey lines are the measures from each section. Dashed vertical lines show approximate borders of the granule cell layer (GCL), external plexiform layer (EPL), and glomerular layer (GL). **C**, Zoom on one glomerulus in the GL (top) and on granule cells in the GCL (bottom). Images from the same section. Scale bars 20 μm . **D**, Average number of dots per cell (top) and percentage of cells with no dot (bottom) in the GL and in the GCL. Each point is from one OB section. Bars indicate the means. **E**, Multiplexed fluorescence ISH of the *Chrm1* transcript (green dots) combined with the immunodetection of CR (magenta) in the GL. Arrow indicates a CR-expressing cells containing >5 fluorescent dots. **F**, Multiplexed fluorescence ISH of the *Chrm1* transcript (green dots) combined with the immunodetection of TH (magenta) in the GL. Arrow indicates a TH-expressing cells containing >5 fluorescent dots. Scale bars in **E** and **F** 10 μm .

Figure 7 – figure supplement 1: Comparison of BF-induced increase of IPSCs in external tufted cells (eTC) and in superficial and middle tufted cells (s/mTC). A-D, Impact of a low frequency (0.5 Hz) photostimulation of the BF cholinergic axons on IPSCs frequency in s/mTC and eTC in control condition (top) and in the presence of 2 μ M pirenzepine (bottom). A and B, average IPSCs frequency per episode (2 s each). Photostimulation of BF axons (1 flash/episode) starts at episode 41. Each line is a cell; colored lines are ensemble averages. C, average IPSCs frequency per cell when BF axons were not stimulated (baseline) and when BF cholinergic afferents were stimulated at 0.5 Hz (stimulation). Blue circles are the means for eTC, magenta circles are the means for s/mTC and opened circles are the ensemble means. D, percent BF-induced increase of IPSCs frequency in eTC vs. in s/mTC. Horizontal lines are the averages. E-G, Impact of a single photostimulation of the BF cholinergic axons on IPSCs frequency in s/mTC and eTC in control condition (top) and in the presence of pirenzepine (bottom). The boxed areas in E show the pre- and post-stimulus periods that were compared in G and H. Data points were all collected in slices from ChAT mice in the presence of NBQX, d-AP5 and mecamylamine (control condition).

Figure 8 – figure supplement 1: Absence of cholinergic excitation in PG cells showing a transient inhibition of spiking. *A*, low frequency photostimulation of the BF axons (1 flash per 2 s-long episode, starting at episode 41). *B*, single photostimulation (blue arrow). Each grey line is a cell, the black line is the ensemble average.

Figure 8 – figure supplement 2: Caveats for the measurements of spiking inhibition duration *A*, Raster plots from three example cells with various spiking activities that were inhibited by the BF input. The duration of the BF-induced inhibition was measured as the average time between the flash (blue bar) and the first spike after the flash (red tick). Histograms show the distribution of the first spike timing in each case and the calculated average duration. *B*, Average first spike timing in all the cells used for Fig.8A as a function of their baseline firing frequency.