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Amygdala Corticofugal Input Shapes Mitral Cell Responses in the Accessory Olfactory Bulb

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Abstract

Interconnections between the olfactory bulb and the amygdala are a major pathway for triggering strong behavioral responses to a variety of odorants. However, while this broad mapping has been established, the patterns of amygdala feedback connectivity and the influence on olfactory circuitry remain unknown. Here, using a combination of neuronal tracing approaches, we dissect the connectivity of a cortical amygdala (PmCo) feedback circuit innervating the mouse accessory olfactory bulb (AOB). Optogenetic activation of PmCo feedback mainly results in feed-forward mitral cell (MC) inhibition through direct excitation of GABAergic granule cells (GC). In addition, LED-driven activity of corticofugal afferents increases the gain of MC responses to olfactory nerve stimulation. Thus, through corticofugal pathways, the PmCo likely regulates primary olfactory and social odor processing.

Significance statement

Olfactory inputs are relayed directly through the amygdala to hypothalamic and limbic system nuclei regulating essential responses in the context of social behavior. However, it is not clear whether and how amygdala circuits participate in the earlier steps of olfactory processing at the level of the olfactory bulb. Unraveling the organization of this circuitry is critical to understand the function of amygdala circuits. Combining cre-dependent viral tracing with optogenetic-assisted patch clamp electrophysiology, the present work maps the synaptic connectivity and physiology of a cortical amygdala pathway innervating primary olfactory circuits.

Introduction

The accessory olfactory system (AOS) plays a crucial role in the detection of sensory signals used for individual recognition in the context of social, reproductive and parental relationships (**Halpern, 1987**; **Winans and Powers, 1977**; **Meredith, 1991**; **Dulac and Wagner, 2006**). AOB neurons processing these chemical signals relay their output directly to the amygdala, which in turn provides feedback projections to AOB circuits (**Raisman, 1972**). Although the precise cell-to-cell connectivity of these circuits is largely unknown, the lack of thalamic relays implies that any refinement of the incoming sensory information must be carried out by either primary AOS circuits, amygdala feedback projections or both.

The AOS detects olfactory information through sensory neurons localized in the vomeronasal organ (VNO). Each sensory neuron innervates multiple glomeruli in the AOB, the most posterior-dorsal bulbar region (**Belluscio et al., 1999**). Here, mitral cells (MCs) integrate inputs from multiple glomeruli (**Wagner et al., 2006**), before relaying this information directly to the medial (MeA) and cortical (PmCo) amygdala subnuclei (**Winans and Scalia, 1970**). Importantly, this connectivity differs dramatically from the main olfactory bulb (MOB), where each MC contacts a single glomerulus comprised of input from sensory neurons expressing the same receptor subclass. Therefore, whereas in the MOB each MC primarily encodes inputs from single odorants, AOB MCs convey to the amygdala related to blends of chemical ligands, which can be as complex as the number of afferent receptor neurons on a given MC. Surprisingly, AOB MCs are capable of highly selective responses to complex individual odor signatures (**Ben-Shaul**

et al., 2010; **Luo et al., 2003**), yet how such narrow tuning is achieved is unclear. Among the possible mechanisms, lateral inhibition through local GABAergic granule interneurons (GCs) has been proposed for both the MOB and AOB (**Geramita et al., 2016**; **Hendrickson et al., 2008**). In the MOB, in addition to these horizontal interactions, GC activity is also strongly modulated by top-down feedback from the piriform cortex (**Boyd et al., 2012**; **Matsutani, 2010**; **Balu et al., 2007**). Not only it has become increasingly evident that this modulatory feedback represents a critical component of olfactory perception (**Boyd et a., 2012**; **Markopoulos et al., 2012**; **Otazu et al., 2015**; **Oettl et al., 2016**), but it is also clear that both mechanisms can interact to generate optimized odor representations by MCs.

Here, we dissect the functional connectivity of a corticobulbar amygdala circuit originating in the posteromedial cortical nucleus (PmCo) and modulating (AOB) output neurons. We show that PmCo input indirectly modulates MC firing through local inhibitory networks. This occurs via enhancement of MC responses to electrically evoked vomeronasal inputs from the periphery. Our results reveal that modulatory feedback from the cortical amygdala is capable of exerting top-down modulation on likely peripheral AOS responses to social stimuli.

Materials and Methods

Animals

Mice were housed in the Children's National Health Center temperature- and light-controlled animal care facility and given food and water *ad libitum*. All animal procedures were approved by the Children's National Institutional Animal Care and

Utilization Committee (IACUC) and conformed to NIH Guidelines for animal use. *nNOS*^{cre} mice (B6.129-Nos1tm1(cre)Mgmj/J; RRID:SCR_014588), RABV mice (B6;129P2-Gt(ROSA)26Sortm1 (CAG-RABVgp4,-TVA)Arenk/J; Stock No: 024708), *GAD*^{cre} (Gad2<tm2(cre)Zjh>/J; RRID:MGI:4418723) and *Dlx5/6*^{cre} mice (Tg(dlx6a-cre)1Mekk/J; RRID:IMSR_JAX:008199) were all obtained from Jackson Laboratories. *Sim1*^{cre} mice were kindly provided by Joel Elmquist (Tg(Sim1-cre)1Lowl/J; RRID:IMSR_JAX:006395) and *Pcdh21*^{cre} animals were kindly provided by Dr. Kevin Briggman (Tg(Cdhr1-cre) KG76Gsat; RRID:MMRRC_036074-UCD)

Viral vectors and stereotaxic injections

The following procedures were followed for each tracer or viral vector injected: Postpubertal mice (postnatal day 30–50) were anesthetized with an IP injection of a 10 µl/g of anesthetic cocktail (8.5 ml sterile saline, 1 ml 100 mg/ml ketamine, 0.5 ml 20 mg/ml xylaxine). Injection sites targeting the PmCo were determined based on the coordinates referred to Bregma: X: -2.5, Y: 2.6, Z: -5.3. Injections (50-100 nl) were made bilaterally using beveled glass pipettes (Kingston Glass) at depths of 5.1-5.3 mm from the pial surface. Viral injections were manually assisted by the use of a Pico Injector (Harvard Apparatus, pli-100), each pressure step delivering ca. 10-20 nL, 1 per minute. Ten minutes after final injection, the glass pipette was withdrawn and the wound sutured. PRV tracing from the AOB was preferably performed using the RABV mouse line due to encountered problems of tissue damage and starter cell viability - especially in AOB GCs.

Cholera toxin subunit-B (Thermo Fisher scientific; Alexa Fluor® 555 Conjugate, C34776; Alexa Fluor® 488 Conjugate, C22841) was diluted 10 µg/µl in sterile PBS, aliquoted and stored at 4 °C until use. The viral vectors used were obtained from: University of North Carolina Vector Core: double floxed reporter, rAAV5/EF1a-DIO-eYFP; University of Pennsylvania Vector Core: double floxed ChR2, AAV9.EF1.dflox.hChR2 (H134R)-mCherry.WPRE.hGH, AddGene20297; CaMKIIa-ChR2, AAV1.CaMKIIa.hChR2 (H134R)-mCherry.WPRE.hGH; Salk Institute Vector Core: PRV, G-deleted-rabies, pseudotyped rabies virus, hereby referred to as "PRV", AddGene 32635 (eGFP), 32636 (mCherry). Each vector was aliquoted and stored at -80 °C until use.

Histology and Immunohistochemistry

Mice were anesthetized with a 4:1 cocktail of ketamine and xylazine (Bayer) and perfused transcardially with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS). Brains were removed, postfixed for 6 hr in 4% paraformaldehyde and incubated overnight in 0.1 M PBS containing 30% sucrose. Cryosections (30 µm thick) were mounted on SuperFrost Plus glass slides for immunofluorescence analysis. Tissue sections were washed (10 min) in PBS, incubated in blocking solution containing 0.5% Triton X-100, 4% horse serum, and PBS (1 hr, room temperature), and incubated overnight at 4°C in blocking solution containing the first primary antibody. Tissue was then washed in PBS (10 min), followed by incubation in secondary antibody for 1hr at room temperature. Primary antibodies used were: anti-Tbr1 (1:500, chicken polyclonal; #AB2261, Millipore), anti-CaMKIIa (1:500, mouse, #SA-

162, Biomol Research Laboratories); anti-Sim1 (1:1000, rabbit, #ab4144, Millipore; RRID:AB_2187608), anti-Cux1 (1:100, mouse, #sc-514008, Santa Cruz; RRID: not available). Secondary antibodies used were Alexa-Fluor 488 donkey anti-mouse (RRID:AB_141607), Alexa fluor 647 donkey anti-chicken (RRID:AB_11194678), Alexa fluor 647 donkey anti-rabbit (RRID:AB_2536183) (all diluted 1:1000).

Brain 3D reconstructions

The 3D reconstructions of injected brains or areas (for the spatial representation of Ct-b staining in **Figure 1** or viral expression in **Figure 5**) were obtained by assembling stacks of images acquired from seriate and consecutive brain sections (30 µm thick), using the Image-J "TrackEM2" plugin. The 3D morphology of Ct-b or viral labelling were captured by 2D thresholded contour delineation. Import of the 3D assembly into the open source software Blender (Blender.org) allowed the editing of shading, transparency, lighting and the 3D rendering of the reconstruction.

Acute Brain Slice Preparation

Acute slices were prepared from 2-4 month old male and female mice. Animals were anesthetized with CO2 and decapitated. Brains were removed quickly and placed in cold (48 °C) sucrose-based oxygenated (95% O2/5% CO2) cutting solution composed of (in mM) sucrose 234, glucose 11, NaHCO3 26, KCl 2.5, NaH2PO4 H2O 1.25, MgSO4 7*H2O 10, and CaCl2 H2O 0.5. Coronal slices containing the PmCo were obtained with a slicing vibratome (VT1200s; Leica, Wetzlar, Germany) by removing the cerebellum with a perpendicular cut to the rostral–caudal plane and gluing the caudal

side down on the vibratome stage submerged in cold cutting solution. Slice thickness was 300 um for all experiments. The slices were immersed in oxygenated (95% O2/5% CO2) artificial cerebral spinal fluid (ACSF) at 34 °C for 30–45 min. ACSF was composed of (in mM) NaCl 126, NaHCO3 26, glucose 10, KCl 2.5, NaH2PO4 H2O 1.25, MgCl2 7*H2O 2, and CaCl2 2*H2O 2, pH 7.4, with osmolarity maintained at 290–300 mOsm.

Slice electrophysiology

Slices were transferred to a recording chamber and superfused with ACSF. All experiments were conducted at room temperature 25-27 °C. Patch-clamp recordings were performed using an upright microscope (Nikon E600 F, Tokyo, Japan), equipped with ×10 and ×60 objectives and DIC optics. Neuron types were identified by their morphology, intrinsic properties, and layering within the different nuclei examined (OB or PmCo). In some recordings biocytin (3-5%, B1592, ThermoScientific) was added to the intracellular solution. This contained (in mM) 130 K-gluconate, 10 NaCl, 10 HEPES, 0.6 EGTA, 2 Na_2ATP, 0.3 Na_3GTP. In some cases, when inhibitory currents were recorded (pair recording experiments) a high chloride solution was used: 70 K-gluconate, 70 KCl, 10 HEPES, 10 EGTA, 2 $MgCl_2$, 2 Na_2ATP, 0.3 Na_3GTP. Recordings were made using a Multiclamp 700B amplifier (Molecular Devices) digitized at 10–20 kHz and acquired using Clampex Software (pClamp 10, Molecular Devices). For most recordings, pipette resistance was 3–6 MOhm. Series resistance was normally <30 MOhm and periodically monitored. Bessel was set at 1 kHz for all voltage clamp and 10 kHz for current clamp experiments. Gain was set at 5 V/V in current clamp recordings.

For experiments involving optogenetic stimulations, a patterned LED light illuminator (Polygon 400, Mightex) was used to illuminate tissue sections (Light source 470 nm, 11 mW, Mightex). Full-field illumination was used unless stated otherwise, setting the LED intensity at 10% of the maximum, which gave us the best control on LED spatial specificity. During mitral cell (MC) recordings, granule cell stimulation was obtained by centering the objective on the granule cell (GC) layer, just below the recorded MCs but far enough to avoid mitral layer stimulations. Full-field illumination did not alter the amplitude of light-evoked responses. The stimulation frequencies used during paired recordings were chosen to mimic odor evoked responses (**Schoppa, 2006**) and, in the case of optogenetic activation, to elicit efficient ChR2-mediated AP propagation while avoiding channel habituation (**Lin, 2011**).

Protocol used for dual VN and PmCo stimulations

Mitral cells were recorded during four different conditions: 1) spontaneous activity was recorded in absence of any stimulation ("baseline"); 2) mitral cell firing was recorded in presence of glomerular electrode stimulation only ("E"), using a stimulation frequency previously used to mimic the physiological activity of olfactory afferents (100 Hz trains at 4 Hz; Schoppa, 2006); 3) MC were recorded during concurrent electrode GL stimulations and light activation of the PmCo afferents reaching the GC layer ("EO"). Optogenetic stimulations were not delivered at frequencies higher than 20 Hz, to avoid ChR2 desensitization (**Lin, 2011**; **Boyd et al., 2012**). Each protocol was run for 5 minutes during which seal resistance was monitored. Typically after seal formation mitral cells were left to stabilize for a few minutes before the recording started. Given

the different duration of single LED and electrical pulses (0.4 and 4 ms, respectively) the two stimuli were not overlapped. However, since the effect of LED stimulation on MC firing was evident on a wider scale (even seconds; Figure 2C), we placed each 20 Hz LED train between the electrode 100 Hz train (40 ms duration) and the end of the following inter-train interval (ca. 200 ms), in order to cover the period in which both direct and indirect (rebound activity) MC responses have been previously observed (electrode train onsets: 81.2, 331.2, 581.2, 831.2 ms; waveform: offset from digitizer output = 0.5 ms, pulse duration = 0.4 ms, after pulse duration 9.1 ms, total pulse duration 10 ms; LED train onsets: 91.2, 141.2 ,191.2 ,241.2 ,291.2 ms; waveform: offset from digitizer output = 10 ms, pulse duration = 4 ms, after pulse duration 36 ms, total pulse duration 50 ms). The firing rates resulting from dual stimulations (EO) were compared to those evoked by LED stimuli alone (O) and calculated as previously described (EO = FRelectrode+LED - FRbaseline)/(FRelectrode+LED + FRbaseline), O = FRLED - FRbaseline)/(FRLED + FRbaseline) ; Boyd et al., 2012). The relative effect of optogenetic stimulation of PmCo afferents (EO) on VN-evoked responses (E), was calculated referring EO to VN-evoked frequency changes (E = FRelectrode - FRbaseline)/(FRelectrode + FRbaseline). Brains in which viral expression was found widespread outside the PmCo (in the MeA and BAOT) were discarded and not included in this analysis.

Statistics and data analysis

All ANOVA analyses are performed with the SPSS software. The Bonferroni correction method was used for the post-hoc tests, when applicable. All indicated data are expressed as means ±SEM.

Statistical table

Experiment	Fig.	Test	Ind. var.	Factors	F	Effect	p
PRV-tracing	Fig.2A_2	t-test	PRV+cells	N.A.	N.A.	genotype	0.018
MC (LED-)	Fig.6C_1	p. t-test	AP freq.	N.A.	N.A.	Stim.	0.019
MC (LED+)	Fig.6C_1	p. t-test	AP freq.	N.A.	N.A.	Stim.	0.002
Evoked resp.	Fig.7B	t-test	amplitude	N.A.	N.A.	cell type	0.0001
(f.stim vs f.post)	Fig.8B	p. t-test	AP freq.	N.A.	N.A.	Stim.	0.05
MC (electrode)	Fig.8B	2wANOVA	AP prob.	I, R	$F_{5,58} = 26.3$	I	0.004
MC (electrode)	Fig.8B	2wANOVA	AP prob.	I, R	$F_{1,58} = 2.6$	R	0.112
MC (electrode)	Fig.8B	2wANOVA	AP prob.	I, R	$F_{5,58} = 0.76$	interaction	0.622
Dual stim., type I MCs	Fig.8F	2wANOVA	AP rate	Protocol, cell type	$F_{4,36} = 2.7$	interaction	0.04
EO/E plot	Fig.8G	X-sq.	Distrib.	N.A.	N.A.	N.A.	$2{,}4 \cdot 10^{-16}$
EO/O plot	Fig.8H	X-sq.	Distrib.	N.A.	N.A.	N.A.	$3{,}5 \cdot 10^{-18}$

Abbreviations: stim., stimulation; resp., responses; distrib, distribution; I, current; R, resistance; freq, frequency; prob., probability; AP, action potential.

Results

The posteromedial cortical amygdala sends corticofugal afferents to the AOB

The AOB is densely innervated by cortical amygdala output neurons (**Raisman, 1972**; **Gutiérrez-Castellanos et al., 2014**; **Oh et al., 2014;** Allen Brain Mouse Connectivity Atlas, experiment #114249084). However, their precise target localization and identity are unknown. To precisely identify the source of neuronal projections to the AOB, we first locally injected the retrograde tracer cholera-toxin (Ct-b; **Figure 1A**). Precise targeting of the AOB, with very limited spread to the MOB (4/10 subjects; **Figure 1B**), consistently resulted in dense labeling of layer II and III in the posterior medial nucleus of the cortical amygdala (PmCo; **Figure 1C,D**).

Layer specific Ct-b injections revealed that Ct-b injections in the GC alone are sufficient to retrogradely label PmCo neurons (data not shown). However, since the tracer can be taken up by passing axon terminals also directed to MCs, this method is not valid to assess the specificity of PmCo targets. To better determine the layer-specificity of ACP afferents to the AOB, we next used a pseudotyped rabies virus as

conditional retrograde tracer (herein referred to as *PRV*; **Wickersham et al., 2007**; **Figure 1E**). In this experiment, PRV was injected in the AOB of a mouse line in which the expression of the protein rabies-G (RABVgp4, required for viral amplification and retrograde PRV trans-synaptic transport) and the avian receptor TVA (required for the virus to access the host cells) were under *cre*-dependent control (RABV mice; **Takatoh et al., 2013**). *RABV* mice were crossed either with mice expressing *cre* recombinase under the control of the MC specific promoter *Pcdh21* (MCs) or the *nNOS* promoter, expressed by GC layer inhibitory neurons (GCs and main accessory cells or *macs*; **Kosaka and Kosaka, 2007**; **Larriva-Sahd, 2008**). As neither *Pcdh21* nor *nNOS* are expressed in the PmCo (**Figure 1F**), both TVA and G expression were limited to the injection site (**Figure 1G$_1$,H$_1$**). This allowed only monosynaptic retrograde tracing (for example, no PRV expression was found in areas two synapses away from AOB starter neurons, such as the hypothalamus or the hippocampus). Although it is possible that TVA/G can be expressed elsewhere due to *cre*-expression outside the AOB (for example *Pcdh21* expression in the anterior piriform cortex, **Nagai et al., 2005**, and *nNOS* expression in the Islands of Calleja, MeA, Cerebellum, CPu, Cortex, hippocampus, hypothalamus), thus inducing neurons outside the AOB to be possible starters for PRV transport to the PmCo. However, this possibility could be ruled out as neither of these regions project to the AOB nor show PRV-expression. Consistent with our Ct-b tracing experiments, retrograde PRV labeling was found in several AOS regions, including the PmCo (**Figure 1G$_2$,H$_2$**) and mainly from infection of *nNOS*^{cre}+ neurons (ratio of PRV-positive cells PmCo/AOB: *Pcdh21*^{cre}+, 0.04 ± 0.02; *nNOS*^{cre}+, 0.54 ± 0.2; N = 4 brains for each strain, ca. 5 sections per animal, 1 tissue section from

283 every 6th). These results confirmed that *nNOS*-expressing GC layer inhibitory neurons
284 (GCs and macs), as opposed to MC neurons, are the major target of ACPs.
285
286 **Sublaminar specificity of PmCo-AOB reciprocal connections**
287 Although, PRV-RABV allows for layer specific retrograde tracing, through this approach
288 is not possible to quantify the relative contribution of different neuronal types (GCs or
289 macs) to the retrograde PRV infection. This limitation also prevents calculation of
290 relative amount of input neurons reaching these neurons from any brain area. To
291 estimate the number of starter neurons in the AOB and the relative contribution of GCs
292 and macs to the retrograde PRV infection, we used a complementary viral approach
293 (**Watabe-Uchida et al., 2012**; **Menegas et al., 2015**) to conditionally express rabies-G
294 and TVA-mCherry in *nNOS*^{cre}-expressing neurons in the OB. This allowed for a more
295 precise quantification of starter cells in the AOB, as those infected by PRV are GFP+
296 and those expressing the molecular component rabies-G and TVA are mCherry-positive
297 (**Figure 2A**). Although double labeled cells were found in both GCs and macs (**Figure**
298 **2A$_1$**), the majority were identified as GCs based on morphological criteria (average
299 percentage of total starters: GC 75,4%, macs 24,5%; N = 4; **Figure 2A$_3$**). Quantification
300 of all PRV-positive cells in the brains of infected animals showed consistent labeling in a
301 restricted range of olfactory and limbic areas (**Figure 2A$_4$**). For each brain region, the
302 relative percentage of traced neurons was calculated over the amount of PRV cells
303 collectively sampled in all brain areas (PRV-region / PRV-brain x 100; N = 4 brains, ca.
304 4-5 sections per animal, 1 tissue section every 6th). The amygdala alone gives rise to
305 9.7% of input neurons to AOB cre-expressing cells (**Figure 2A$_4$**). Of these, 83.5% are

localized in the PmCo, with the majority arising from layer III (**Figure 2A$_4$**). These findings were consistent with our above Ct-b tracing experiments (**Figure 1C,D**). Overall, these results reveal that ACPs represent a major source of top-down feedback mainly targeting GCs in the AOB.

Molecular phenotype and connectivity of ACPs

To define the molecular phenotype of ACPs, we conducted multi-channel immunohistochemistry on tissue sections from AOB Ct-b injected brains. We found that almost all PmCo Ct-b-positive neurons co-expressed the excitatory neuronal markers CaMKIIa and Tbr1 (ca. 90%; **Figure 3A**), with no co-expression of markers of inhibitory neurons such as GAD or Dlx5/6 (**Figure 3B,C**). Retrogradely traced amygdala corticobulbar projection neurons (ACPs) also expressed Ctip2 and Cux1 (ca. 30% overlap; **Figure 3D**), similar to other subpopulations of corticobulbar neurons in the piriform cortex (**Diodato et al., 2016**). A large majority (82.4%) of Ct-b positive ACPs also co-expressed Sim1, a limbic system marker (**Semple and Will, 2018**; **Figure 3E**).

Interestingly, corticobulbar projection neurons in the piriform cortex have been shown to extend axon collaterals to other subcortical and cortical targets (**Diodato et al., 2016**). This implies the existence of top-down inputs from other high order olfactory areas such as the PmCO. Specific gene expression patterns in piriform corticobulbar projections have been associated with this top-down cortical circuit (**Diodato et al., 2016**). In particular, Cux1/Ctip2 expressing piriform cortex neurons have been shown to project to both the OB and prefrontal cortical areas (PFC, **Diodato et al., 2016**). Thus, to evaluate the presence of ACPs axon collaterals to other brain regions, we injected

Ct-b coupled with different fluorophores into both the AOB (Ct-b 555) and other known targets of PmCo efferent projections (Ct-b 488; **Gutiérrez-Castellanos et al., 2014**; **Figure 4A**). We found unbiased 555/488 dual labeling only when the MeA (7.6%), the medial prefrontal cortex (1.7%, mPFC) and the entorhinal cortex (2.8%, Ent) were targeted together with the AOB (N = 3; **Figure 4G**,**I**,**J**). Since no detectable differences were found using either tracer in single Ct-b injection experiments, we are confident that ACPs mainly target the AOB with very limited collateral axonal projections to the MeA, Ent and mPFC. This result also reveals similarities between ACPs and the sub-populations of other corticobulbar projection neurons in the piriform cortex (**Diodato et al., 2016**).

To further validate these findings, we performed anterograde viral tracing experiments using a *CaMKIIa*-specific adeno-associated virus, exploiting the high expression levels of *CaMKIIa* in ACPs. When viral injections were restricted to the PmCo (N = 6; **Figure 5A-D**), there was negligible or no viral expression in any targets of PmCO efferent projections such as the BAOT, basolateral amygdala, olfactory tubercle and mPFC (**Figure 5B-D**). Negligible or no evidence of viral expression was detected in the MeA, Ent and PFC (all receiving minimal ACP collateral input; **Figure 4F-K**), with the strongest expression in the AOB GC and along the stria terminalis (**Figure 5A,B**). Overall, as shown by different retrograde and anterograde tracing methods, these results confirm that the AOB is the major target of ACPs. This implies that ACPs might be predominantly functioning in the modulation of AOB output.

ACPs synapse onto AOB GABAergic interneurons

From our viral tracing experiments, AOB GCs appear to be the main target of ACPs in the AOB. However, from this analysis it was not possible to evaluate the relative weight of ACP synaptic inputs onto either cell type or to assess the impact on the physiology of AOB circuits. To analyze these properties, we expressed channelrhodopsin (ChR2) specifically in ACPs through conditional viral delivery in the PmCo of *Sim1*^{cre} mice (N = 28; **Figure 6A**), as Sim1 is expressed by the majority of these neurons (**Figure 3E**). Four to six weeks after viral injection, ChR2 was strongly expressed in the PmCo (**Figure 6A$_1$**), along the stria terminalis (**Figure 6A$_2$**) and in the AOB GC layer (**Figure 6A$_3$**). Perisomatic stimulation with blue light evoked excitatory responses in GCs with relatively fast kinetics and low onset variability (4.8 ± 0.2 ms, N = 23), consistent with a direct excitatory input from the PmCo (**Figure 6B**). This was further confirmed by 4-AP mediated rescue of evoked excitatory events, initially blocked with TTX (onset 8.3 ± 0.7 ms, amplitude reduction 77.9 ± 23.2 pA, N = 6; **Figure 6B**; **Petreanu et al., 2009**). Excitatory input was instead completely eliminated by blockers of AMPA and NMDA glutamatergic transmission, DNQX and AP5, respectively (**Figure 6B**). The absence of light evoked IPSCs recorded at the reversal potential for excitation (0 mV) indicated a lack of indirect inhibitory transmission between the PmCo and AOB GCs (N = 23; **Figure 6B**). MC layer or glomerular layer light stimulation did not result in any response (neither excitatory nor inhibitory) in either GCs (N = 23) or MCs (N = 28). Conversely, light activation of PmCo afferents to the GC layer evoked disynaptic IPSCs in MCs (**Figure 6C**). These responses were approximately three times slower than those evoked in GCs (onset 17.1 ± 0.3 ms, N = 28) and completely abolished by bicuculline, revealing their polysynaptic nature, likely resulting from GABA release from PmCo

activated GCs. Accordingly, trains of light pulses on GCs (4 ms at 20 Hz) induced distinct effects on MC firing (holding = - 45 mV): either a sharp and transient decrease (ca. 70% reduction: 1s before LED vs 1s after LED, found in N = 9/27 cells; paired t-test "before" vs "after" p = 0.019) or a gradual increase in the normalized spike frequency (ca. 30% increase: 1s before LED vs 1s after LED, found in N = 4/27 cells; paired t-test "before" vs "after" p = 0.002; **Figure 6C$_1$**).

In the MOB, deep short axon cells (dSACs) are a type of inhibitory GABAergic neuron that provides feed-forward inhibition to multiple GCs. dSACs are also the main recipient of Pir excitatory feedback which in turn results in strong GC inhibition (**Boyd et al., 2012**; **Markopoulos et al., 2012**). Therefore, while direct GC-mediated inhibition can result in reduction of MC firing rate, both dSAC-mediated disinhibition and GC-induced rebound firing (**Balu and Strowbridge, 2007**; **Desmaisons et al., 1999**) can explain the slow increase in MC firing we observed. In the AOB, macs have an analogous connectivity and function as dSACs (**Larriva-Sahd, 2008**). Surprisingly, activation of PmCo afferents on macs (onset 6.9 ± 0.6 ms, N = 10 cells) elicited excitatory events much lower in amplitude when compared to GCs (amplitude: 103.1 ± 21.9 pA GCs N = 23; 20.9 ± 3.1 macs N = 10, t-test p = 0.005; **Figure 6B**). The persistence of very low magnitude responses detected in the presence of TTX and 4-AP (**Figure 6D**) revealed the occurrence of direct synaptic connectivity with PmCo afferents, however, under these conditions (peri-somatic or wide field LED illumination), they were not sufficient to induce detectable light-evoked feed-forward inhibition of GCs (**Figure 6B**).

Given that our tracing experiments suggested a lower extent of PmCo-MC connectivity (**Figure 1G$_2$**), the lack of light-evoked EPSCs in MCs was unexpected. One possibility is that PmCo projections to MCs are either *Sim1*-negative or simply too scarce to be detected. To rule out these possibilities we used a *CaMKIIa*-specific viral vector as described above to target ChR2 expression to the highest possible number of corticobulbar projection neurons in the PmCo, (CaMKIIa/Ct-b = 92.2%; **Figure 3A**). In this case, during perisomatic LED stimulations, direct and fast excitatory responses were sometimes detectable in MCs (onset 1.3 ± 0.2 ms; **Figure 7**). However, by a thorough survey of all injected brains used in these experiments, we were able to rule out the origin of excitatory afferents to MCs in the PmCo: fast monosynaptic excitatory currents were only detected when *CaMKIIa*-expressing neurons in the BAOT – which also projects to the AOB **Figure 1D** - were also infected (**Figure 7** and **Table 1**). No other type of excitatory events (slower in onset) were detected on MCs. Collectively these results conclusively validate the observation that ACPs innervate the AOB GC layer only and further confirm that this input is mainly directed to AOB GCs (**Figure 6E**).

ACPs enhances AOB mitral cell excitatory output

In the MOB, GC mediated inhibition has been proposed to be responsible for tuning MC responses to different odor inputs by sharpening their molecular receptive range through suppression of non-specific neuronal responses and facilitating relevant output (i.e. providing contrast enhancement; **Yokoi et al., 1995**; but see also **Fukunaga et al., 2014**). In-vivo experiments have shown that AOB MC firing can either increase or decrease in response to different odor stimuli (**Luo and Katz, 2003**), suggesting the

presence of similar tuning mechanisms also in the AOB. In our experiments, corticofugal PmCo inputs induced either inhibitory or disinhibitory effects on AOB MCs (**Figure 6C$_1$**), which may potentially indicate a contribution to MC odor coding through contrast enhancement. To study this further, we conducted cell-attached recordings from AOB MCs during concurrent electrical stimulations of the vomeronasal nerve (VN) and blue light excitation of PmCo afferents (**Figure 8A**). VN stimulations consisted of a series of 4 x 100 Hz trains of 0.4 ms pulses delivered in 2s trials (**Schoppa, 2006**). Light stimuli were partially interleaved with electrical pulses and consisted of 4 x 4/20 Hz trains of 4 ms light pulses delivered onto PmCo afferents in the AOB GC layer. Most cells (16/23) were responsive to a single electrical pulse, as the current used for the stimulations was tuned each time to reach firing threshold (equal or above 0.4 mA for both low, 50 MOhm, and high, 1 GOhm, resistance seals; 2 way ANOVA, factors: current (levels: 0.04, 0.08, 0.4, 0.8, 4, 8 pA), resistance (levels: 1 GOhm, 50 MOhm); current effect $p < 0.005$; significant pairwise post-hoc comparisons ($p < 0.05$): 0.04 pA and 0.08 pA vs. 0.4, 0.8, 4, 8 pA; **Figure 8B**). As expected, AOB MCs showed either excitatory or inhibitory responses to VN input stimulation alone (**Figure 8C,D**). Comparing the firing rates during and after VN stimulations (40 ms ON vs. 200 ms OFF) we selected excited (type I) and inhibited (type II) cells to further analyze the effect of PmCo feedback in relation to different VN inputs (paired t-test, $p \leq 0.05$; **Figure 8D**). This categorization accounted for the relative changes in firing rates between baseline activity and evoked responses and was referred to a more restricted sample of cells (16/23). Inhibited cells were the most represented (type II cells were ca. 50% - 10/23 – while type I cells only about 26% - i.e. 6/23), probably due to the high number of

443 inhibitory neurons recruited by electrode stimulations of the VN. Cells which responses
444 did not fall into either of the two categories were considered not to have any statistically
445 significant change in firing rate ("no change", **Figure 8C**). When current intensity was
446 kept at subthreshold levels (0.08 mA), only type II MCs were observed (**Figure 8C**).
447 Taken together these results suggest that the threshold for MC excitability is determined
448 by both VN input and the extent of concurrent activation of local inhibitory circuits.

449 When trains of light stimuli were delivered to PmCo afferents in the GC layer,
450 type I MC firing rate was increased upon concurrent VN afferent stimulation, while type
451 II MC responses remained low in frequency or were even slightly reduced (ca. 2Hz; 2
452 way ANOVA, factors: type (levels: I, II, *no change*), protocol (levels: E, EO4Hz,
453 EO20Hz); interaction effect $F_{4,36} = 2.78$ $p = 0.04$; type effect $p < 0.005$; protocol effect p
454 = 0.03; post-hoc comparisons: E vs. EO4Hz p = 0.014, E vs. EO20Hz p = 0.018; N type
455 I = 4, N type II = 10, N *no change* = 7; **Figure 8E,F**). In other words, considering only
456 the two subsets of MCs characterized by significant VN-evoked firing rate changes (type
457 I, II), the addition of optogenetically evoked PmCo input was mainly evident in the type I
458 MCs. This implies the predominant inhibitory effect of PmCo feedback on MCs
459 observed during voltage clamp experiments (**Figure 6C-C_1**) might be limited by the
460 excitatory effects induced by the concurrent activation of VN afferents in both type I and
461 II responses. Conversely, since both the activation of PmCo corticofugal afferents as
462 well as electrical VN stimulation can induce GC-mediated rebound excitation (**Schoppa,**
463 **2006**), or other disinhibitory mechanisms enacted by local GABAergic circuits, these two
464 effects might be additive in other cases, possibly explaining the more significant effect
465 of dual stimulations on type I responses. Thus, in contrast to the generic and

466 homogeneous impact of piriform afferents to MOB circuits (**Boyd et al., 2012**; but see
467 also **Otazu et al., 2015**), the effect of PmCo feedback depends on the polarity of VN-
468 evoked responses in AOB MCs. Accordingly, photoactivation of PmCo afferents did not
469 shift MC firing rates towards excitation during subthreshold VN stimulations (data not
470 shown). However, this analysis was limited to the two extremes of the VN-response
471 range (type I and type II). To test whether the effect of dual PmCo/VN activation could
472 be generalized to all VN-evoked responses, we compared the relative frequency
473 changes $((F_{evoked} - F_{baseline})/(F_{evoked} + F_{baseline})$; see methods) during VN stimulations
474 (electrode-evoked vs. baseline firing rates = E) to those evoked during dual VN/PmCo
475 stimulations, in all recorded MCs (electrical + optical stimulation = EO). Firing rates
476 during dual stimulations were either shifted towards excitation (in 62.5% of all cells for
477 which $F_{EO} > F_{baseline}$, $F_{EO} > F_E$) in case of positive VN-evoked responses or inhibition (in
478 60.7% of all cells for which $F_{EO} < F_{baseline}$, $F_{EO} < F_E$) in case of negative ones ($E < 0$; Chi-
479 square tests run to compare the effect of stimulations to random data distributions
480 yielded p-values lower than 0.0001). Thus, the effect of PmCo input on MC gain to VN-
481 evoked activity appears to be conserved in most recorded neurons in our sample
482 (**Figure 8G**). Importantly, the differential effect of cortical input on MC VN-evoked firing
483 does not depend on PmCo input alone since very low correlation was found ($R^2 = 0.2$)
484 comparing the effect of dual stimulations (EO) to the one of light stimulations alone (O,
485 **Figure 8H**). Light-evoked firing rate changes (in absence of paired electrical
486 stimulations) were broadly inhibitory ($F_O < F_{baseline} = 65.8$ %, similar rates were found in
487 current clamp experiments: $N_{inhibited}/(N_{inhibited} + N_{excited}) \times 100 = 69.2\%$). Taken together
488 these results indicate that PmCo feedback exerts differential and input-specific effects

on MCs. This leads to an increase in the gain of MC responses to incoming stimuli, which is a typical functional requirement for odor discrimination by olfactory circuits. Finally, our study shows important similarities between the corticofugal pathways arising in the cortical amygdala and those in the piriform cortex. These data, together with our electrophysiological analysis, suggests that similar to what occurs in piriform circuits, amygdala corticobulbar neurons might play a crucial role in shaping odor processing by the AOS through experience or brain state dependent feedback.

Discussion

In this study we dissected the functional connectivity of a cortico-bulbar circuit originating in the posteromedial nucleus of the amygdala (PmCo) and innervating the accessory olfactory bulb (AOB). We show that the PmCo receives direct input from the AOB and in turn establishes direct synaptic connections with AOB GABAergic neurons, eliciting feed-forward modulation of MC firing. Optogenetic activation of PmCo corticofugal afferents during stimulation of VNO input to the AOB enhances MC output activity indicating a possible role of amygdala corticofugal circuits in odor processing by the AOS.

Functional dissection of the PmCo cortico-bulbar circuit

From our Ct-b and retroviral tracing experiments, we find that higher order brain input to the AOB mainly originates in the PmCo. At the synaptic level, our experiments show that optogenetic stimulation of the PmCo-AOB afferents evokes direct excitation onto GC layer neurons only. This finding is consistent with previous studies showing that the

predominance of piriform cortical and amygdala centrifugal inputs is directed to the granule cell layer (**Gutiérrez-Castellanos et al., 2014**; **Matsutani, 2010**; **Balu et al., 2007**). Even though PRV infections in the AOB of *Pcdh21*^{cre} mice yielded some retrograde tracing to the PmCo, suggesting connectivity between PmCo and MCs, we believe this might be due to either recombination leakiness in the *RABV* mouse or nonspecific PRV transport (which occurrence was minimal even in absence of Cre-expression and could be caused by local leak of helper adeno-associated viruses; **Miyamichi et al., 2013**), rather than direct connectivity. The fact that neither *Sim1*^{cre} nor *CaMKIIa*-driven conditional ChR2 expression limited to the PmCo led to MC activation, supports this interpretation.

Role of PmCo feedback on AOB circuit activity

Compared to the PmCo-AOB circuit analyzed here, corticobulbar projections from the piriform cortex to the MOB display an analogous connectivity. Within the granule cell layer, piriform afferents mainly reach MOB dSACs (**Boyd et al., 2012**). These have been proposed to regulate MC inputs in a center-surround fashion acting via ensembles of connected interneurons (**Geramita et al., 2016**; **Willhite et al., 2006**; **Kim et al., 2011**). GCs instead provide a more narrowly tuned inhibitory drive onto MCs (**Boyd et al., 2012**). In the AOB we find that the amount of excitation delivered to macs (homologous to dSACs; **Larriva-Sahd, 2008**) by PmCo afferents is much lower compared to the input onto GCs (**Figure 6**) and we never observed inhibitory responses in GCs upon light-activation of PmCo afferents, as would occur in case of strong and diffuse PmCo-macs-GC connectivity. This could indicate a narrower tuning of

corticofugal circuits directed to the AOB and the presence of a lower degree of lateral interactions between MCs and GCs (**Moriya-Ito et al., 2009**; **Castro et al., 2007;** but see also **Hendrickson et al., 2008**; **Guo and Holy, 2005**). Consistent with this view, periglomerular cells - the very first layer of horizontal integration of incoming input to MCs - are scarcer in the AOB than in the rest of the bulb (**Meisami and Bathnagar, 1998**). In addition, AOB GCs are mainly connected to MC apical dendrites (**Moriya-Ito et al., 2009**; **Castro et al., 2007**) where they likely shunt inputs converging on a single MC rather than regulating adjacent mitral cell circuits, as in the MOB (**Geramita et al., 2016**). It follows that GC mediated selective inhibition - as opposed to rebound excitation or mac mediated disinhibition - of MC output might be the dominant mechanism by which inhibitory feedback triggered by PmCo projections regulates MC activity in the AOB. This view is supported by the fact that MC responses to VN stimuli are more shifted towards suppression (type II) rather than excitation (type I; **Figure 8C**): type II MC responses represent 43% of all MC VN-evoked responses, while type I only the 26% (indicating a marked recruitment of local feedback inhibitory circuits in the response to VN stimuli). Because the effect of ACP feedback does not change the ratio of the response type (**Figure 8G**), their effect on MC gain is also more tuned towards suppression, if one considers the whole sample of recorded cells (not only type I and II).

Therefore, our results are in agreement with a GC-centered wiring of PmCo projections (as opposed to the dSAC-centered organization of piriform afferents in the MOB). These inputs are likely to act preferentially on a much narrower scale, consistent with their hypothesized role in tuning the highly selective odor responses of AOB MCs. Conversely, in the MOB, the broader innervation of inhibitory circuits by piriform

afferents might explain their more generalized inhibitory action on MC firing, with limited or no dependence on incoming peripheral stimuli (**Boyd et al., 2012**). In this case, given the broader tuning of MOB MCs to single odor molecules, coding fidelity of odor information might be achieved by coordinating the activity of larger ensembles of MCs, properly matching their activity patterns to different odor inputs (for example decorrelating odor responses; **Otazu et al., 2015**). Conversely, in the AOB this correspondence might be theoretically more precise since MCs receive a highly specific but heterogeneous set of inputs. However, due to their heterotypic glomerular connectivity, overlap might exist in the set of inputs each MC is tuned to. We propose that the functional organization of the PmCo corticobulbar pathway is suitable to improve coding fidelity through contrast enhancement of odor representations by a very limited set of MCs.

Role of amygdala corticofugal circuits in the encoding of social signals

Our results reveal in detail that the cortical amygdala and the AOB are directly interconnected. This has two important implications: first, the amygdala modulates the early processing of sensory information through corticobulbar input; second, this modulatory role might occur early (i.e. in the AOB) before any valence-related processing by either the amygdala or parallel circuits. The amygdala circuits such as the medial, basal and central nuclei might be implicated in the further elaboration of value and motivational aspects of these inputs (**Moncho-Bogani et al., 2005**, **McCarthy et al., 2017**; **DiBenedictis et al., 2015**).

Within the MOS, the most prominent cortical top-down modulation occurs through corticofugal projections from the piriform cortex, which provides a crucial feedback for the earliest stages of olfactory processing (**Boyd et al., 2012**; **Otazu et al., 2015**). The organizational similarities shared by these pathways and the PmCo corticobulbar circuits highlighted by our study, not only confirm the proposed role of the PmCo as primary vomeronasal cortical area (**Gutiérrez-Castellanos et al., 2014; Mucignat-Caretta et al., 2006**), but also suggests that these inputs maybe instrumental to the attribution of behavioral relevance to only a selected range of signals. This would imply a role of ACPs in the fine tuning of highly selective AOB responses to social odors. However, since some ACPs send axon collaterals to the MeA, PFC and Ent (**Figure 5**), these could play a more complex role than simply shaping odor processing by the AOB.

Importantly, a recent study showed that chemogenetic silencing of the MeA results in impairments in social odor processing (expressed as a decreased difference in male vs. female odor investigation shown by female subjects; **McCarthy et al., 2017**), likely due to defects in receptive behaviors and in the motivation to investigate sex odors, rather than solely sensory deficits (**DiBenedictis et al., 2012**). Indeed, since MeA output mainly targets hypothalamic nuclei involved in mid- to long-term hormonal, motivational and consummatory consequences of social odor perception (**Bian et al., 2008**; **Bergan et al., 2014**), it appears that ACP-AOB collaterals to the MeA might have more of a relay function rather than tuning incoming odor input.

In addition, although both the prefrontal (**Li et al., 2010**) and the entorhinal cortex (**Mayeaux and Johnston, 2004**; **Hargeaves et al., 2005**) have been implicated in the encoding of key aspects of odor perception, lesion studies revealed these areas might

be dispensable for odor discrimination (**Koger and Mair, 1994**; **Mayeaux and Johnston, 2004**). Thus, both may be more involved in the multimodal elaboration of odor-associated inputs and odor value (**Alvarez and Eichenbaum, 2002**; **Schoenbaum et al., 1999**; **Rolls, 2001; Chapuis et al., 2013**; **Ferry et al., 2015**). The PmCo – which is directly connected to the AOB - can process relevant odor information within the same time scale of primary odor processing (**Mucignat-Caretta et al., 2006**; **Maras & Petrulis, 2008**), with ACPs mainly providing a direct feedback to the earlier steps of AOB-mediated sensory processing, as opposed to other value-associated functions. ACPs might also integrate more complex information, related to brain states or aspects of social odor perception. Because ACPs are reminiscent of piriform-MOB connections, our data contribute to the notion of the potential importance of direct and fast cortical input relaying brain state related information back to all primary olfactory circuits in order to optimize odor perception (**Boyd et al., 2012**; **Markopoulos et al., 2012**; **Otazu et al., 2015**; **Rothermel et al., 2014**; **Smith et al., 2015**).

Overall, our results highlight the importance of rethinking olfactory-based responses as functions that are integrated at a system level, with significant cross-talk and feedback interactions, as opposed to be simply the outcome of unidirectional computations by segregated olfactory or amygdala sub-circuits. Future studies are required to extend this concept to other sensory systems and understand how valence and saliency of social cues might develop or change, adapting to different brain states or pathophysiological conditions.

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779

780 **Figure Legends**

781 **Figure 1. Amygdala corticobulbar projections (ACPs) to the AOB arise in the**
782 **PmCo.** A) Cholera toxin (Ct-b) injections targeting the AOB (B) result in retrograde
783 labeling in the PmCo (C). D) 3D serial section reconstruction of the medial and cortical
784 amygdala showing the extent of a typical Ct-b injection in the AOB. E) Pseudorabies
785 (PRV) retrograde viral tracing strategy used to identify the putative synaptic targets of
786 PmCo projection neurons in the AOB. Cre-expressing "starter cells" are defined as
787 those activating PRV retrograde infection in the AOB. As Cre-expression is limited to
788 AOB starter cells and is absent in the PmCo (F), PRV spreads retrogradely across one
789 synapse only. Starter neurons are either mitral cells (MCs, in *Pcdh21^{cre};RABV* mice; G)
790 or GABAergic nNOS-expressing cells in the granule cell layer (GC, in *nNOS^{cre};RABV*
791 mice, H). PRV injections in the AOB of *Pcdh21^{cre};RABV* or *nNOS^{cre};RABV*cre mice
792 results in local infection (G_1,H_1) and monosynaptic retrograde spread to the PmCo
793 (G_2,H_2). (Scale bars: 200 µm in all panels except, 50 µm in C, 500 µm in D).
794 Abbreviations: MeA, medial amygdala; BAOT, bed nucleus of the accessory olfactory
795 tract; GL, glomerular layer; AHi, amygdala hippocampal transition area.

796

797 **Figure 2. ACPs are synaptically connected to cells in the AOB GC layer.** A)
798 Conditional PRV tracing with helper adeno-associated viruses used to identify starter
799 cells in the AOB GC layer. A_1) Both granule (GCs) and main accessory cells (macs) are
800 identified as starters (TVA-G/PRV positive). A_2) The histograms show the number of

starter cells in the AOB GC layer and the total number of input neurons in the rest of the brain of 4 injected brains. A_3) Estimate percentage contribution of each cell type (macs or GCs) to the total amount of starter cells in the GC layer (paired t-test, $p < 0.05$). A_4) The upper histograms (light gray) show the percentage of input neurons (PRV-labeled) to the AOB GC layer per brain area, referred to the total amount of PRV-labeled neurons in the brain (N = 4 brains). The histogram below (dark gray) show the percentage of input neurons relative to the amount of PRV-labeled neurons within the amygdala only. Layer II and III of the PmCo provide the highest amount of inputs. Scale bars: 200 µm and 20 µm. Abbreviations: PlCo, posteriorlateral cortical amygdala; BLA, basolateral amygdala; ACo, anterior cortical amygdala; aav, anterior amygdala, ventral subdivision.

Figure 3. Molecular phenotypes of PmCo-AOB projection neurons. A) PmCo cortico-bulbar projection neurons (ACPs) labeled with Ct-b after AOB retrograde tracing. Ct-b-labeled PmCo neurons (red) express the excitatory markers CaMKIIa (cyan, 92.2%) and Tbr1 (gray, 93.3%). B-C) Ct-b labeled neurons in the PmCo do not express YFP in *GAD^{cre};Ryfp* or *Dlx5/6^{cre};Ryfp* mouse lines (cyan), confirming their excitatory phenotype. D) PmCo-Ct-b labeled neurons express Cux1 (cyan, 37.1%) and Ctip2 (gray, 94.5%), typical cortical neurons markers in layer 2-4 and 5-6, respectively. All Cux1-positive PmCo neurons co-expressed Ctip2. E) There is also high co-expression with Sim1 (82.4%) in PmCo Ct-b-positive cells. For each count, tissue collected (3-4 sections) from 3 Ct-b injected mice was used. (Scale bars 20 µm and 200 µm).

Figure 4. Collateral projections of PmCo corticobulbar neurons.

A) Dual Ct-b injections were used to identify possible additional target areas of the PmCo neurons retrogradely labeled from the AOB (Ct-b 555). Tracing was considered reliable in case of clear separation of the two injection sites and 555/488 co-labeling of the same region (sr) or the same cells (sc). We considered *nonspecific* (ns) tracing experiments those in which the two tracers showed partial overlap near the two injection sites or in case of Ct-b 488 injections adjacent to the stria terminalis (for reference see Allen Brain Connectivity Atlas, exp. #114249084) where AOB-directed ACPs course (e.g. G,I): in such cases Ct-b 488 would be likely taken up by passing fibers and yield false-positive results (compare H to I and G to F to see how Ct-b overlap in the PmCo decreases as the injection site is moved either dorsally or rostrally, respectively). Injections of green Ct-b 488 (cyan) were targeted to different AOS regions, known main targets of the PmCo: the olfactory tubercle (Tu, B), the paraventricular nucleus (PVN, C,D), the bed nucleus of the stria terminalis (BNST, D), the ventro-medial hypothalamic nucleus (VMH, E), the medial nuclei of the amygdala (MeA and MePD, G and H, respectively), the basolateral amygdaloid nucleus (BLA, F), the endopiriform nuclei (K) and the medial prefrontal cortex were targeted (I, J). The pie charts next to the injection sites indicate the co-expression of Ct-b 555 in Ct-b 488 fibers and therefore possible biases due to *non-specific* tracing (tracing reliability is indicated in the same charts). The co-expression percentage of double labeled Ct-b 488+/Ct-b 555+ cells in the PmCo

(second panel associated to each injection site) was calculated and indicated in the pie charts in the corresponding panel. Absolute values of Ct-b 555 and 488 are averaged and indicated in the high magnification insets relative to regions showing higher co-expression. Other abbreviations used: HDB, Nucleus of the diagonal band of Broca, ZI, zona incerta, BSTMP, bed nucleus stria terminalis medial division posterior part, PeF, perifornical nucleus, LH, lateral hypothalamic nucleus, opt, optic tract, M2, secondary motor cortex, Cg, cingulate cortex, IL, infralimbic cortex, DP, Dorsal peduncular cortex, Den, dorsal endopiriform nucleus, VEn, ventral endopiriform nucleus. (Scale bars are 20 µm and 200 µm). Data are means ± SEM.

Figure 5. Anterograde tracing of PmCo projections using a CamKIIa-specific adenovirus. A) Stack of brain sections showing the extent of viral labeling in the brain (mCherry) following AAV injection in the PmCo: the corticobulbar tract coursing through the stria terminalis (st) and reaching the AOB GC layer shows the most intense mCherry expression. In the lower panels: digitized version of the image stack showing from frontal and top views the course of PmCo fibers in the brain. The pattern of mCherry expression was reconstructed by selecting on each image pixels having values of Hue/Intensity/Brightness equal or higher than those in the AOB GC layer. In the frontal view the route of the injection is indicated by the white dotted line. In the top view the sectioning planes relative to the images in B,C,D are in indicated by the dashed yellow lines. B-D) Single images taken from the stack represented in A showing the extent of mCherry expression at the lateral levels indicated by the coordinates in the

lower left corners of each panel. The AOB GC, the st and the PmCo show the highest level of mCherry expression. (Scale bars are 500 μm).

Figure 6. ACPs establish direct synaptic contact with AOB granule cells (GCs). A) Injection of double-floxed channelrhodopsin (ChR2) mCherry-expressing adeno-associated virus in the PmCo (A_1) of *Sim1*^{cre} mice results in labeling (red) of the corticobulbar circuit coursing through the stria terminalis (st, A_2), and terminating in the AOB GC layer only (as shown by the color histogram on the side, A_3). B) Light stimulation of PmCo afferents to the GC layer induced TTX-sensitive excitatory responses in GCs at resting potential (-65mV, onset 4.8 ± 0.2 ms, N = 23). These are rescued by TTX-4AP bath application, indicating direct synaptic connectivity. C) No evoked post-synaptic current (EPSCs) is detected in MCs under the same conditions, while inhibitory currents (IPSCs, onset 17.1 ± 0.3 ms, N = 28) are visualized using a high chloride intracellular solution. C_1) Effects of repeated optogenetic stimulation of the GC layer on MC firing (5 overlapped trials are shown for each effect): MC activity is either temporarily suppressed or facilitated (20Hz light pulses, 4ms each; frequency was compared 1s before vs 1s after LED stimulus onset). D) Main accessory cells (macs) receive direct PmCo inputs of lower amplitude compared to GCs. E) Circuit diagrams showing the putative effects of PmCo feedback on AOB MC firing: GC-mediated feed-forward inhibition and mac-GC mediated MC disinhibition. Above each trace, the recorded cell type (black) and the site of LED stimulations (blue) are indicated. Other abbreviations: a.u., arbitrary units, VN, vomeronasal nerve, GL, glomerular layer. (Scale bars are 100 μm for A_3 and 500 μm for A_1,A_2).

Figure 7. Optogenetically evoked MC excitatory responses are induced by activation of BAOT afferents. A-B_2) Spread of *CaMKIIa-ChR2* virus to the BAOT results in expression of ChR2 not only in the PmCo projection neurons but also in the BAOT. Infection of this region results in mCherry labeling in the AOB GC and MC layers (see color histogram on the side, indicating the localization of mCherry labeling). C) Optogenetic stimulation of the MC layer evoked excitatory responses in MCs (onset 1.3 ± 0.2 ms, 14 cells) only when the BAOT was infected. These direct responses (not blocked by TTX-4AP bath application) result in disynaptic excitatory events detectable in GCs (D) which are slower (onset 7.7 ± 0.4 ms, 17 cells) compared to PmCo direct inputs elicited by GC stimulation (4.8 ± 0.2 ms, 23 cells, t-test $p < 0.0005$, **Figure 1B**).

Figure 8. PmCo feedback enhances MC responses to vomeronasal inputs. A) Diagram showing the configuration used during recordings: electrical stimuli were targeted onto the VN, light stimuli where delivered onto the GC layer and the firing rate of MCs recorded in cell-attached mode. B) Tuning curves of MCs in response to current stimuli of different intensity were obtained at different seal resistance levels: 1 GOhm, black dots, 50 MOhm, circles. This preliminary test was made to define the threshold current for MC firing, used in the rest of the experiments. C) Percentage of the different types of MC responses following electrical VN stimulations above (0.8 mA) and below (0.08 mA) threshold. D) Raster plots of type I and type II MC activity during electrode stimulation (red dots). E) Average firing rate (across units and trials) of MCs divided by type (I or II) and stimulation (E electrical stimulation; EO4Hz, EO20Hz, joint electrical

and optical stimulation at 4 and 20Hz respectively. Red dots mark electrical stimuli, blue dots mark optical stimuli). F) Mean firing rate changes induced in MCs by optogenetic activation of PmCo feedback. The red lines represent the firing frequency at unstimulated baseline levels. G) Relative increase and decrease in firing rates during joint electrical and optical stimulation (y-axis) and electrical only stimulation (x-axis) both referred to baseline levels. Firing rate was computed during 40 ms of stimulation. Two LED stimulation protocols are color coded in darker (EO20Hz) or lighter (EO4Hz) red (for excitation) or blue (for inhibition). Percentage values refer to either values of $E > 0$ or $E < 0$. H) Scatter plot showing the lack of correlation between the relative effect of dual electrical and light stimulations on light-evoked responses alone. Data are means ± SEM.

Tables

AAV expression		Evoked EPCs		
PmCo	**BAOT**	**MC**	**GC**	**mac**
yes	N/D	no	yes	no
no	**N/D**	**yes**	N/D	
no	**N/D**	**yes**	no	
yes	**N/D**	**yes**	no	
yes	**N/D**	**yes**	yes	yes
yes	no	N/D	yes	
yes	no	N/D	yes	
yes	no	N/D	no	
yes	no	N/D	N/D	yes
yes	no	no	yes	
yes	no	no	yes	
yes	no	no	N/D	
yes	no	no	no	
no	no	no	no	
yes	no	no	N/D	
no	no	no	no	
yes	no	no	yes	
yes	no	no	yes	
yes	no	no	N/D	yes
yes	no	no	yes	
yes	yes	N/D	N/D	
yes	**yes**	**yes**	no	
yes	**yes**	**yes**	N/D	
yes	**yes**	**yes**	N/D	
yes	**yes**	**yes**	yes	
yes	**yes**	**yes**	N/D	yes
yes	**yes**	**yes**	yes	yes
yes	**yes**	**yes**	N/D	yes

Table 1: Summary of CaMKIIa optogenetic stimulations experiments. Viral delivery of Chr2 to the PmCO was accomplished using a CaMKIIa specific viral vector (see methods) to target excitatory neurons. When the PmCo was efficiently targeted (first left column, PmCo, value = “yes”), excitatory responses were evoked in AOB granule cells (GC). Similar responses were observed also in AOB MCs (MC) but only when the

931 BAOT was labeled (second left column, BAOT, value = “yes”; N = 7). In other cases (N
932 = 4) MCs were also responsive to perisomatic light stimulations but, due to tissue
933 damage during brain tissue harvest, the area corresponding to the anterior portion of
934 the injection site (in proximity with the BAOT) was not available. This survey was used
935 to assess the likelihood of PmCo-to-GC and BAOT-to-MC connectivity.

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