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JNeurosci
THE JOURNAL OF NEUROSCIENCE

Research Articles | Cellular/Molecular

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https://doi.org/10.1523/JNEUROSCI.1867-23.2024

Received: 2 October 2023
Revised: 13 December 2024
Accepted: 21 December 2024

Calretinin-expressing neurons in the basal forebrain specifically contact granule cells in the olfactory bulb and modulate odor learning

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Running Title: The role of the basal forebrain in odor processing

Keywords: olfactory bulb, granule cells, basal forebrain, horizontal diagonal band of Broca, magnocellular preoptic nucleus, calretinin, interneurons, odor discrimination, odor learning

Number of pages: 34

Number of figures: 7

Number of words: Abstract – 228; Introduction – 701; Discussion – 1691

Abstract

GABAergic neurons in basal forebrain (BF) nuclei project densely to all layers of the mouse main olfactory bulb (OB), the first relay in odor information processing. However, BF projection neurons are diverse and the contribution of each subtype to odor information processing is not known. In the present study, we used retrograde and anterograde tracing methods together with whole-brain light-sheet analyses, patch-clamp recordings coupled with optogenetic and chemogenetic approaches during spontaneous odor discrimination, and go/no-go odor discrimination/learning tests to characterize the synaptic targets in the OB of BF calretinin-expressing (CR+) GABAergic cells and to reveal their functional implications. We used mice of either sex to show that OB-projecting CR+ neurons innervate the bulbar granule cell layer but not the glomerular layer. Optogenetic stimulation of CR+ axonal projections in OB slices elicited monosynaptic GABAergic currents in granule cells (GCs). Retrograde rabies virus-based transsynaptic tracing experiments confirmed these synaptic connections and further suggested that CR+ neurons provide the principal, if not the unique, BF input onto GCs. Chemogenetic inhibition of CR+ neurons in the BF of male mice did not affect odor discrimination in habituation/dishabituation tasks but led to impairment in odor learning during go/no-go odor-associative tasks. Our results revealed a subtype-specific projection pattern in the OB of a select population of BF neurons and suggested that distinct BF GABAergic projections have distinct effects on odor information processing and learning.

Significance statement

The basal forebrain projects densely into the olfactory bulb and plays an important role in odor processing. The basal forebrain contains neurochemically distinct cellular populations but the contribution of each subtype to odor information processing is not known. We identified predominant, if not unique, synaptic connections between neurons in the basal forebrain that are characterized by the expression of calretinin (CR) and granule cells in the olfactory bulb. The detailed morpho-functional characterization of these connections based on anterograde, retrograde, and rabies-based transsynaptic labeling, patch-clamp recordings, optogenetics, and behavioral analyses indicated that

these connections are GABAergic in nature and suggested that CR-expressing neurons in the basal forebrain are involved in odor learning.

Introduction

The olfactory system is essential for the survival of many animal species. It provides vital information about the surrounding world and influences social and sexual behaviors. In mammals, odor information is conveyed by olfactory sensory neurons, which are embedded in the olfactory epithelium, into the olfactory bulb (OB) where it is transmitted to mitral and tufted (M/T) cells, the principal output neurons. Bulbar principal cells convey the information to other brain regions such as the olfactory cortex, olfactory tubercle, amygdala, entorhinal cortex, and elsewhere, with no thalamic relay (Davis, 2004). Odor information processing in the OB is supported by a wide variety of interneurons, principally granule cells (GCs) in deep layers, periglomerular cells (PGCs) in the glomerular layer, and short-axon cells distributed in different layers. GCs are the most abundant population in the OB and have a very specific morpho-functional identity (Breton-Provencher & Saghatelyan, 2012). These axonless neurons mediate reciprocal inhibition through reciprocal dendro-dendritic synapses with the lateral dendrites of M/T cells (Isaacson & Strowbridge, 1998; Schoppa *et al*, 1998) and regulate fast neuronal synchronization in the bulbar network and odor discrimination (Abraham *et al*, 2010; Dalal & Haddad, 2022; Fukunaga *et al*, 2014; Lepousez & Lledo, 2013; Nunes & Kuner, 2015). GCs in the OB are sub-divided into distinct subtypes based on their localization patterns (Lemasson *et al.*, 2005) and molecular profiles (Malvaut & Saghatelyan, 2016). These subtypes have distinct morpho-functional properties (Lemasson *et al*, 2005) and regulate specific types of olfactory behavior (Hardy *et al*, 2018; Malvaut *et al*, 2017).

Information processing in the OB is regulated not only by local interneurons but also by long-ranging excitatory, inhibitory, and neuromodulatory projections from other brain regions. All bulbar layers are densely innervated by centrifugal inputs from the basal forebrain (BF), particularly from two adjacent nuclei, the horizontal limb of the diagonal band of Broca (HDB), and the magnocellular preoptic nucleus (MCPO) (Gracia-Llanes *et al*, 2010; Niedworok *et al*, 2012; Zaborszky *et al*, 1986). Projections from the HDB/MCPO

are cholinergic and GABAergic and impinge onto most OB neuronal subtypes including GCs and PGCs (Case *et al*, 2017; De Saint Jan, 2022; Hanson *et al*, 2020; Nunez-Parra *et al*, 2013; Sanz Diez *et al*, 2019; Villar *et al*, 2021) as well as M/T cells (Zhou *et al*, 2023). Pharmacogenetic inhibition of the whole population of BF GABAergic neurons impairs odor discrimination in spontaneous habituation/dishabituation tasks (Nunez-Parra *et al.*, 2013). However, HDB/MCPO GABAergic neurons are diverse and the exact topological organization of their projections in OB layers and their functional roles in odor information processing are not well understood.

HDB/MCPO neuronal subtypes are distinguished by their molecular marker profiles, morpho-functional properties, and projection patterns (Gritti *et al*, 2003; McKenna *et al*, 2021). Distinct HDB/MCPO GABAergic subtypes can be defined based on the expression of Ca^{2+}-binding proteins such as parvalbumin (PV), calbindin (CB), and calretinin (CR) (Gritti *et al.*, 2003; McKenna *et al.*, 2021; Zaborszky *et al*, 2005) or by neuropeptides such as somatostatin (SOM) (Anaclet *et al*, 2018; Freund & Gulyas, 1991). Release properties and the impacts of HDB/MCPO GABAergic inputs on bulbar interneurons are diverse and target-specific (De Saint Jan, 2022; Sanz Diez *et al.*, 2019), suggesting that select subtypes of HDB/MCPO GABAergic neurons may innervate specific targets in the OB. To shed more light on the function and topological organization of HDB/MCPO neurons in the OB, we used anterograde and retrograde tracing approaches together with light-sheet analyses of OB-projecting CR+ neurons in the whole brain, patch-clamp recordings combined with optogenetics, behavioral tests, and pharmacogenetic approaches. We identified three subtypes of GABAergic neurons in the HDB/MCPO that project to the OB. Moreover, we found a specific projection pattern for CR-expressing HDB/MCPO neurons that principally innervate the GCL of the main OB but not the glomerular layer. We demonstrated that CR+ HDB/MCPO neurons make GABAergic synaptic connections with GCs and that pharmacogenetic inactivation of OB-projecting CR+ neurons locally, in the HDB/MCPO, alter odor learning in an operant-conditioning go/no-go odor discrimination/learning task but not odor discrimination in a habituation/dishabituation task. These results suggested that distinct neuronal subtypes in the HDB/MCPO contribute in their own and specific way to odor information processing based on their pattern of synaptic connections with OB interneurons.

Materials and methods

Animals

The experiments were performed using 2- to 4-month-old C57Bl/6 wild-type mice, CR-EGFP mice (Caputi *et al*, 2009), and Calretinin-Cre mice (CR-Cre; B6(Cg)-*Calb2*$^{tm1(cre)Zjh}$/J; RRID:IMSR_JAX:010774) of either sex. All animal experiments were approved by the Université Laval and University of Ottawa animal care and protection committees according to the guidelines of the Canadian Council on Animal Care (CCAC) and by the French Ministry and local ethic committee for animal experimentation (CREMEAS at the University of Strasbourg, and CETEA at the Institute Pasteur). One to four mice per cage were kept on a 12-h light/dark cycle at a constant temperature (22°C) with food and water *ad libitum,* except for the behavioral experiments, where the mice were housed individually and were partially water-deprived.

Stereotaxic injections

The mice were anesthetized either with an intraperitoneal injection of a ketamine (100 mg/kg), acepromazine (3 mg/kg), and medetomidine (1 mg/kg) mix or with isoflurane (2-2.5% isoflurane, 1 L/min of oxygen) and were placed in a stereotaxic apparatus. Lidocaine (1 mg/kg) was subcutaneously injected over the skull 3-5 min before the surgery. The animals were placed on a heating pad set at 37°C, and their corporal temperature was continuously monitored with a rectal probe. Paw and ocular reflexes were periodically checked during surgery to determine the depth of the anesthesia. After suturing the incision, the mice received an intraperitoneal injection of Metacam 100 (meloxicam; 5-10 mg/kg) or caprofen (20 mg/kg) and were rehydrated with a 0.5-1 mL subcutaneous injection of 0.9% NaCl. Antisedan (atipamezol; 0.4 mg/kg) was also injected intraperitoneally in mice anesthetized with ketamine/acepromazine/medetomidine. The mice were then placed under a heating lamp during the awakening phase.

Retrograde tracer injections in the OB were given to CR-GFP (n=4) and C57BL6 mice (n=4) of either sex. The injection site was approximately in the center of the OB rostral to the anterior venous sinus and lateral to the central scissure. A solution containing red fluorescent latex microbeads (200 to 600 mL, RetroBeads™; Lumafluor) diluted to 25%

or 50% in 0.9% NaCl or AlexaFluor™ 555 cholera toxin subunit B conjugate (Invitrogen, Thermo Fisher Scientific #C34776) was injected 200-500 µm deep from the dura using a glass pipette. The mice were sacrificed 3 to 6 days post-injection.

For the whole-brain light-sheet analyses of OB-projecting CR+ cells, retrograde AAV viral particles (pAAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHp, #20298-AAVrg, Addgene) were injected into the OB of CR-Cre male mice (n=5). Two injections per OB to target the ventro-anterior and dorso-posterior regions were given at the following coordinates (relative to bregma): for the ventro-anterior part of the OB, anterior-posterior (AP) 5.5 mm, medio-lateral (ML) ±0.9 mm, and dorso-ventral (DV) 1.8 mm; for the dorso-posterior part of the OB, AP 5.3 mm, ML ±0.5, and DV 0.9. Two weeks post-injection, the animals were transcardially perfused with ice-cold PBS supplemented with 10 U/mL of heparin, followed by ice-cold 4% PFA. The brains were extracted and were incubated in a 4% PFA solution at 4°C for 24 h with gentle shaking. The brains were then washed twice in PBS and were stored in PBS containing 0.02% sodium azide prior to shipping to LifeCanvas Technologies (Cambridge, USA) for clearing and light-sheet imaging.

For the optogenetic and axonal tracing experiments, viruses were injected into the HDB/MCPO of CR-Cre mice of either sex. Small holes were drilled at the following coordinates (relative to the bregma): AP 0.2-0.6 mm, ML ±1.2-1.4 mm, and DV 5.2-5.5 mm) and 200-500 nL of either AAV9-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-HGH (University of Pennsylvania Viral Vector Core), AAV9-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA (Addgene #20298), or AAV5.EF1a.DIO.ChETA-EYFP (Addgene #26968) were injected. The mice were sacrificed 2-4 weeks post-injection.

For the retrograde rabies virus-based transsynaptic tracing, a retroviral construct was injected into the rostral migratory stream (RMS) of neonatal mice pups at P6 (n=3) to label GCs, as previously described (Grelat *et al*, 2018). Briefly, P6 male pups were anesthetized with isoflurane (3.5%; 372 mL/min; Iso-Vet, Piramal Healthcare) and were positioned in a stereotaxic frame using a homemade cast. Small craniotomies were drilled above the injection sites with a needle, and bilateral viral injections (350 nL per site) were

made into the RMS at the following coordinates: AP 2.4 mm, ML ±0.6 mm, and DV 2.7 mm from the skull surface. The skin was closed with adhesive cyanoacrylate (Vetbond; 3M). The pups were returned to their mother after recovery (30-60 min) on a warm pad. The retrovirus used in this study expresses DsRed, a fluorescent red marker, and the G and TVA proteins necessary for a RABV vector secondary infection and propagation (Retro-G-TVE-DsREd; $1.85x10^8$ infectious particles/mL; 350 nL per site; produced by VVGT-SFR Necker). Rabies viral vector expressing GFP (RABV-GFP; $3.5x10^8$ ffu/mL; 250 nL per site; a gift from the University of Munich, Germany) was injected into the OB at the following coordinates AP 4.8 mm, ML ±0.8 mm, and DV 1 and 1.5 mm from the brain surface, 16 weeks after the retro-G-TVA-DsRed injections. The animals were sacrificed 7 days after the RABV injections. Immunostaining was performed to ensure that GFP and DsRed fluorescence could be visualized. For each mouse, the staining was performed on 1 out of 3 sagittal brain sections from one hemisphere to verify the specificity of the monosynaptic tracing. Only mice in which starter cells were exclusively located in the granule cell layer were included in the analysis.

Pharmacogenetic AAV construct injections in the HDB/MCPO and CNO administration

To study the role of BF CR+ interneurons using a go/no-go olfactory discrimination task, we inactivated CR+ cells in the HDB/MCPO using the Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) pharmacogenetic approach. CR-Cre male mice were anesthetized with isoflurane (2-2.5% isoflurane, 1 L/min of oxygen) and were placed on the stereotaxic frame as described above. The Cre-dependent AAV 2/8 EF1α-DIO-hM4D(Gi)-mCherry or AAV 2/8 EF1α-DIO-GFP viral vectors were bilaterally injected into the HDB/MCPO of the CR-Cre mice at AP 0.6 mm, ML ±1.2 mm, and DV 5.2 mm (relative to bregma). The mice were subjected to go/no-go olfactory discrimination/learning training 4 weeks after the injection.

To specifically inhibit OB-projecting CR+ neurons in the HDB/MCPO of a different cohort of CR-Cre male mice (n=14), we injected retrograde Cre-dependent DREADDs (pAAVrg-hSyn-DIO-hM4D(Gi)-mCherry, # 44362-AAVrg, Addgene) or control (pAAVrg-hSyn-DIO-

mCherry, #50459-AAVrg, Addgene) viral particles into the OB. Small craniotomies were bilaterally drilled above the injection sites, and the viral injections (300 nL per site) were made into the OB at the following coordinates (relative to the bregma): for the ventro-anterior part of the OB, AP 5.5 mm, ML ±0.9 mm, and DV 1.8 mm; and for the dorso-posterior part of the OB, AP 5.3 mm, ML ±0.5, and DV 0.9. During the same surgery, fluid cannulas (Doric Lenses, Canada) were installed bilaterally above the HDB/MCPO at the following coordinates: AP 0.4-0.6 mm, ML 1.4 mm, and DV 5.2 mm. After allowing for viral expression and recovery of the animals for 4 weeks, the animals were then subjected to behavioral training.

DREADDs receptors were activated either by an intraperitoneal injection of clozapine-N-oxide (CNO, 2 mg/kg; Tocris, Cat# 4936) or by an intracerebral CNO injection just above the HDB/MCPO through fluid cannulas (100 µM; Tocris, Cat# 4936). For the behavioral experiments, both the experimental and control groups of mice received either an intraperitoneal injection of CNO every day, 25-30 min before starting the go/no-go task, which lasted approximately 60 min for each animal, or an intracerebral CNO injection via cannulas every day 5-10 min before starting the go/no-go task, which lasted approximately 45 min for each animal. No CNO was given during the training.

To estimate the specificity of the viral targeting approach and the percentage of DREADDs-infected CR+ cells in the entire CR+ HDB population, we estimated the percentage of CR-immunolabeled cells among the DREADDs-infected cells (i.e., CR+ among the mCherry+ virally labeled cells) and calculated the percentage of DREADDs-infected CR+ cells in the entire CR+ cell population in the HDB (i.e., mCherry+ virally labeled cells among the CR+ immunolabeled cells in the HDB). To do so, confocal images were taken, and the co-expression of markers was verified in several optical sections.

Tissue preservation and clearing, immunolabeling, and imaging

PFA-fixed samples were preserved using SHIELD reagents (LifeCanvas Technologies) according to the manufacturer's instructions (Park *et al.*, 2018). Samples were delipidated using LifeCanvas Technologies Clear+ delipidation reagents. Following delipidation, the

samples were labeled using eFLASH (Yun *et al.*, 2019) technology, which integrates stochastic electro-transport (Kim *et al.*, 2015) and SWITCH (Murray *et al.*, 2015), using a SmartBatch+ (or SmartLabel) device (LifeCanvas Technologies). We used primary goat polyclonal anti-GFP (EnCor Biotechnology RRID: AB_2737371, 10 µg/brain) and secondary donkey anti-goat 488 (Jackson Immunoresearch, RRID: AB_2336933) to boost the signal. Propidium iodide (ThermoFisher Scientific, P3566, 48 µL/brain) labeling was also used to perform the registration and alignment of sections to the Allen Brain Atlas. After immunolabeling, the samples were incubated in 50% EasyIndex (RI = 1.52, LifeCanvas Technologies) overnight at 37°C followed by a 24-h incubation in 100% EasyIndex for refractive index matching. After index matching, the samples were imaged using a SmartSPIM axially-swept light sheet microscope using a 3.6x objective (0.2 NA) (LifeCanvas Technologies).

Acquired images were registered in the Allen Brain Atlas (Allen Institute: https://portal.brain-map.org/) using an automated process (alignment performed by LifeCanvas Technologies). A propidium iodide channel for each brain was registered to an average Syto16 atlas (generated by LifeCanvas Technologies using previously registered samples). Registration was performed using successive rigid, affine, and b-spline warping algorithms (SimpleElastix: https://simpleelastix.github.io/).

Automated cell detection was performed by LifeCanvas Technologies using a custom convolutional neural network created with the Tensorflow python package (Google). The cell detection was performed by two networks in sequence. First, a fully-convolutional detection network (https://arxiv.org/abs/1605.06211v1) based on a U-Net architecture (https://arxiv.org/abs/1505.04597v1) was used to find possible positive locations. Second, a convolutional network using a ResNet architecture (https://arxiv.org/abs/1512.03385v1) was used to classify each location as positive or negative. Each cell location was projected onto the Allen Brain Atlas in order to count the number of cells for each atlas-defined region using the previously calculated Atlas Registration.

Acute brain slice preparation

For the electrophysiological recordings in the OB, the mice were deeply anesthetized (ketamine/xylazine, 10 and 1 mg/mL, respectively, 0.1 mL per 10 g of body weight) 3-5 weeks after the stereotaxic injections and were transcardiacally perfused with modified oxygenated artificial cerebro-spinal fluid (ACSF) containing the following (in mM): 210.3 sucrose, 3 KCl, 2 $CaCl_2.2H_2O$, 1.3 $MgCl_2.6H_2O$, 26 $NaHCO_3$, 1.25 $NaH_2PO_4.H_2O$, and 20 glucose. The OBs were then quickly removed, and 250-µm-thick horizontal sections were cut using a vibratome (Microm HM 650V; ThermoFisher Scientific). The sections were placed in oxygenated ACSF containing the following (in mM): 125 NaCl, 3 KCl, 2 $CaCl_2.2H_2O$, 1.3 $MgCl_2.6H_2O$, 26 $NaHCO_3$, 1.25 $NaH_2PO_4.H_2O$, and 20 glucose.

For the electrophysiological recordings in the HDB/MCPO, the brains were removed, and coronal sections were prepared in ice-cold oxygenated ACSF containing the following (in mM): 83 NaCl, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 3.3 $MgSO_4$, 0.5 $CaCl_2$, 70 sucrose, and 22 D-glucose (pH 7.3, osmolarity 300 mOsm/L). Horizontal slices (300 µm) were cut using a vibratome (HM 650V; Microm International GmbH, Germany) in the same solution. The sections were incubated for 30-40 min at 34°C and were then stored at room temperature in oxygenated ACSF containing the following (in mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$ and 25 D-glucose.

Electrophysiological recordings

Patch-clamp whole-cell recordings were performed in oxygenated ACSF superfused at a rate of 2 mL/min at approximately 35°C. Neurons were examined by differential interference contrast (DIC) using a Slicescope microscope (Scientifica, UK) coupled to a camera (CoolSNAP EZ, Photometrics, USA). Cells were recorded using glass pipettes (resistance 3-6 MΩ for neurons in the HDB/MCPO, 7-9 MΩ for GCs in the OB) filled with an intracellular solution containing the following (in mM): 135 K-gluconate, 2 $MgCl_2$, 0.025 $CaCl_2$, 1 EGTA, 4 Na-ATP, 0.5 Na-GTP, 10 HEPES (all from Sigma Aldrich, USA), and 1 mg/mL of neurobiotin (Vector Labs, UK) or biocytin (Sigma-Aldrich, B4261) (pH 7.2, 290 mOsm). Atto 594 (Sigma Aldrich) or Alexa Fluor 594 (Life Technology) (5-20 µM) was added to the intra-pipette solution to visualize the recorded cell. Recordings

were performed with a Multiclamp 700B amplifier (Molecular Devices, USA) and Axograph X acquisition software at an acquisition frequency of 20 kHz. Series resistances (Rs), membrane resistances (Rm), and membrane capacitances (Cm) were calculated by fitting an exponential curve to the relaxation phase of a current generated by a potential jump (–10 mV/15 ms) in voltage-clamp mode. Series resistances did not exceed 10% of the membrane resistances and were not compensated. Discharge patterns were examined in the current-clamp mode by injecting currents of increasing magnitude and a duration of 0.5-1 s. To assess the activation threshold of the voltage-gated Na^+ current (I_{Na+}) and its peak amplitude, we delivered depolarizing pulses ranging from –100 to 30 mV in 10-mV increments. Sodium currents were isolated by subtracting the traces obtained with and without the application of TTX (1 µM, Tocris). For optogenetic experiments, blue light stimuli were delivered from a LED lamp (wavelength 490 nm) through the 40x objective. Evoked IPSCs were isolated by the bath application of 6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione (NBQX) (50 µM; Tocris) and D-2-amino-5-phosphonopentanoic acid (D-AP5, 50 µM; Tocris) to block glutamatergic activity, while bicuculline methiodide (BMI) (50 µM; Tocris), a $GABA_A$ receptor antagonist, was used to block GABAergic transmission. The latency of opto-induced currents was measured by subtracting the time of the stimulus onset from the onset of the recorded currents in the GCs. We also measured the jitter of optogenetically induced currents that reflects variations in the latencies of currents in response to repeated (20-s intervals) blue light pulses.

To reveal the morphology of the recorded cells, the patch pipette was slowly retracted after the recording to avoid damaging the cell body. The sections were then fixed in 4% PFA overnight, washed three times in PBS, and incubated in blocking buffer (0.5% Triton X-100 and 4% skim milk diluted in PBS) at room temperature for 90 min. The sections were then incubated for 2 h in VECTASTAIN® ABC-HPR (PK-4000 kit; Vector Labs) and, after three washes in PBS, were stained with Streptavidin Alexa Fluor™ 546 conjugate (S11225; ThermoFisher) at room temperature for 90 min. DAPI was used to label nuclei. The sections were mounted in fluorescence mounting medium (Dako) and were kept at 4°C before imaging using a confocal microscope (Olympus).

Immunohistochemistry

Adult mice were deeply anesthetized by an intraperitoneal injection of a mixture of xylazine (20 mg/kg) and ketamine (100 mg/kg) and were intracardially perfused with PBS or 0.9% NaCl followed by 4% PFA. The brains were removed and were kept in 4% PFA overnight. Sections (50 µm) were cut using a vibrating blade microtome (Leica VT 1000S). The sections were blocked for 2 h in a PBS solution containing 4% BSA or 5% goat serum and 0.3% Triton X-100. They were then incubated overnight at 4°C with the following primary antibodies: goat anti-choline acetyl transferase (ChAT, 1/500; Merck Millipore, Darmstadt, Germany; cat. no. AB144P, RRID:AB_2079751), mouse anti-somatostatin (SOM; 1/500; Genetex, Irvine, USA; cat. no. GTX71935, RRID: AB_383280), mouse anti-parvalbumin (PV; 1/1000; MilliporeSigma cat. no. P3088, RRID:AB_477329), rabbit anti-calretinin (CR; 1/1000; Swant, Switzerland; cat. no. 7697, RRID:AB_2721226), mouse anti-calbindin D28K (CB; 1/1000; MilliporeSigma cat. no. C9848, RRID:AB_476894), rabbit anti-GFP (A-11122, 1/1000, 24 h; ThermoFisher Scientific), chicken anti-GFP (GFP-1020, 1/1000, 24 h; Aves or Abcam), or rabbit anti-mCherry (5993-100, 1/1000; 24 h; Biovision). After three washes in PBS, the sections were incubated for 2 h at room temperature with the corresponding secondary antibodies. They were then washed three times with PBS and were mounted in Prolong Diamond Antifade Mountant (P36961; ThermoFisher Scientific). Images were acquired using either a TCS SP5 II confocal microscope (Leica), an FV-1000 confocal microscope (Olympus), an LSM880 AxioObserver Z1; confocal microscope (Zeiss), or a NanoZoomer (Hamamatsu). Immunostained, EYFP-expressing and CR-expressing cells were manually counted using the Fiji software Cell Counter plugin. For the rabies experiments, images were acquired using an LSM980 confocal microscope (LCI Plan-Neofluor 25X/NA 0.8; Zeiss). GFP+ and GFP+/CR+ double-positive cells were counted manually throughout the entire stack of optical slices cells in the HDB/MCPO (1-3 stacks per slice; 4-6 slices per mouse).

Go/no-go olfactory discrimination learning

The DREADDs (AAV 2/8 EF1α-DIO-hM4D(Gi)-mCherry or pAAVrg-hSyn-DIO-hM4D(Gi)-mCherry) or control GFP (AAV 2/5 CAG-GFP or pAAVrg-hSyn-DIO-mCherry) viral vector-injected mice were partially water-deprived until they reached 80 to 85% of their initial body weight before starting the go/no-go training. Three-to-four days before beginning the go/no-go training sessions, they were provided with 2 mL of water per day. This time is usually sufficient for the mice to reach 80-85% of their body weight. They were weighed twice every day and an additional amount of water was provided if the weight loss was greater than 20%. After the start of the go/no-go training and test sessions, the animals were still water-deprived and received water only during the training/test sessions through the licking port (3 µL/lick). At the end of the training/test day, the animals received a water reward that did not exceed 1.5 mL of water through the same water port. After completion of the go/no-go tests, the mice received water *ad libitum*. The animals were first trained to insert their snouts into the odor sampling port and to lick the water port to receive a 3-µL water reward. The reward-associated odor (S+) was then introduced. The mice initiated each trial by breaking the light beam across the odor sampling port, which opened an odor valve. The duration of the opening was increased progressively from 0.1 to 1 s. The main flowmeter was set at 1950 cc air/min, while the odor flowmeter, which carries the flow from the odor vials to the water port, was set at 50 cc air/min. The mice with a minimum sampling time of 50 ms received a water reward. They usually successfully completed the training in one or two sessions. No differences between groups were observed. All the mice went through one day of training/shaping. The animals had to lick a water port through which they received the water reward when an odor was presented to them. The time between the odor presentation and when the water reward was delivered, was gradually increased from 0.1 s to 1.1 s. When the mice reached the 1.1-s threshold and successfully licked 20 times, their training was considered complete. All the animals spent approximately 40 min for the training/shaping session in one day. Following the training procedure, the mice were subjected to the go/no-go odor discrimination task. To ensure that they had successfully learned the task, the first session consisted of 30 trials during which only the S+ odor was presented. Mice that reached at least an 80% success rate were then exposed randomly to a reward-associated odor (S+) or to a no reward-associated odor (S−) for several blocks of 20 trials each (random exposure to 10

S+ and 10 S−). Correct responses consisted of correct hits (mouse licking the water port after the S+ exposure) and correct rejections (mouse not licking the water port after the S− exposure). False responses consisted of the mouse licking the water port after the S− exposure or not licking the water port after the S+ exposure. The percentage of correct responses for each block of 20 trials was calculated. The mice were considered to have successfully completed the go/no-go olfactory discrimination task if they reached ≥85% correct responses. The sessions lasted 2 to 6 days, depending on the odor pairs. The following two odor pairs were used: odor pair 1, 0.1% octanal (S+ odor) vs. 0.1% decanal (S− odor) in mineral oil and odor pair 2, 0.6% (+)-limonene + 0.4% (−)-limonene (S+ odor) vs. 0.4% (+)-limonene + 0.6% (−)-limonene (S− odor) in mineral oil. The control and experimental groups were both subjected to the same behavioral protocol with the same exposure to the S+ and S− odors.

Habituation/dishabituation odor discrimination task

We performed an habituation/dishabituation spontaneous odor discrimination task with CR-Cre male mice (n = 13 mice) that had been injected with Cre-dependent retrograde AAV expressing either DREADDs (pAAVrg-hSyn-DIO-hM4D(Gi)-mCherry) or, as a control, mCherry (pAAVrg-hSyn-DIO-mCherry) in the OB. The fluid cannulas for local intracerebral delivery of CNO (100 µM) just above HDB/MCPO were installed in both hemispheres during the same surgery. The stereotaxic injection and cannula installation were performed as described above. The habituation/dishabituation odor discrimination task was performed 4-5 weeks after viral injection. It consisted of three successive 4-min trials with 6-min intervals between each exposure when the mice were presented with the habituation odor (limonene(+), diluted 10^{-3}) followed by the dishabituation odor (limonene(-), diluted 10^{-3}). During successive exposures to limonene(+), the total investigation time progressively decreased as the mice became habituated to the odor. The mice were considered to be able to discriminate the novel dishabituation odor (limonene(-)) from the odor they were habituated to if the investigation time during the dishabituation exposure was longer than the investigation time recorded during the third

and final habituation phases. CNO was injected into both groups of mice 5-10 min before beginning the test.

Statistics

Data are expressed as means ± SD unless otherwise indicated in the text. A Student's t-test was used to assess the statistical difference between paired datasets with a normal distribution, a non-parametric Wilcoxon-Mann-Whitney rank sum test to assess the statistical difference between unpaired datasets, and a Kruskal-Wallis test for datasets with more than two variables. The exact value of n and its representation (cells, animals) are indicated in the text. No statistical methods were used to predetermine the sample size. Equality of variance for the unpaired t-test was verified using the F-test. The levels of significance were as follows: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. When possible, the investigator was blinded to the experimental conditions. Most of the experiments reported herein were confirmed independently in two different labs, one at Université Laval and the other at Université de Strasbourg.

Results

Multiple neuron subtypes in the HDB/MCPO project to the OB

To identify BF neurons that project to the OB, we first employed a retrograde tracing approach (**Fig. 1A**) in which fluorescent microbeads (MB) (**Fig. 1B**) or Alexa Fluor conjugates of the Cholera Toxin subunit B (CTB) (**Fig. 1C-D**) were injected into one OB. Both tracers retrogradely labeled neuronal cell bodies in several regions of the brain, as previously described (Hook & Puche, 2023; Santiago & Shammah-Lagnado, 2004; Shipley & Adamek, 1984; Zaborszky *et al.*, 1986). Along a rostro-caudal axis, a high density of MB+ or CTB+ neurons was found bilaterally in the anterior olfactory nucleus and ipsilaterally in the piriform cortex in BF nuclei and in the nucleus of the lateral olfactory tract (regions posterior to the nLOT were not examined). In the BF, retrogradely labeled OB-projecting neurons were found along a ~1-mm-long antero-posterior axis, with the highest density of labeled cells in the HDB/MCPO at the level of the crossing of the anterior commissure (**Fig. 1B-C**).

The OB receives dense GABAergic and cholinergic axonal projections from the HDB/MCPO. To characterize which subtypes of HDB/MCPO neurons project to the OB, we performed immunolabeling for CR, CB, PV, and SOM to label four non-overlapping cell populations (Gritti *et al.*, 2003; Yang *et al*, 2017). These four generic markers essentially label some of the BF GABAergic neurons (Yang *et al.*, 2017), but CR and CB are also expressed in subpopulations of BF glutamatergic neurons (McKenna *et al.*, 2021). We also used choline acetyltransferase (ChAT) staining to label cholinergic neurons. These experiments were done on brain sections from the CTB-injected mice. CR-expressing neurons constituted the largest population of retrogradely labeled HDB/MCPO neurons (19% of the CTB+ neurons, n=56 CR+ cells out of 294 CTB+ neurons, n=4 sections from 3 mice) (**Fig. 1C**). Eleven percent of CTB+ neurons were PV+ (n=42 PV+ neurons out of 383 CTB+ neurons, 7 sections from 3 mice) and 7% were SOM+ (n=26 SOM+ neurons out of 362 CTB+ neurons, n=4 sections from 3 mice) (**Fig. 1D**). Very little co-labeling was observed in CB-expressing HDB/MCPO neurons, with only 2% of the OB-projecting CTB+ neurons labeled with CB (n=10 CB+ cells out of 469 CTB+ neurons, n=8 sections from 4 mice). Co-labeling of CTB with ChAT was found in 9% of

HDB/MCPO retrogradely labeled neurons (n=61 ChAT+ neurons out of 678 CTB+ neurons, 9 sections from 3 mice). These data indicated that at least three known sub-populations of BF GABAergic neurons project to the OB, indicating that these projections are quite diverse.

We then concentrated on studying a single OB-projecting BF sub-population, i.e., those expressing CR. We first performed rabies virus-based monosynaptic tracing to determine whether CR+ neurons in the HDB/MCPO establish direct synaptic inputs with GCs, the most abundant cells in the OB. As the majority of GCs are born during the early postnatal period (Hinds, 1968; Lemasson *et al.*, 2005; Mirich *et al*, 2002), we injected the retro-TVA-G-DsRED vector into the RMS on postnatal day 6 (P6) to infect neuroblasts migrating to the OB. We injected a RABV virus into the GCL 120 days later to trace first-order pre-synaptic partners of the previously labelled neuroblasts that differentiated into GCs (**Fig. 2A**). With this protocol, GCs in the OB that had integrated both the G-TVA retro and the RABV became the starter cells that allowed retrograde RABV propagation to their presynaptic monosynaptic inputs located in different brain regions. This protocol made it possible to specifically label GCs but not principal neurons or short-axon cells located in the GCL or PGCs located in the glomerular layer (**Fig. 2B-C**). To assess brain regions that are monosynaptically connected to GCs, we analyzed serial sagittal brain sections and observed dense labelling in the HDB/MCPO (**Fig. 2D-E**). Next, we performed immunolabeling for CR (**Fig. 2E**) and observed that the vast majority (86.3%; n=416 CR+ out of 482 GFP+ cells, 50 slices from 3 mice) of presynaptic partners of GCs in the HDB/MCPO are CR+ (**Fig. 2F**). These data indicated that CR+ neurons in the HDB/MCPO establish monosynaptic connections with GCs in the OB and are thus a major neuronal subtype by which neuronal assemblies of BF may regulate the activity of GCs.

We next investigated the localization and axonal pattern of CR+ cells that project to the OB throughout the entire brain. To do so, we injected retrograde Cre-dependent AAVs into the GCL of the OB of CR-Cre mice, followed by whole-brain clearing and light-sheet imaging (**Fig. 3A**). We observed dense labeling of axons in the GCL of the OB together with some labeled GCs (**Fig. 3B-C**). Interestingly, we also observed neuronal labeling in

several brain regions such as the entorhinal cortex (**Fig. 3D**), subiculum (**Fig. 3E**), piriform cortex (**Fig. 3F-G**), and different cortical areas, including the gustatory, somatosensory, and agranular insular cortices (**Fig. 3H**). In line with our previous results from retrograde tracer labeling (**Fig. 1**) and monosynaptic rabies tracing (**Fig. 2**), we also observed dense neuronal labeling in the HDB/MCPO (n=3 mice; **Fig. 3F, 3I**).

Optogenetic stimulation of CR-expressing HDB/MCPO neurons elicit monosynaptic inhibitory currents in the GCs of the OB

The results of the viral tracing suggested that CR-expressing neurons are an important sub-population of HDB/MCPO neurons projecting to the OB. We thus examined the projection pattern and functional connectivity of HDB/MCPO CR+ neurons within the OB using an optogenetic approach. We injected an AAV encoding a Cre-inducible ChR2–EYFP fusion protein into the HDB/MCPO of CR-Cre mice (**Fig. 4A)**. To validate the specificity of the Cre-dependent recombination, we performed immunolabeling for CR and observed that the majority of ChR2–EYFP-expressing cells in the HDB/MCPO are also immunolabeled for CR (80 ± 6%, n = 221 cells from 3 mice; **Fig. 4B**). Next, we characterized the functional properties of HDB/MCPO neurons that express ChR2-EYFP by performing whole-cell patch-clamp recordings in acute coronal slices (**Fig. 4C**). All the cells tested (n=11 from 3 mice) responded to a blue light photo-stimulation (1-300-ms duration), with a large inward current in the voltage-clamp configuration (peak amplitude 695 ± 354 pA at Vh = –75 mV, 100-ms pulses). In the current-clamp mode, photo-stimulations evoked an irregular train of action potentials (mean frequency 46.7 ± 9 Hz in response to 300-ms blue light pulses) (**Fig. 4D**) similar to the responses evoked by the injection of a depolarizing current step (**Fig. 4E**). The capacitance and membrane resistance of the recorded cells were, respectively, 14.6 ± 4.1 pF and 465 ± 190 MOhms (n=11 cells). CR+ cells had lower capacitance values than the general population of retrogradely labeled neurons in the HDB (26 ± 7 pF, n=15 cells, $p<0.001$, Wilcoxon-Mann Whitney test), suggesting that CR+ neurons are in general smaller than other OB-projecting HDB/MCPO neurons.

We next examined the projection pattern of HDB/MCPO CR+ neurons in OB sections from fixed brain slices in which an AAV encoding a Cre-inducible ChR2–EYFP fusion protein had been injected into the HDB/MCPO (n=3 CR-Cre mice). We observed EYFP-labeled axons in the entire GCL. Occasionally, a few fibers crossed the mitral cell layer and entered the external plexiform layer but rarely, if ever, penetrated into the glomerular layer (n=13 sections from 3 mice, **Fig. 5A)**. These results suggested that CR+ neurons in the HDB/MCPO selectively target interneurons in the GCL of the OB.

To assess the synaptic properties of the connections made by ChR2-expressing CR+ HDB/MCPO neurons within the GCL, we used whole-cell voltage-clamp recordings from GCs in acute OB sections (**Fig. 5B-D)**. Stimulating CR+ ChR2-expressing BF axons with brief (1-5 ms) flashes of blue light evoked outward synaptic currents in GCs voltage-clamped at 0 mV (**Fig. 5C)**. Photo-evoked currents exhibited fast onset latencies (2.2 ± 0.78 ms) and little jitter (274 ± 170 μs), which is consistent with monosynaptic transmission. To selectively isolate the source of these currents, we first recorded GC responses under baseline conditions and then, following the application of NBQX and D-AP5, the AMPA and NMDA receptor antagonists, respectively. No changes in the amplitude of photo-evoked currents were observed following the NBQX and D-AP5 applications (n=10 GCs from 7 mice; **Fig. 5C**). Light-induced currents were, however, completely abolished by bicuculline methiodide (BMI), the $GABA_A$ receptor antagonist (n=10 GCs, **Fig. 5C**). These results are in line with the monosynaptic rabies viral tracing and indicated that OB GCs receive monosynaptic GABAergic inputs from CR+ neurons in the HDB/MCPO. Of the 32 GCs recorded in the GCL, 13 (40.6%) responded to the photostimulation with an IPSC (n=7 mice). To assess whether GCs receiving synaptic inputs from CR+ HDB/MCPO neurons are characterized by electrophysiologically distinct properties compared to GCs that did not respond to the optogenetic stimulation, we recorded passive and active membrane properties. No differences in the passive and active membrane properties of responding and non-responding cells were observed (**Fig. 5D)**. Overall, our results with viral tracing and electrophysiological analysis identified a specific synaptic connection from CR+ neurons in the HDB/MCPO onto GCs in the OB.

Pharmacogenetic inhibition of CR+ GABAergic neurons in the HDB/MCPO affect olfactory discrimination and/or learning

It has been previously shown that different subsets of GCs play distinct roles in odor behavior (Hardy *et al.*, 2018; Malvaut *et al.*, 2017) and that HDB GABAergic projections are involved in passive odor discrimination (Nunez-Parra *et al.*, 2013). However, the HDB/MCPO GABAergic population is heterogeneous and it is not known how CR+ cells are involved in odor discrimination and olfactory learning. To address this issue, we employed a pharmacogenetic approach that specifically inhibits CR+ GABAergic neurons in the HDB/MCPO and subjected the mice to the habituation/dishabituation odor discrimination and go/no-go odor discrimination/learning tasks (**Fig. 6-7**). The Cre-dependent AAVs expressing DREADDs coupled with mCherry were injected into the HDB of the CR-Cre mice 4 weeks before beginning the behavioral task (**Fig. 6A-B**). We used DREADDs coupled with a Gi protein. DREADDs-Gi activation by CNO leads to GIRK-mediated K+ ion extrusion and hyperpolarization of the cell, which inhibits the activity of these neurons. The control group of mice was injected with Cre-dependent AAV-GFP. The two groups received a CNO injection 30 min prior to the task. Almost all the DREADDs-infected cells were immunostained for CR (95.8 ± 0.9%), which made up 10.2 ± 0.7% (n = 996 cells from 4 mice; **Fig. 6C**) of all HDB/MCPO CR+ GABAergic neurons.

We next subjected the mice to a go/no-go odor discrimination/learning task using different odor combinations that progressively increased the difficulty of the task from a simple odor pair (octanal vs. decanal, see Materials and Methods) to a more complex odor pair [(0.6% (+)-limonene+0.4% (−)-limonene (S+ odor) vs. 0.4% (+)-limonene+0.6% (−)-limonene (S− odor)] (**Fig. 6D-E**). As expected, the number of blocks needed to reach the criterion of 85% correct responses increased with the difficulty of the discrimination task for both the control and the DREADDs groups. Interestingly, inhibiting CR+ neurons in the HDB/MCPO affected odor discrimination and/or learning in both the simple and the complex tasks (**Fig. 6D-E**).

Although all the mice learned the task to criterion, the mean number of blocks required to reach the 85% criterion was significantly higher for the DREADDs group than for the control group when the mice were exposed to a simple task (the mean number of blocks

584 to reach the 85% criterion was 9 for the DREADDs group and 6 for the control group, n=7
585 and 5 mice, respectively, **Fig. 6D**). Statistically significant differences were also observed
586 in individual blocks beginning from block 5 to block 8 (**Fig. 6D**). The differences became
587 even more striking when the difficulty of the task was further increased with a complex
588 odor pair (**Fig. 6E**). The ability of the mice in the DREADDs group to discriminate the
589 odors was significantly lower than that of the mice in the control group (**Fig. 6E**). The
590 number of blocks needed to reach the 85% criterion was higher for the mice in the
591 DREADDs group. The mean number of blocks to reach the 85% criterion was 38 and 29
592 for the experimental and control groups, respectively (n=8 and 7 mice, respectively) (**Fig.**
593 **6E**). Significant differences in individual blocks were observed beginning from block 27
594 (**Fig. 6E**).

595 Although these results indicated that the HDB/MCPO CR+ neurons are involved in the
596 modulation of odor discrimination and/or learning in both the simple and complex go/no-
597 go tasks, the major drawback of these experiments is that the entire population of CR+
598 neurons in the BF is inhibited. It has been previously shown that HDB/MCPO neurons
599 innervate distinct cortical and subcortical brain areas to regulate arousal, motivation,
600 attention, learning, memory, and decision-making (Everitt & Robbins, 1997; Hanson *et al*,
601 2021; Klinkenberg *et al*, 2011; Lin *et al*, 2015; Nunez-Parra *et al*, 2020). It is thus possible
602 that the effects observed in the go/no-go odor discrimination/learning task following
603 systemic injection of CNO and the inhibition of the entire CR+ population in HDB/MCPO
604 is independent of the activity of GCs in the OB and is related to the changes in attention,
605 motivation, and/or reward-seeking behavior.

606 To address this caveat, we specifically inhibited OB-projecting CR+ cells in the
607 HDB/MCPO. To do so, we injected a retrograde Cre-dependent AAV expressing
608 DREADDs coupled with mCherry into the OB of CR-Cre mice. As a control, we used a
609 retrograde Cre-dependent AAV-mCherry. As the brain clearing and light-sheet analysis
610 had revealed several other brain regions in addition to HDB/MCPO that contain CR+ cells
611 projecting into the OB, we also installed fluid cannulas just above HDB/MCPO for the
612 local intracerebral delivery of CNO (100 µM) in both groups of mice (**Fig. 6F**). This made
613 it possible to avoid inhibiting CR+ neurons in other brain areas. Both groups of mice

received comparable days and blocks of training with the go/no-go odor discrimination/learning task and spent equal amounts of time to complete the training (**Fig. 6G**). The viral injection resulted in specific labeling of CR+ cells in the HDB/MCPO as 94.8 ± 1.4% of mCherry+ cells were also immunolabeled for CR (n=185 cells, **Fig. 6H**). Importantly, intracerebral CNO infusion resulted in a significant decrease in mCherry+/cfos+ cells in mice injected with DREADDs viral constructs, indicating that the CNO infusion results in an effective inhibition of control and DREADDs-injected mice (35.2 ± 1.1% and 15.8 ± 1.4% for the control and DREADDs-injected mice, respectively; n=339 and 537 mCherry+ cells analyzed, respectively, from 3 mice per group; **Fig. 6I-J**). We next subjected the mice to a go/no-go odor discrimination/learning task using a simple odor pair (octanal vs. decanal) followed by a more complex odor pair [(0.6% (+)-carvone+0.4% (−)-carvone (S+ odor) vs. 0.4% (+)-carvone+0.6% (−)-carvone (S− odor)] (**Fig. 6K-L**). Interestingly, inhibiting OB-projecting CR+ neurons in the HDB/MCPO affected odor discrimination/learning only in the complex task but not in the simple go/no-go task (**Fig. 6K-L**). The mean number of blocks required to reach the 85% criterion for a simple go/no-go odor discrimination/learning task was comparable for both groups of mice (n=6 and 8 for control and DREADDs-injected mice, respectively) (**Fig. 6K**). No statistically significant differences were observed in individual blocks (**Fig. 6K**). In contrast, statistically significant differences were observed in the complex go/no-go odor discrimination/learning task (**Fig. 6L**). Pharmacogenetic inhibition of OB-projecting CR+ cells by intracerebral administration of CNO in the HDB/MCPO resulted in an increase in the number of blocks needed to reach the 85% criterion (the mean number of blocks to reach the 85% criterion was 22 and 32 for the control and experimental groups, respectively, n=6 and 7 mice) (**Fig. 6L**), with significant differences in individual blocks between both groups of mice (**Fig. 6L**)).

These results suggested that local intracerebral inhibition of OB-projecting CR+ neurons in the HDB/MCPO affects animal performance in the complex associative odor discrimination/learning task. It remains, however, unclear whether these differences are due to affected odor discrimination or learning. To address this issue, we subjected mice to a passive habituation/dishabituation odor discrimination task (**Fig. 7**). In this task, both groups of mice received an intracerebral injection of CNO (100 µM) 5-10 min before

beginning the task and were exposed to the same odor (limonene(+)) using three consecutive 4-min exposures with 6-min intervals between each exposure. The investigation time during each consecutive exposure (habituation) decreased progressively, indicating that the mice became habituated to the odor. For the final presentation, the mice were presented with a novel odor (limonene(-)) alone for 4 min and the investigation time was measured (**Fig. 7A**). Our results indicated that the pharmacogenetic inhibition of OB-projecting CR+ neurons in the HDB/MCPO does not affect the odor discrimination abilities of mice (**Fig. 7B**), suggesting that the differences observed in the go/no-go odor associative task are due to affected odor learning abilities, rather than odor discrimination.

Overall, our viral tracing, electrophysiological, and behavioral data indicated that long-ranging CR+ cells in the HDB/MCPO establish monosynaptic GABAergic connections with GCs in the OB and are required for learning complex odor mixtures.

Discussion

Local and long-ranging inhibitory circuits in the OB play an essential role in bulbar network functioning and olfactory behavior. Odor information processing in the OB is under the control of local interneurons such as PGCs and GCs that regulate the theta and gamma rhythms in the bulbar network, respectively (Fukunaga *et al.*, 2014; Villar *et al.*, 2021). Each of these two classes of local interneurons can be further subdivided into heterogeneous cell populations based on their morpho-functional and molecular properties that modulate different types of olfactory behavior in different ways (Hardy *et al.*, 2018; Malvaut *et al.*, 2017; Malvaut & Saghatelyan, 2016). A growing body of evidence has also revealed that long-ranging inhibitory projections, especially the GABAergic inputs from the BF to OB neurons, play a role in bulbar network functioning and odor processing (Bohm *et al*, 2020; Nunez-Parra *et al.*, 2013; Sanz Diez *et al.*, 2019). However, although the diversity of GABAergic neurons in the basal forebrain and, specifically in the HDB/MCPO, is largely recognized (Freund & Gulyas, 1991; Gracia-Llanes *et al.*, 2010; Gritti *et al.*, 2003; Hook & Puche, 2023; McKenna *et al*, 2013; Niedworok *et al.*, 2012; Nunez-Parra *et al.*, 2013; Sanz Diez *et al.*, 2019; Yang *et al.*, 2017; Zaborszky *et al.*,

675 2005), the contribution of specific inhibitory neuron subtypes in these regions is unknown.
676 In the present study, we identified specific HDB/MCPO GABAergic projections defined by
677 the expression of CR that are synaptically wired to GCs in the GCL of the main OB.
678 Optogenetic activation of HDB-derived CR+ axons in the OB elicited monosynaptic
679 GABAergic currents in GCs, while chemogenetic inactivation of the CR+ neurons in the
680 HDB/MCPO during spontaneous habituation/dishabituation odor discrimination and
681 go/no-go odor discrimination/learning tasks revealed an impairment in odor learning, but
682 not odor discrimination. Our results revealed that a particular cellular type in the
683 HDB/MCPO makes a specific contribution but, together with previous findings (Bohm *et*
684 *al.*, 2020; Nunez-Parra *et al.*, 2013; Sanz Diez *et al.*, 2019), also indicated that
685 HDB/MCPO GABAergic projections into the OB may have multiple and distinct
686 involvements in odor information processing and olfactory behavior.

687 Our morphometric retrograde and anterograde tracing results showed that there is a cell-
688 type-specific innervation of a subpopulation of GCs in the GCL by CR+ axons projecting
689 from the HDB/MCPO. This contrasts with the labeling pattern observed after targeting all
690 GABAergic neurons in the HDB/MCPO that results in the dense innervation of all bulbar
691 layers (Gracia-Llanes *et al.*, 2010; Hook & Puche, 2023; Niedworok *et al.*, 2012; Nunez-
692 Parra *et al.*, 2013; Sanz Diez *et al.*, 2019). The HDB/MCPO also contains a large
693 population of cholinergic neurons that mostly target the glomerular layer and the internal
694 plexiform layer (IPL) of the OB (Case *et al.*, 2017; De Saint Jan, 2022; Hamamoto *et al*,
695 2017; Salcedo *et al*, 2011). Recently, it has also been shown that HDB/MCPO cholinergic
696 and GABAergic projections contact different subtypes of PGCs and regulate their activity
697 in distinct ways (De Saint Jan, 2022). These results indicated that different bulbar
698 sublayers and OB cell subtypes are targeted by distinct HDB/MCPO neuronal subtypes
699 based on their molecular identities.

700 Interestingly, not all GCs in the GCL were synaptically connected to CR+ cells in the
701 HDB/MCPO. Our combined optogenetic stimulations of CR+ axons and
702 electrophysiological recordings from GCs showed that about 40% of these cells receive
703 a monosynaptic GABAergic input from CR+ cells in the HDB/MCPO. This is in part due
704 to an inherent technical limitation of our local injections, with viruses likely infecting only

a fraction of the OB-projecting CR+ neurons in the HDB/MCPO. These BF nuclei extend for more than 1 mm on the rostro-caudal axis along which CR+ neurons are heterogenously distributed, making it difficult to infect the entire population (Zaborszky *et al.*, 2005). However, we cannot exclude the possibility that HDB/MCPO CR+ neurons target specific GC subtypes in the OB. GCs in the GCL are indeed heterogeneous and can be distinguished based on their morphology (Merkle *et al*, 2014) or by the expression of molecular markers such as 5T4, CR, or CaMKIIα (Hardy *et al.*, 2018; Malvaut *et al.*, 2017; Murata *et al*, 2011). In the present study, we did not distinguish between the molecular phenotypes of OB target cells during recording sessions, and it is unclear whether GCs receiving cell-type specific connections from CR+ neurons in the HDB/MCPO are distinct in terms of their molecular make-up or not. It is also conceivable that CR+ HDB neurons contact early-born and adult-born GCs differently. We did not distinguish between these subtypes, which have different synaptic (Lemasson *et al.*, 2005; Valley *et al*, 2013) and intrinsic membrane (Belluzzi *et al*, 2003; Carleton *et al*, 2003) properties and play distinct roles in odor behavior (Alonso *et al*, 2012; Breton-Provencher *et al*, 2009; Magavi *et al*, 2005).

How do CR+ cells in the HDB affect olfactory behavior? To address this issue, we used a pharmacogenetic approach and olfactory behavioral tests. We first used a go/no-go operant-conditioning odor discrimination/learning paradigm in which the animals had to discriminate between two distinct (simple task) or similar (difficult task) odors to receive a water reward. The mice in which the activity of all CR+ cells in the HDB/MCPO was inhibited through systemic injections of CNO displayed reduced odor discriminatory/learning capabilities in both the simple and the complex odor tasks. The reduced odor discrimination/learning in a simple task when the animals needed to distinguish two very different odors was, however, intriguing. One clue may come from the fact that HDB/MCPO neurons innervate distinct cortical and subcortical brain areas to regulate arousal, attention, learning, memory, and decision-making (Everitt & Robbins, 1997; Hanson *et al.*, 2021; Klinkenberg *et al.*, 2011; Lin *et al.*, 2015; Nunez-Parra *et al.*, 2020). Both GABAergic and cholinergic neurons in the BF are recruited during go/no-go odor discrimination tasks (Hanson *et al.*, 2021; Nunez-Parra *et al.*, 2020) and play a role in sustaining attention (Hangya *et al*, 2015) or encoding reward and motivational

information (Avila & Lin, 2014; Hanson *et al.*, 2021; Nguyen & Lin, 2014). Interestingly, their responses are bi-phasic and increase during reward seeking behavior and decrease with reward delivery in a go/no-go odor discrimination task (Hanson *et al.*, 2021). It is thus conceivable that the role of HDB/MCPO CR+ cells in the go/no-go odor discrimination/learning task goes beyond the modulation of bulbar network activity through inhibitory contacts with GCs and may be due to their role in attention and reward-seeking behavior through their connections with non-olfactory areas. To reveal the role of OB-projecting CR+ neurons in the HDB/MCPO, we labeled these cells with retrograde viral injections in the OB and inhibited their activity with the local intracerebral infusion of CNO through fluid cannulas installed in the HDB/MCPO. Interestingly, inhibiting OB-projecting CR+ neurons in the HDB/MCPO affected the complex but not the simple odor discrimination/learning task. These data revealed, on the one hand, that CR+ neurons in the HDB/MCPO make a specific contribution to modulating the odor discrimination/learning of complex odor mixtures through their monosynaptic contacts with OB GCs and, on the other hand, that other subtypes of CR+ neurons in the HDB/MCPO may also affect the attention, reward-seeking, and motivation of mice through their connections with the cortical and subcortical areas. This may influence behavioral outcomes in both simple and complex go/no-go odor discrimination/learning tasks when the entire population of CR+ cells in HDB/MCPO is inhibited. The reduction in odor discrimination/learning following the local intracerebral inhibition of OB-projecting CR+ cells in the HDB/MCPO was likely because the learning abilities of mice are affected given that the pharmacogenetic inhibition of these cells did not affect odor discrimination in the habituation/dishabituation task. Interestingly, previous studies have shown that the pharmacogenetic inhibition of the entire GABAergic population of HDB/MCPO affected odor discrimination in the spontaneous habituation/dishabituation task (Nunez-Parra *et al.*, 2020; Nunez-Parra *et al.*, 2013). As the projection patterns of CR+ and other GABAergic neurons of HDB/MCPO into OB are markedly different, and target exclusively the GCL or the entire bulbar layer, respectively, these data suggested that the modulation of the activity of these populations of cells may result in distinct behavioral outcomes.

The specific involvement of OB-projecting CR+ neurons in the HDB/MCPO in the odor learning, but not habituation/dishabituation odor discrimination, is interesting as previous

studies have shown that inhibiting different subtypes or numbers of bulbar interneurons affects complex odor discrimination/learning (Alonso *et al.*, 2012; Hardy *et al.*, 2018; Lepousez & Lledo, 2013; Malvaut *et al.*, 2017), while optogenetic activation of GCs improves complex task learning (Alonso *et al.*, 2012; Gschwend *et al*, 2015) and odor-reward association (Grelat *et al.*, 2018). It should be noted that, in all these experiments, the pharmacogenetic, optogenetic, or pharmacological manipulation of GCs occurred in the OB. In contrast, in the present study, we inhibited the activity of a select subtype of GABAergic neurons in the HDB/MCPO, which should in turn disinhibit the activity of GCs. How can an increase in the inhibition or disinhibition of GCs lead to the same behavioral outcome? One clue may come from the subtype of GCs that receive selective inhibitory inputs from CR+ cells in the HDB/MCPO. Depending on the molecular phenotype or morpho-functional properties, different GC subtypes may play distinct roles in principal cell activity in the OB (Malvaut & Saghatelyan, 2016; Merkle *et al.*, 2014). For example, several new subtypes of GCs in the superficial GCL have been described and, depending on the location of their synaptic outputs, either in the somatic or dendritic domains of principal cells, it has been proposed that they can affect odor information processing in different ways (Merkle *et al.*, 2014). Our results provided additional evidence and point to a major involvement of specific cell types in the HDB/MCPO defined by the expression of CR in odor learning. This also warrants an investigation of the subtypes of OB GCs that receive this specific synaptic input from CR+ cells in the BF as well as the roles of other HDB/MCPO interneurons in olfactory behavior.

In conclusion, our experimental results showed that HDB/MCPO GABAergic projections can have multiple and complex involvements in odor information processing and that the HDB/MCPO projections defined by CR expression provide selective inhibitory inputs to GCs in the OB and strongly influence odor learning.

Acknowledgments

This work was supported by a Canadian Institute of Health Research (CIHR) grant to A.S. and by a Centre National de la Recherche Scientifique (CNRS) grant to D.D.S-J,. We are

grateful to Dr. Karl-Klaus Conzelmann and Alexandru Adrian Hennrich (Max Von Pettenkofer Institute Virology and Gene Center, Medical Faculty, Ludwig-Maximilians-University Munich, Germany) for the generous gift of the (EnvA)SAD-ΔG-mCherry virus and Dr. Bennedikt Bennigner (University Medical Center of the Johannes Gutenberg University, Mainz, Germany) for the retro-TVA-G plasmid construction. We thank the Cell Biology and Image Acquisition (CBIA) Core Facility (RRID:SCR_021845) at the University of Ottawa for the use of the various imaging systems and the Viral Vector for Gene Transfer core facility of the Structure Fédérative de Recherche Necker for the retroviral vector production. We also thank Aurelia Ces and Pierre Hener (ComptOpt platform and morpho-functional analysis platform, respectively, Institut des Neurosciences Cellulaires et Intégratives, Strasbourg) for their technical support.

Declaration of interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Retrograde labeling of OB-projecting neurons in the HDB/MCPO.

A: Schematic of retrograde tracer injections in the OB. CTB = Alexa 555-coupled cholera toxin subunit B. MB = red microbeads. **B**: Coronal section at about bregma from a brain in which microbeads were injected in the left OB. Retrogradely-labeled neurons can be seen in the HDB/MCPO and in the piriform cortex (boxed area on top, enlarged in the middle). Bottom, examples of calretinin-expressing retrogradely-labeled MB+ neurons (arrowheads). **C**: Coronal section at about bregma +0.3 mm from a brain in which Alexa 555-coupled CTB was injected in the left OB. This section was immunostained for calretinin (CR, green). Bottom, zoom of the boxed area in the HDB/MCPO. Scale bar 100 µm. Captions on the right show the colocalization of CTB and CR in select neurons from the boxed area (arrows). Scale bar 20 µm. **D**: Colocalization of CTB and parvalbumin (PV), CTB and somatostatin (SOM), and CTB and choline acetyl transferase (ChAT) in select neurons (arrows) in the HDB/MCPO. Scale bars 20 µm.

Figure 2. CR+ neurons in the HDB/MCPO establish monosynaptic contacts with GCs in the OB.

A: Experimental timeline for the rabies tracing experiments. **B**: Sagittal section of the OB at P133 showing DsRed+/GFP+ starter cells (yellow). DAPI staining (blue) delimits layers. Note that all cells are located in the granule cell layer (GCL). The mitral cell layer (MCL), the external plexiform layer (EPL), and the glomerular layer (GL) are shown. The boxed area depicts the region in the GCL shown at a higher magnification in panel **C**. **C:** High magnification image of the boxed area in **B**. **D**: Sagittal sections showing GFP+ cells (green) in the MCPO. **E**: Sagittal section showing cells expressing GFP+ cells (green), immunolabeling for CR+ (red), and GFP+/CR+ double-positive cells (yellow) in the HDB/MCPO. **F**: Proportion of HDB/MCPO neurons contacting OB GCs (GFP+ cells) that also express CR.

Figure 3. Whole-brain light-sheet imaging of OB-projecting CR+ neurons.

A: Schematic drawing showing the experimental procedure for labeling OB-projecting CR+ neurons and whole-brain light-sheet imaging. **B:** Low magnification image of a single plan (4-µm-thick) horizontal section of a CR-Cre mouse brain previously injected with AAVrg in the GCL of the OB. The image shows dense labeling of axonal projections in the OB and retrograde-labeled neurons in other brain regions. Boxed areas depict regions for which high magnification images are shown. **C-E:** High magnification images of the OB (**C**), entorhinal cortex (**D**), and subiculum (**E**) showing retrograde-labeled neurons that project to the OB. The images were obtained from 500-µm max projection images, except for the OB, that show axonal projections in a 4-µm-thick section. **F:** Low magnifications image of a single plan (4-µm-thick) horizontal brain section at the level of the BF and the piriform cortex of a CR-Cre mouse injected with AAVrg in the GCL of the OB. Boxed areas depict regions for which high magnification images are shown. **G-I:** High magnification images of the piriform cortex (**G**), the insular cortex (**H**), and the HDB/MCPO (**I**) showing retrogradely labeled neurons and axons that project to the OB. The images were obtained from 500-µm max projection images. The orientation of sections for B, C and F is shown. A, P, M, and L indicate anterior, posterior, medial and lateral.

Figure 4. Conditional expression of ChR2 in CR+ cells in the HDB/MCPO.

A: Schematic representation of the AAV injection protocol to label CR+ cells in the HDB/MCPO of CR-Cre transgenic mice. **B:** Expression of ChR2-EYFP (green) in CR-expressing neurons (red) in the HDB/MCPO. **C:** Experimental procedure for characterizing CR-expressing neurons in the HDB/MCPO. **D:** Photo-evoked whole-cell responses recorded in the same cell in the current-clamp mode (top) and in the voltage-clamp mode (Vh = –75 mV, bottom). **E:** Membrane voltage responses in the same neuron as in **D** in response to depolarizing and hyperpolarizing current steps injected through the patch pipette.

Figure 5. Optogenetic stimulation of CR+ axons in the OB induce monosynaptic GABAergic responses in GCs.

A: Coronal section of the ipsilateral OB 3 weeks after the conditional viral infection of CR+ neurons in the HDB/MCPO. The right panel is a zoom of the boxed area. Fluorescent axons (yellow) are concentrated in the granule cell layer (GCL). Arrowheads point to the few fibers crossing the mitral cell layer (MCL) and entering the external plexiform layer (EPL). However, none enter the glomerular layer (GL). DAPI staining (blue) delimits the layers. **B**: Example of a recorded GC filled with biocytin (red). **C:** Synaptic currents induced by the optogenetic stimulation of ChR2-expressing CR+ axons in the GC shown in B (top) and average IPSC amplitudes over time for 10 GCs (bottom). Photo-evoked IPSCs persisted in the presence of NBQX and D-AP5 but were blocked by bicuculline (BMI). **D**: Passive and active membrane properties of GCs responding and not responding to the optogenetic stimulation of CR+ axons. No differences in the parameters were noted.

Figure 6. Pharmacogenetic inhibition of CR+ neurons in the HDB/MCPO affects odor discrimination/learning in the go/no-go operant conditioning task.

A: Schematic representation of a Cre-dependent AAV injection leading to the expression of either DREADDs or GFP in CR+ cells in the HDB/MCPO of CR-Cre mice. **B:** Schematic drawing of the go/no-go odor discrimination/learning paradigm. The water-restricted mice were randomly exposed to reward-associated and non-reward-associated odors (S+ and S-, respectively), and the percentage of correct responses (hits + correct rejections) was calculated for every 20 trials. The mice were considered to have successfully discriminated between the two odors if they reached the 85% criterion of correct responses. **C:** Low (left) and high (right) magnification images showing that that injection of Cre-dependent AAV EF1α-DIO-hM4D(Gi)-mCherry into the HDB/MCPO of CR-Cre mice results in the selective labeling of CR-expressing mCherry+ cells (red). The sections were counterstained with DAPI. **D-E:** The mean scores in percentages for each block of 20 trials obtained from the control and DREADDs AAV-injected mice for the go/no-go odor discrimination/learning task using odor mixtures of different complexity. The odor pairs used were 0.1% octanal and 0.1% decanal (**D**), and 0.6% (+)-limonene + 0.4% (-)-limonene and 0.4% (+)-limonene + 0.6% (-)-limonene (**E**). The dashed lines represent the 85% criterion score. *p<0.05 and **p<0.01 with an unpaired t-test. **F:** Schematic

representation of the protocol for AAVrg injections in the OB and fluid cannula installation above the HDB/MCPO to label and modulate the activity of OB-projecting CR+ cells in the HDB/MCPO of CR-Cre mice. **G**: The time spent for control (black) and experimental (grey) mice during the training stage. Note that both groups of mice performed similarly during the go/no-go training stage. **H:** Low and high (inset) magnification images showing specific labeling of CR+ cells in the HDB/MCPO by retrograde AAV injections into the OB. **I:** High magnification confocal images of HDB/MCPO CR+ neurons from the control and DREADDs-injected mice showing labeling for mCherry (red) and an early immediate gene, cfos (green). **J:** The percentage of mCherry++/cfos+ cells in the HDB/MCPO of control and DREADDs AAVrg-injected mice. ***p<0.001 with unpaired t-test. **K:** The mean scores of correct responses (in percentages) of control and DREADDs AAVrg-injected mice subjected to the go/no-go odor discrimination/learning task using odor mixtures of different complexity. The odor pairs used were 0.1% octanal and 0.1% decanal (**K**), and 0.6% (+)-carvone + 0.4% (-)-carvone and 0.4% (+)-carvone + 0.6% (-)-carvone (**L**). *p<0.05 and **p<0.01 with an unpaired t-test).

Figure 7. Pharmacogenetic inhibition of OB-projecting CR+ neurons in the HDB/MCPO do not affect odor discrimination.

A: Schematic representation of the AAVrg injection and fluid cannula installation protocol to label and modulate the activity of CR+ cells in the HDB/MCPO of CR-Cre mice. Intracerebral injections of CNO just above the HDB/MCPO were performed 5-10 min before the test. **B:** Investigation time of control- (black) and DREADDs- (red) injected mice during habituation and dishabituation sessions. The inset shows the experimental protocol of the habituation/dishabituation odor discrimination task. Note the similar performances of both groups of mice and the significant increase in the investigation time during presentation of the dishabituation odor.