Immature olfactory sensory neurons provide complementary input in the healthy olfactory system

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Abstract

Adult neurogenesis of olfactory sensory neurons (OSNs) in the rodent olfactory system provides the unique opportunity to understand how new neurons functionally integrate into existing circuitry and contribute to behaviors. Immature OSNs express odorant receptors (ORs), form dendritic knobs with short cilia, and project their axons into the olfactory bulb (OB) where they form functional synapses. Furthermore, immature OSNs respond selectively to odorants and exhibit graded responses in a higher concentration range than mature OSNs, suggesting that they provide a distinct odor input stream. Finally, in mice that lack mature OSNs, sensory input from immature OSNs is sufficient to mediate odor detection and discrimination behavior. What remains unknown, however, is how these immature OSNs contribute to odor-guided behavior in the healthy, intact olfactory system. Here we show, using male and female mice, that chemogenetically silencing immature OSNs impairs odor detection ability without affecting their odor discrimination ability. Furthermore, immature OSN silencing reduces the amplitude of odor-evoked dendritic calcium responses of OB neurons *in vivo*. Together, these findings suggest that immature OSNs provide distinct odor input that complements mature OSN input to contribute to odor-guided behaviors in the healthy, intact olfactory system.

Introduction

Most mammalian neurons are generated embryonically or early in postnatal development and cannot be replaced. In contrast, newborn olfactory sensory neurons (OSNs) are generated from basal stem cells in the olfactory epithelium (OE) throughout life (Graziadei & Graziadei, 1978; Harding et al., 1977). Globose basal cells generate neuronally committed intermediate cells that undergo a developmental program to produce mature OSNs (Verhaagen et al., 1989; Huard & Schwob, 1995; McIntyre et al., 2010; Nickell et al., 2012). One to two days after terminal cell division, nascent OSNs transition to immature OSNs marked by the expression of both GAP43 and Gγ8 (Verhaagen et al., 1989; Tirindelli & Ryba, 1996; McIntyre et al., 2010; Rodriguez-Gil et al., 2015). Seven to eight days after terminal division, OSNs downregulate their expression of GAP43 and Gγ8 and upregulate expression of olfactory marker protein (OMP) (Monti Graziadei et al., 1977; Miragall & Monti Graziadei, 1982; Kondo et al., 2010; Savya et al., 2019).

Mature OSNs typically express one of ~1,100 odorant receptor (OR) genes (Monahan & Lomvardas, 2015; Mombaerts, 2004), with mature OSN axons expressing the same OR converging into the same glomeruli in the olfactory bulb (OB) (Serizawa et al., 2000; Shepherd, 2004). Here, they form synaptic connections with OB neurons, generating a highly organized odor input map. As existing OSNs undergo apoptosis, newborn OSNs are generated concurrently. This constant turnover creates a highly plastic environment in which newborn OSNs must integrate into pre-existing circuitry to contribute to olfaction. Given that OSNs are also generated throughout life in humans, it is important to understand how ongoing OSN neurogenesis contributes to odor processing, plasticity, and odor-guided behavior.

It has been assumed that mature OSNs provide the sole source of odor input to the OB. However, mounting evidence suggests that immature OSNs also contribute to olfaction. OSNs begin to express odorant receptors (ORs) four days after terminal cell division (Rodriguez-Gil et al., 2015), well before the onset of OMP expression. Furthermore, immature OSNs strongly upregulate neurite outgrowth genes (Nickell et al., 2012) and late-stage immature OSNs both project axons into glomeruli in the OB and form dendritic knobs with short cilia (Menco & Farbman, 1985; Marcucci et al., 2011; Cheetham et al., 2016; McClintock et al., 2020, Huang et al., 2022). Moreover, immature OSNs can detect odorants and form synaptic connections with OB projection neurons in mice (Cheetham et al., 2016; Huang et al., 2022). Importantly, odor selectivity is similar for immature and mature OSNs (Huang et al., 2022). However, compared to mature OSNs, immature OSNs have higher thresholds for odorant detection but continue to encode concentration information at high odorant concentrations at which mature OSN responses are saturated (Huang et al., 2022). Furthermore, sensory input from immature OSNs is sufficient to mediate odor detection and discrimination at early recovery time points in mice that have undergone methimazole-mediated OSN ablation (Huang et al., 2022). Together, these data suggest that immature OSNs provide behaviorally relevant information to the olfactory system.

What remains unknown is what contribution, if any, immature OSNs make in the healthy, intact olfactory system. We hypothesize that immature and mature OSNs provide complementary, but distinct, odor input to OB neurons. Here, we used selective chemogenetic silencing (Stachniak et al., 2014; Salvi et al., 2019) of immature OSNs to address two key questions. First, do immature OSNs contribute to odor-guided behaviors in healthy mice? Second, do immature OSNs provide odor input to the OB that cannot be supplied by mature OSNs? Silencing immature OSNs impairs odor detection ability but does not affect odor discrimination. Furthermore, acutely silencing immature OSNs reduces the amplitude of

odorant-evoked responses in OB neurons *in vivo*. Together, our data suggest that immature and mature OSNs provide complementary odor input to the OB that contributes to distinct aspects of odor-guided behavior.

Results

Selective expression of hM4Di silences ~60% of immature OSNs

To determine the contribution of immature OSNs to olfaction in the healthy, intact olfactory system, we bred Gγ8-tTA;tetO-hM4Di (referred to hereafter as Gγ8-hM4Di) and tetO-hM4Di mice. In Gγ8-hM4Di mice, immature OSNs express hM4Di, which silences activity in the presence of a ligand such as clozapine-N-oxide (CNO), specifically in immature OSNs due to tetracycline transactivator (tTA) expression under control of the Gγ8 promoter (Tirindelli & Ryba, 1996; Fig. 1A). We validated the specificity of hM4Di expression in immature OSNs via co-immunostaining for GAP43 to mark immature OSNs (Verhaagen et al., 1989) and the hemagglutinin (HA) tag attached to the hM4Di receptor (https://www.jax.org/strain/024114) (Fig. 1B). tetO-hM4Di mice were included as negative controls throughout this study and did not express the hM4Di receptor marked by HA staining (Fig. 1C). Approximately 60% of immature OSNs expressed the hM4Di receptor in Gγ8-hM4Di mice, whereas tetO-hM4Di controls, which lack tTA expression, did not express hM4Di (Fig. 1D).

Silencing immature OSNs reduces mitral cell activity in response to odors throughout the OB

We first sought to validate our model to acutely silence immature OSNs and determine the effects of immature OSN silencing on odor-evoked mitral cell (MC) activity. We stained the OB for the immediate early gene phospho-S6 (PS6) in saline- and CNO-treated mice after a 1-hour odor exposure session (Fig. 1E,F). We then calculated the linear density of PS6+ cells in the mitral cell layer (MCL) for each mouse. This showed that silencing immature OSNs significantly decreased the density of PS6-expressing MCs, indicating that MC activity was reduced as a result of immature OSN silencing (Fig. 1G; saline-treated Gγ8-hM4Di n = 4, CNO-treated Gγ8-hM4Di n = 4). Recent evidence has shown that the ventral OE contains a larger proportion of immature OSNs than the dorsal OE (Guan, Martens, and Schwob, 2022). Given the topography of OE-OB projections, this led us to ask whether MC activity in the dorsal vs. ventral OB is differentially affected by immature OSN silencing. Subdividing the MCL showed that immature OSN silencing had a significant effect on MC activity in both dorsal and ventral regions (Fig. 1H,I). This reduction in MC PS6 staining was not present in CNO-treated tetO-hM4Di control mice (Fig. 1J-L), thus validating our strategy to acutely silence immature OSN input throughout the OB.

Immature OSN silencing impairs odor detection behavior

Odor detection ability was tested using a buried food assay wherein mice are given 10 minutes to complete the task. In Gγ8-hM4Di mice, all saline-injected mice successfully uncovered the buried food within the 10-minute period (n = 7 mice), whereas 2/8 CNO-injected Gγ8-hM4Di mice failed to uncover the buried food (Fig. 2A). The time taken to uncover the buried food was significantly longer for CNO-treated than for saline-treated Gγ8-hM4Di mice (Fig. 2A). There was no effect of CNO treatment on the time spent digging during the five-minute acclimation period for Gγ8-hM4Di mice (Fig. 2B). In tetO-hM4Di control mice, 3/5 saline-injected and 2/3 CNO-injected mice uncovered the buried food. There was no difference in the time to complete the task between saline- (n=5) and CNO-treated (n=5) tetO-hM4Di mice (Fig. 2C). There was also no difference in acclimation period digging time between saline-

and CNO-treated tetO-hM4Di mice (Fig. 2D). Together, these data show that odor detection ability is impaired when immature OSNs are silenced.

Immature OSN silencing does not affect odor discrimination in a habituation-dishabituation task

To test whether odor discrimination ability is impacted by silencing immature OSNs, we performed an odor habituation-dishabituation task using Gγ8-hM4Di mice (Huang et al. 2022; Fig. 2E). We found no effect of immature OSN silencing via CNO administration on total investigation time (Fig. 2F), habituation index (Fig. 2G) or dishabituation index (Fig. 2H). We also found no effect of CNO administration on odor habituation-dishabituation in tetO-hM4Di control mice (Fig. 2I); total investigation time (Fig. 2J), habituation index (Fig. 2K) and dishabituation index (Fig. 2L) were unaffected. These data suggest that silencing immature OSNs does not impact odor discrimination ability in this task.

Immature OSN silencing reduces the amplitude of odor-evoked responses in the GL of the dorsal OB

To investigate the impact of immature OSN silencing on odor-evoked responses in OB neurons, we implanted acute cranial windows over the right OB of 3–5-week-old Gγ8-hM4Di and tetO-hM4Di mice that had received a neonatal injection of AAV5-syn-GCaMP6s directly into the OB (Fig. 3A). This allowed us to record odor-evoked responses in the glomerular layer (GL) of the olfactory bulb *in vivo* before and after CNO injection. Neonatal AAV5-syn-GCaMP6s injection resulted in GCaMP6s expression in neuronal types throughout the OB, but expression was greatest in the MCL and the external plexiform layer (EPL) with apical dendrites of putative MCs and tufted cells (TCs) expressing GCaMP6s also projecting into the GL (Fig. 3B-D). Thus, MC and TC dendrites are likely to be a major contributor to the GL odor-evoked calcium responses that we report using AAV5-syn-GCaMP6s.

We found that silencing immature OSNs significantly reduces the amplitude of odor-evoked glomerular responses across a panel of seven odorants (Fig. 3E). Hence, immature OSNs make a meaningful contribution to the odor information that is supplied to the OB in healthy adolescent mice. To further evaluate the odor selectivity of these responses before and after immature OSN silencing, we calculated the lifetime sparseness (S_L) of glomerular odorant responses. An S_L value of 1 indicates that a glomerulus responds to a single odorant (highly selective), whereas an S_L value of 0 indicates that a glomerulus responds equally to all seven odorants in our panel (non-selective). We found that odor selectivity is not impacted by immature OSN silencing (Fig. 3F). CNO administration in tetO-hM4Di control mice did not impact the amplitude of odor-evoked responses *in vivo* (Fig. 3G) and did not affect odor selectivity (Fig. 3H). Together, these results suggest that immature OSNs contribute to the odor input received by OB neurons in the healthy, intact olfactory system.

Discussion

In this study we sought to define the contribution of immature OSNs to olfaction in the healthy, intact olfactory system. We showed that chemogenetically silencing immature OSNs in awake, behaving mice impairs odor detection ability without hindering odor discrimination in a habituation-dishabituation assay. Furthermore, silencing immature OSNs results in decreased neuronal activity marked by immediate early gene expression in MCs throughout the OB and a broad reduction in the amplitude of odor-evoked responses across multiple glomeruli *in vivo* without affecting odor selectivity. Together, these data suggest that immature OSNs provide complementary odor input to that supplied by mature OSNs in the intact olfactory system. Hence, our findings provide further evidence that endogenously generated

postnatally born neurons, once integrated into the existing circuitry, provide functional information for odor processing.

An important question to address is what proportion of sensory input to the OB is being silenced by CNO treatment in Gγ8-hM4Di mice. Our approach to express the inhibitory DREADD hM4Di utilized a tetracycline operator (tetO) sequence activated by expression of tetracycline transactivator (tTA) in immature OSNs under control of the Gγ8 promoter. Validation of our model revealed that approximately 60% of GAP43+ (i.e. immature) OSNs express hM4Di, as marked by hemagglutinin (HA) tag expression (Fig. 1B-D). Data from a previous study (Cheetham, Park, and Belluscio, 2016) that assessed linear densities of immature and mature OSNs in the OE shows that Gγ8+ OSNs make up approximately 29% of the OSN population in 8-week-old mice (this value excludes nascent OSNs as they do not provide sensory input to the OB). Additionally, immature OSNs express GAP43 and Gγ8 from 1-7 days after terminal cell division (Verhaagen et al., 1989; Tirindelli and Ryba, 1996, Savya et al., 2019),which defines the population of immature OSNs in which hM4Di is expressed. However, immature OSNs do not project to the OB until 5 to 7 days after terminal cell division (McIntyre, Titlow, and McClintock, 2016, Huang et al., 2022). This limited window of both Gγ8 expression (which drives hM4Di expression in only 60% of immature OSNs) and the 2-3 day timeframe during which immature OSNs project to the OB before reaching maturity as defined by OMP expression (a fraction of 3/7 of Gγ8-expressing OSNs, assuming a uniform neurogenesis rate) results in an estimate that we are silencing a maximum of 12% of OSNs projecting to the OB at a given time (Gγ8+ account for 29% of all OSNs and 3/7 of these project to the OB = 12%). To that end, reducing OSN input into the OB seems to have surprisingly significant effects on olfactory-guided behaviors and odor-evoked responses, suggesting that their input may be distinct from that provided by mature OSNs.

We have shown previously that a subset of mice can perform odor detection and discrimination tasks, including the tasks used in the current study, using only immature OSNs following the ablation and subsequent regeneration of OSNs via methimazole treatment (Huang et al., 2022). In that previous study, timepoints at which mature OSNs are absent from the olfactory epithelium were included in testing (Huang et al., 2022). However, an important remaining question was whether immature OSN activity is necessary for odor-guided behaviors in the healthy olfactory system. Here, we demonstrated that silencing immature OSNs impairs odor detection ability in the buried food task (Fig. 2A) without affecting odor discrimination ability in an olfactory habituation-dishabituation task (Fig. 2E-H). This suggests that immature OSN input may be less important for odor discrimination behavior, although additional assays should be performed to strengthen this conclusion. Why do immature OSNs play a particularly important role in buried food detection? Our previous finding that immature OSNs encode odor concentration information high in the concentration range, when mature OSN responses have already saturated (Huang et al., 2022), provides a potential explanation: immature OSNs may play an important role in odor source localization. Hence, sensory input from mature OSNs may play a more important role far from the buried food, whereas immature OSN input is important, or in some cases necessary, to pinpoint the exact location.

Our calcium imaging data showed that silencing immature OSNs reduces the amplitude of odor-evoked responses in postsynaptic OB neurons *in vivo*. Our AAV5-based strategy to introduce GCaMP6s into OB neurons does result in expression throughout all layers of the OB, but GCaMP6s is preferentially expressed in the MCL and EPL of the OB (Fig. 3B-D). Thus, we reason that MC and TC dendritic

responses are a major contributor to the GL GCaMP6s responses that we imaged. A similar effect of immature OSN silencing on GCaMP6s responses was seen across our odor panel, which includes the major odorant functional groups of esters (ethyl butyrate, isoamyl acetate, ethyl valerate, methyl valerate, and methyl butyrate), an aldehyde (hexanal), and an amine (phenethylamine) (Fig. 3E). Additionally, we sampled from a random population of glomeruli across the dorsal surface of the OB in each mouse. These factors together suggest that the reduction in odor-evoked response amplitude elicited by immature OSN silencing is a generalized phenomenon in glomeruli populating at least the dorsal surface of the OB. This suggests that immature OSNs that project to the OB GL contribute to ongoing odor processing in the healthy olfactory system.

We also found that immature OSN silencing did not affect the odor selectivity of odor-evoked responses in OB neurons (Fig. 3F). This finding agrees with our previous study, which found that the selectivity of odor-evoked responses was similar in immature and mature OSN axons (Huang et al., 2022). In contrast, single cell RNA sequencing studies found that some immature OSNs express multiple PR transcripts (Hanchate et al., 2015; Tan, Li, and Xi, 2015), which might be expected to reduce odor selectivity. The solution to this discrepancy is provided by a recent study describing a Mex3a-dependent post-transcriptional regulatory mechanism that represses translation of multiple ORs in immature OSNs (Duffié et al., 2025).

A limitation of this study is that imaging was restricted to dorsal OB glomeruli, due to their optical accessibility. Hence, while silencing immature OSNs reduces the amplitude of dorsal GL odor-evoked responses, does silencing have a similar impact in other OB regions? Our immediate early gene staining of odor-evoked MC activity shows a significant reduction in the number of PS6 positive cells throughout the OB (Fig. 1G). We also found that immature OSN silencing had a similar effect on MC activity in the dorsal and ventral halves of the OB (Fig. 1H,I). This was surprising in light of a recent study showing that ventral OE regions have a greater proportion of immature OSNs than dorsal OE regions (Guan, Martens, and Schwob, 2022), which might be expected to result in greater immature OSN innervation of ventral vs. dorsal OB. While more sensitive assays of neuronal activity, that also assess other OB neuron types, might detect such a difference, the key finding here is that immature OSN input is sufficient throughout the OB to impact MC activity when it is silenced.

Together, our data highlight the important contribution of postnatally born OSNs to normal odor processing even while these cells are in development. It was once believed that newborn OSNs did not functionally contribute to olfactory circuity until they had reached maturity. In contrast, we have shown that immature OSNs not only make up a contribution to odor-evoked responses but also provide odor input that is useful in performed odor-guided behaviors, and cannot be supplied by mature OSNs, in the healthy olfactory system. Hence, we reason that immature OSNs provide complementary input to that supplied by mature OSNs that is essential for olfactory processing and odor-guided behaviors that are important for survival.

Methods

Experimental animals

All animal procedures conformed to the National Institute of Health guidelines and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Mice were housed on a 12-hour light/dark cycle in individually ventilated cages at 22°C and 48% humidity with unrestricted access

to food and water unless otherwise stated. Mice were group-housed if same sex littermates were available. A total of 56 mice was used in this study.

Generation of the Gγ8-tTA (Nguyen et al., 2007; Huang et al., 2022; Cheetham, Park, and Belluscio, 2016) and tetO-hM4Di (Salvi et al., 2019) (Jackson Laboratory strain #024114) mouse lines has been published. Separate cohorts of experimental mice were used for the following: behavioral testing (buried food and habituation/dishabituation tests) (age range: 6 to 8 weeks), *in vivo* 2-photon calcium imaging (age range: postnatal days 24 to 35 [P24-35]) and immunohistochemistry (age range: 6 to 8 weeks). All mice used were either Gγ8-hM4Di [Gγ8-tTA$^{+/-}$;tetO-hM4Di$^{+/-}$] or tetO-hM4Di$^{+/-}$ genotypes. Each experimental group comprised roughly equal numbers of male and female mice. Mice of each genotype were randomly assigned to each experimental group for behavioral testing. Mice were genotyped by PCR using previously validated primers (Huang et al., 2022; https://www.jax.org/Protocol?stockNumber=024114&protocolID=26393).

Clozapine-N-Oxide (CNO) treatment to silence immature OSNs

30 minutes prior to starting behavioral or 2-photon calcium imaging experiments (to allow for CNO to begin taking effect), mice received a single intraperitoneal injection of either saline (vehicle) or CNO (1 mg/kg; 10 µl/g). All experiments were conducted 30 minutes after injection to allow for the CNO to have taken effect well but remain within its ~1-hour half-life (Manvich et al., 2018; Jendryka et al., 2019).

Odor exposure assay for immediate early gene phospho-S6 immunohistochemistry

30 minutes prior to odor exposure, mice (age range: 6 to 8 weeks) received an i.p. injection of either CNO or saline and were housed in a separate empty home cage to allow the drug to take effect. After these 30 minutes passed, mice were then placed individually into new empty home cages with the cage lid on and were exposed for 1 hour to a glass vial with a perforated lid containing a mixture composed of 30 µL each of the following odorants diluted 1:10 in mineral oil: isoamyl acetate, ethyl butyrate, methyl valerate, and isovaleric acid. Mice underwent transcardial perfusion immediately after the 1-hour exposure.

Transcardial perfusion and cryosectioning

Mice were deeply anesthetized using 4% isoflurane in 1L/min O_2 and transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The OBs of these mice were then dissected out, cryoprotected in a 30% sucrose solution, embedded in 10% gelatin, fixed/cryopreserved, flash frozen, and sectioned on a cryostat for immunostaining as described previously (Huang et al., 2022).

Immunohistochemistry in OB and OE sections

Free floating OB and OE sections were treated with 1% sodium borohydride, washed with PBS, and then permeabilized for one hour in blocking buffer (5% normal donkey serum [NDS]/0.5% Triton X-100 in PBS). 40 µm OB sections were then stained with a primary antibody to detect the immediate early gene (IEG) phospho-S6 (PS6). 50 µm OE sections were co-stained with primary antibodies to detect immature OSNs via growth-associated protein 43 (GAP43) and the hM4Di receptor via its hemagglutinin (HA) tag. All sections were incubated in primary antibody solutions (3% NDS, 0.2% Triton X-100, 0.01% sodium azide in PBS) as shown in Table 1. Following primary antibody application,

sections were washed in PBS and then incubated in secondary antibody solution (3% NDS, 0.2% Triton X-100 in PBS) for one hour at room temperature. Sections were then washed in PBS and mounted with Vectashield containing DAPI (Vector Labs).

Antibody	Dilution	Species	Incubation conditions	RRID	Manufacturer catalog #
GAP43	1:500	Mouse	96 h | 4 °C	AB 2533123	ThermoFisher 33-5000
Anti-HA	1:250	Rabbit	96 h | 4 °C	AB 1549585	Cell Signaling Technology #3724
Phospho-S6	1:400	Rabbit	96 h | 4 °C	AB 916156	Cell Signaling Technology #4858
Rabbit-AF488	1:500	Donkey	1 h | room temp.	AB 2535792	ThermoFisher A21206
Rabbit-AF 546	1:500	Donkey	1 h | room temp.	AB 2534016	ThermoFisher A10040
MouseAF-546	1:500	Donkey	1 h | room temp.	AB 11180613	ThermoFisher A10036

Table 1: Primary and secondary antibody information

Confocal microscopy

Images of stained OE sections were acquired using an Olympus Fluoview 3000 confocal microscope with an Apo 60x/1.4 NA oil-immersion objective and Fluoview imaging software (Olympus). Laser excitation and bandpass emission filters were: 405 nm and 450/50 for DAPI, 488 nm and 525/50 for AF488, and 561 nm and 595/50 for AF546. Z series images were collected with 2 μm steps between each z plane. Images were 212.132 x 212.132 μm (1024 x 1024 pixels) collected with a 2.0 μs/pixel sampling speed.

Widefield fluorescence microscopy

Images of OB sections were collected using ECHO imaging software (Echo) on a Revolve microscope equipped with LED excitation (Echo) and a Plan Apo 10x/0.4 NA air objective (Olympus). Images were stitched together in Affinity Photo software (Affinity). Bandpass excitation/emission filter wavelengths were (in nm): DAPI (450/50) and Phospho-S6-AF546 (590/50). Detection settings were identical for all images collected for this dataset.

Image analysis in OB and OE sections

Image analysis for both PS6 intensity in OB sections and colocalization of GAP43 and HA in OE sections was performed in Fiji (ImageJ) (Schindelin et al., 2012). OB sections were subdivided into dorsal and ventral halves by horizontally bisecting the image halfway along a vertical line extending from the center of the dorsal surface to the center of the ventral surface. MCs, defined as large neurons within the MCL, that expressed PS6 were counted and the length of the MCL/EPL border was measured, for dorsal and ventral halves of each section. PS6+ MC linear density (MCs per mm) was then calculated separately for each half, and as a total value for the entire section. OSNs expressing either GAP43 or HA were marked separately in z-stacks, and the proportion of immature OSNs in the OE that expressed the hM4Di receptor was calculated by dividing the number of co-stained (GAP43+ and HA+) cells by the total number of immature (GAP43+) OSNs and converting it to a percentage.

Odor detection and discrimination assays and analysis

A buried food assay (Yang and Crawley, 2009; Huang et al., 2022) was used to test odor detection ability following either CNO or saline injection. Two days before testing, a Froot Loop (Kellogg's) was placed into the home cage to familiarize the mice with the target odor. 14 hours prior to testing, mice were placed in a new home cage and food restricted until testing. During testing, 6-8-week-old mice were placed in an empty cage (L: 29.21 cm x W: 19.05 cm x H: 12.7 cm) with 1.5 cm of autoclaved bedding for five minutes to acclimate to the environment. Mice were then removed so that the Froot Loop could be buried in the center of the cage. The mice were then placed back into the cage and given 10 minutes to complete the task. Their behavior during both the acclimation and testing periods was monitored via video recording software (pylon Viewer, Basler). The amount of time spent digging in each 10-minute test session was recorded for each mouse. The time that it took for the mouse to uncover the buried food was reported. Mice failed the task if they did not uncover the buried food within 10 minutes. If mice failed to complete the task within the allotted ten minutes, they were removed from the cage, assigned a time of 10 minutes, and given the cereal to eat in their home cage. Mice that uncovered the buried food within the allotted 10 minutes of the test were allowed to eat the cereal before being returned to their home cage.

An odor habituation-dishabituation assay was used to test odor discrimination ability in saline or CNO-injected mice, using some modifications from previous protocols (Yang and Crawley, 2009; Huang et al., 2022). Mice were acclimated for 30 minutes to the test cage, which was an empty cage (length: 30.48 cm x width: 19.05 cm x height: 12.7 cm) with a metal tea ball secured to the center of the cage floor. Mice were then presented with a series of odorant-soaked filter paper squares placed in the tea ball for two minutes each. The series of odorants consisted of three 2-minute trials each of water, almond extract, mint extract, and vanilla extract (all diluted 1:100 in water). Live recordings were captured via Pylon viewer software, and investigation of the tea ball during each odor presentation was analyzed using EthoVision tracking software (Noldus). Time spent investigating the tea ball for each odor and total investigation time across the entire test period were calculated. A habituation index value was calculated by subtracting the amount of time the mouse spent sniffing during the second trial from the first trial of each odorant. A dishabituation index value was calculated by subtracting the amount of time the mouse spent sniffing the third presentation of an odorant from the amount of time they spent sniffing the first presentation of the subsequent odorant.

Neonatal AAV injections in mouse olfactory bulbs

At P2, Gγ8-hM4Di and tetO-hM4Di mice were anesthetized by acute hypothermia (Phifer and Terry, 1987) until immobile and 1 ul of AAV5-syn-GCaMP6s (Addgene #100843-AAV5) was injected into the right OB via a glass micropipette (Cheetham, Grier, and Belluscio, 2015). Mice then recovered on a heating pad until awake and mobile in a separate holding container before being returned to their home cage until they reached imaging age (P24-P35).

Acute window preparation for *in vivo* 2-photon calcium imaging

P24-P35 Gγ8-hM4Di mice were anesthetized with isoflurane (1.5 - 2% in 1L/min oxygen), administered ketoprofen (5 mg/kg) as an analgesic, and received a ~1 mm diameter craniotomy over the right OB where a glass coverslip cranial window was implanted (Cheetham, 2018; Fig. 3A). A head bar was also attached to the skull between the coronal and lambdoid sutures.

In vivo 2 photon calcium imaging and analysis

After acute cranial window implantation and head bar affixation, mice received dexmedetomidine (0.5 mg/kg; 10 µl/g) for sedation while undergoing imaging. Mice were imaged using ThorImage 4.1 software (ThorLabs) and a Bergamo II 2-photon microscope with a resonant scanner and GaAsP detectors (ThorLabs) using a Semi/Apo 20x/1.0NA water-immersion objective (Olympus), and an Insight X3 IR laser (Newport) mode-locked at 935 nm to image GCaMP fluorescence. An imaging field of view was selected through GCaMP6s expression identified via widefield 490 nm LED (ThorLabs) illumination across the region of the dorsal OB surface visible through the cranial window.

Odorant stimulation was performed using a custom-built Arduino-controlled olfactometer. Stimulation and image acquisition timing were controlled and recorded using Python, ThorSync, and ThorImage (ThorLabs). A panel of seven odorants known to stimulate dorsal surface OB glomeruli was used. The following odorants were used at a 10% concentration diluted in mineral oil (v/v): ethyl butyrate (EB), isoamyl acetate (IAA), hexanal, ethyl valerate (EV), methyl butyrate (MB), methyl valerate (MV), and 2-phenethylamine (PEA). Saturated vapor from odorant vials, or a blank deodorized air control, were delivered via an oxygen carrier stream (1 L/min flow rate). All stimuli were 1 s in duration and were delivered via a tube positioned ~5 mm from the mouse's nose. All image trials were 20 s in duration (3.5 s baseline, 1 s stimulus, 15.5 s post-stimulus). Three trials were collected for each stimulus, with odorants and blank stimuli delivered in a pseudo-randomized order with a 60 s inter-trial interval.

Images were analyzed in Fiji. Glomeruli were manually outlined in each field of view, and these outlines were used for analysis of all stimulus-evoked responses. Fluorescence intensity over time was calculated for each glomerulus for each trial. Mean values from the three trials for each stimulus were then calculated for each glomerulus. Additionally, the mean value across all glomeruli was calculated for each mouse. The fluorescence intensity change (ΔF) was calculated as the difference between a 1s baseline prior to stimulus onset and peak fluorescence within 3s after stimulus onset. A glomerulus was defined as responding to a stimulus if the ΔF amplitude was greater than three standard deviations of the baseline period. $\Delta F/F$ (%) was reported as $(\text{baseline} - \text{peak})/\text{baseline} \times 100$ for significant responses only. For blank subtraction, the $\Delta F/F$ in response to the blank (deodorized air) stimulus for a glomerulus was subtracted from the $\Delta F/F$ in response to each odorant.

Lifetime sparseness (S_L) was calculated to measure the degree of odor selectivity of each glomerulus before and after CNO treatment. S_L was calculated as $(1 - (\Sigma_{i=1,n}(r_i/n)^2)/(\Sigma_{i=1,n}(r_i^2/n)))/(1 - 1/n)$, where r_i is the $\Delta F/F$ of the glomerular response to odorant i and n is the total number of odorants (Huang et al., 2022). A highly odorant-selective glomerulus had an S_L of 1 and a glomerulus that was equally responsive to all odors in the panel had an S_L of 0.

Statistics

Statistical analyses were conducted in Prism 9 or Prism 10 software (GraphPad). All data were tested for normality (Shapiro-Wilk or Kolmogorov-Smirnov test) and equal variance (Browne-Forsyth test) to determine whether parametric tests could be employed. Co-staining for the HA tag on the hM4Di receptor in immature OSNs (identified via GAP43 expression) in olfactory epithelia of Gγ8-hM4Di and tetO-hM4Di mice was assessed using an unpaired t-test. Two-way ANOVA was used to compare PS6 fluorescence staining intensity between saline- and CNO-treated Gγ8-hM4Di and tetO-hM4Di mice. Two-way ANOVA was used to compare PS6 fluorescence staining intensity between saline- and CNO-

treated Gγ8-hM4Di and tetO-hM4Di mice. For the habituation/dishabituation task, unpaired t-tests were used to compare total investigation time between saline- and CNO-treated Gγ8-hM4Di and tetO-hM4Di mice. For the buried food task, unpaired t-tests were conducted on the time to success. Odor-evoked calcium responses before and after CNO treatment were analyzed using a two-way ANOVA. Finally, a paired t-test was used to compare lifetime sparseness before and after CNO treatment.

Figures

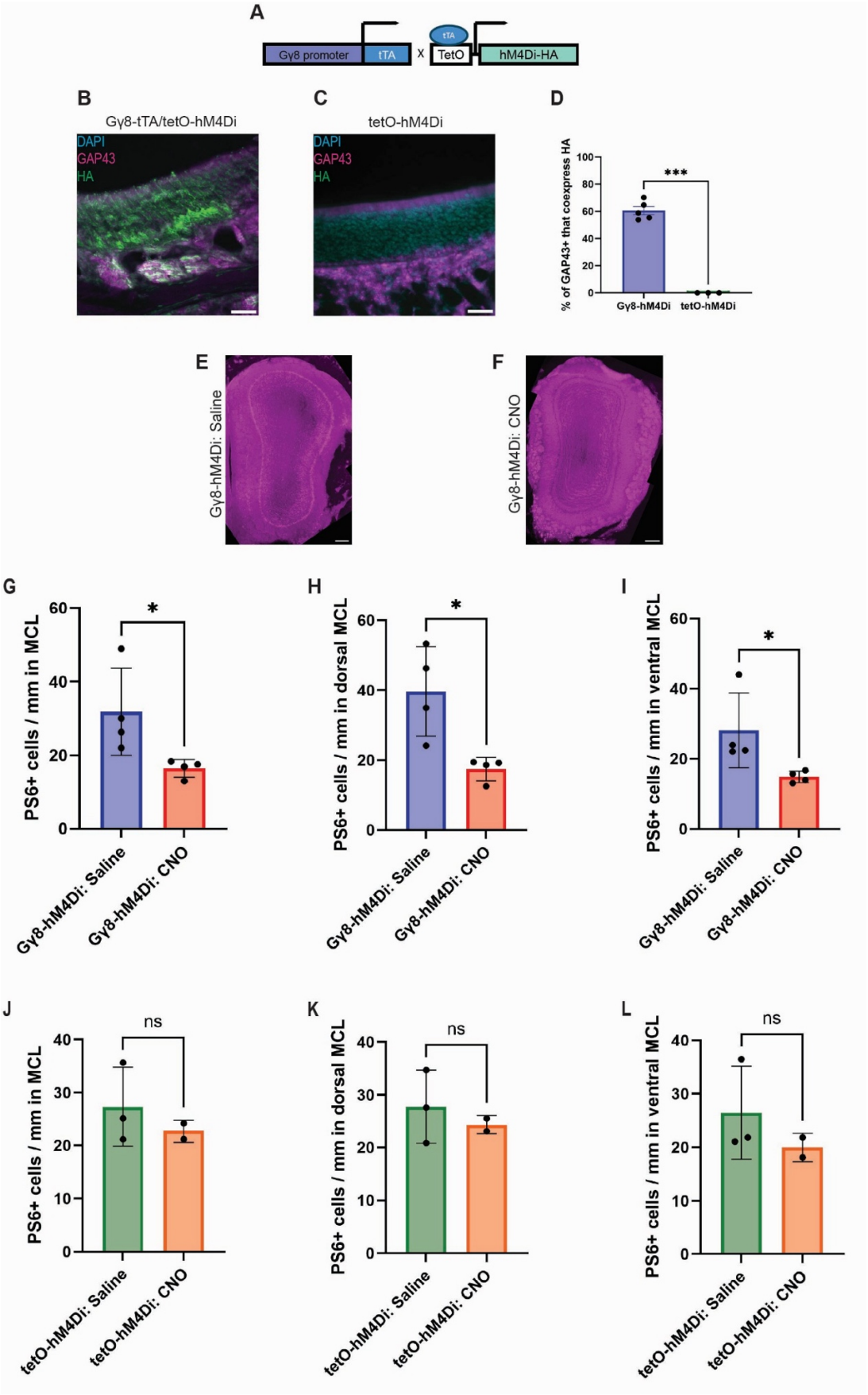
A
Gγ8 promoter
tTA
X
tTA
TetO
hM4Di-HA
B
Gγ8-tTA/tetO-hM4Di
DAPI
GAP43
HA
C
tetO-hM4Di
DAPI
GAP43
HA
D
% of GAP43+ that coexpress HA
***
Gγ8-hM4Di
tetO-hM4Di
E
Gγ8-hM4Di: Saline
F
Gγ8-hM4Di: CNO
G
PS6+ cells / mm in MCL
*
Gγ8-hM4Di: Saline
Gγ8-hM4Di: CNO
H
PS6+ cells / mm in dorsal MCL
*
Gγ8-hM4Di: Saline
Gγ8-hM4Di: CNO
I
PS6+ cells / mm in ventral MCL
*
Gγ8-hM4Di: Saline
Gγ8-hM4Di: CNO
J
PS6+ cells / mm in MCL
ns
tetO-hM4Di: Saline
tetO-hM4Di: CNO
K
PS6+ cells / mm in dorsal MCL
ns
tetO-hM4Di: Saline
tetO-hM4Di: CNO
L
PS6+ cells / mm in ventral MCL
ns
tetO-hM4Di: Saline
tetO-hM4Di: CNO

Figure 1. Validation of transgenic approach to silence immature OSNs. A. Breeding strategy to generate Gγ8-tTA;tetO-hM4Di experimental and tetO-hM4Di control mice. **B,C.** 60x magnification maximum intensity projection confocal images of OE stained for DAPI (blue), HA (green), and GAP43 (magenta) in a Gγ8-hM4Di mouse (**B**) and a tetO-hM4Di mouse (**C**). Scale bar: 100 µm. **D.** Percentage of GAP43-positive OSNs that co-express the hM4Di receptor marked with the HA tag (Gγ8-hM4Di n = 5; tetO-hM4Di n = 3; Welch's t-test: $t(4) = 19.9$, $P < 0.001$). **E,F.** Example images of OBs from a saline-treated Gγ8-hM4Di mouse (**E**) and a CNO-treated Gγ8-hM4Di mouse (**F**) stained for PS6. Scale bars: 100 µm. **E',F'. G.** Linear density of PS6+ cells in the MCL is decreased by silencing immature OSN input (unpaired t-test: $t(6) = 2.54$, $P = 0.044$). **H,I.** Reduced PS6+ MC density when immature OSNs are silenced during odor exposure in dorsal (**H**) (unpaired t-test: $t(6) = 3.35$, $P = 0.015$) and ventral (**I**) (unpaired t-test: $t(6) = 2.46$, $P = 0.049$) MCL. **J.** Linear density of PS6+ cells in the MCL is not affected by CNO administration in tetO-hM4Di control mice (unpaired t-test: $t(3) = 0.81$, $P = 0.48$). **K, L.** No effect of CNO administration during odor exposure on linear density of PS6+ MCs in dorsal (**K**) (unpaired t-test: $t(3) = 0.65$, $P = 0.57$) or ventral (**L**) (unpaired t-test: $t(3) = 0.98$, $P = 0.40$) MCL in tetO-hM4Di control mice. Bars: mean and standard deviation; symbols: values for individual mice.

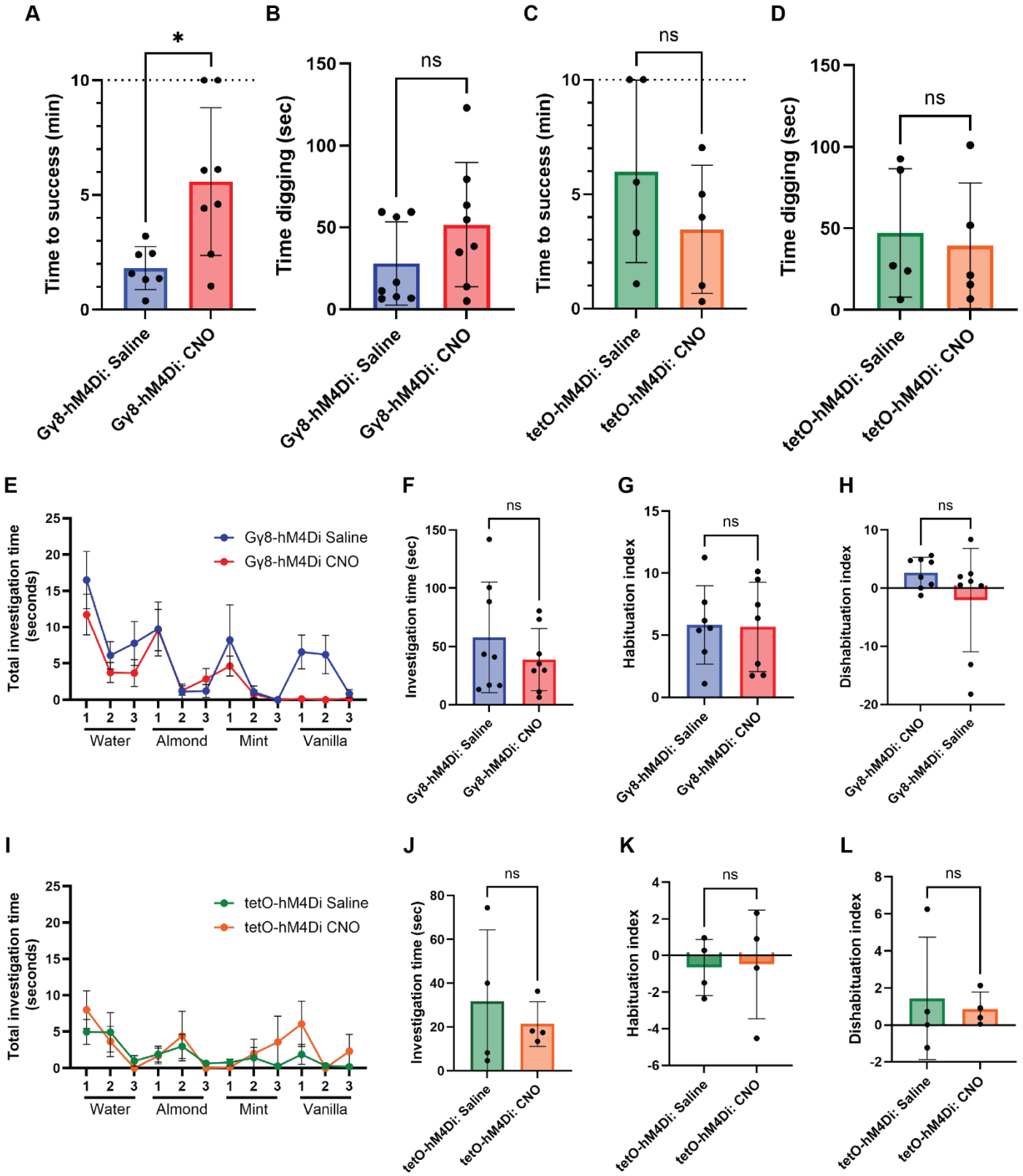

Figure 2. Immature OSNs contribute to odor detection, but not odor discrimination, abilities in the intact olfactory system. A. Silencing immature OSN input via CNO injection increases time to success in buried food task (unpaired t-test: $t(13) = 2.98$, $P = 0.011$, saline-treated Gγ8-hM4Di mice n

= 7, CNO-treated Gγ8-hM4Di mice n = 8). Bars: mean with standard deviation. Symbols: individual mice. **B.** Time spent digging in the buried food task did not differ between saline- and CNO-treated Gγ8-hM4Di mice (unpaired t-test: t(14) = 1.47, P = 0.17). Bars: mean with standard deviation. Symbols: individual mice. **C.** CNO administration into tetO-hM4Di control mice does not affect odor detection abilities (unpaired t-test: $t(8) = 1.16$, $P = 0.28$, saline-treated tetO-hM4Di mice n = 5, CNO-treated tetO-hM4Di mice $n = 5$). Bars: mean with standard deviation. Symbols: individual mice. **D.** Time spent digging in the buried food task did not differ between saline- and CNO-treated tetO-hM4Di mice (unpaired t-test: t(8) = 0.32, P = 0.76). Bars: mean with standard deviation. Symbols: individual mice. **E** Investigation time in an odor habituation-dishabituation assay for saline- and CNO-treated Gγ8-hM4Di (n = 8 per group). Symbols: mean, bars: standard deviation. **F** Total investigation time during odor discrimination assay is not affected by CNO administration in Gγ8-hM4Di mice (unpaired t-test: $t(14) = 1.00$, $P = 0.34$). Bars: mean with standard deviation. Symbols: individual mice. **G** Mean habituation index across odors is unaffected by CNO administration in Gγ8-hM4Di mice (unpaired t-test: $t(12) = 0.088$, $P = 0.93$). Bars: mean with standard deviation. Symbols: individual mice. **H.** Mean dishabituation index is unaffected by CNO administration in Gγ8-hM4Di mice (unpaired t-test: $t(14) = 1.43$, $P = 0.18$). Bars: mean with standard deviation. Symbols: individual mice. **I.** Investigation time in an odor habituation-dishabituation assay for tetO-hM4Di mice (n = 4 per group). **J.** Total investigation time during odor discrimination assay is not affected by CNO administration in tetO-hM4Di control (unpaired t-test: $t(6) = 0.62$, $P = 0.56$). Bars: mean with standard deviation. Symbols: individual mice. **K.** Mean habituation index across odors is unaffected by CNO administration in tetO-hM4Di mice (unpaired t-test: $t(7) = 0.098$, $P = 0.93$). Bars: mean with standard deviation. Symbols: individual mice. **L.** Mean dishabituation index is unaffected by CNO administration in tetO-hM4Di mice (unpaired t-test: $t(7) = 0.33$, $P = 0.76$). Bars: mean with standard deviation. Symbols: individual mice.

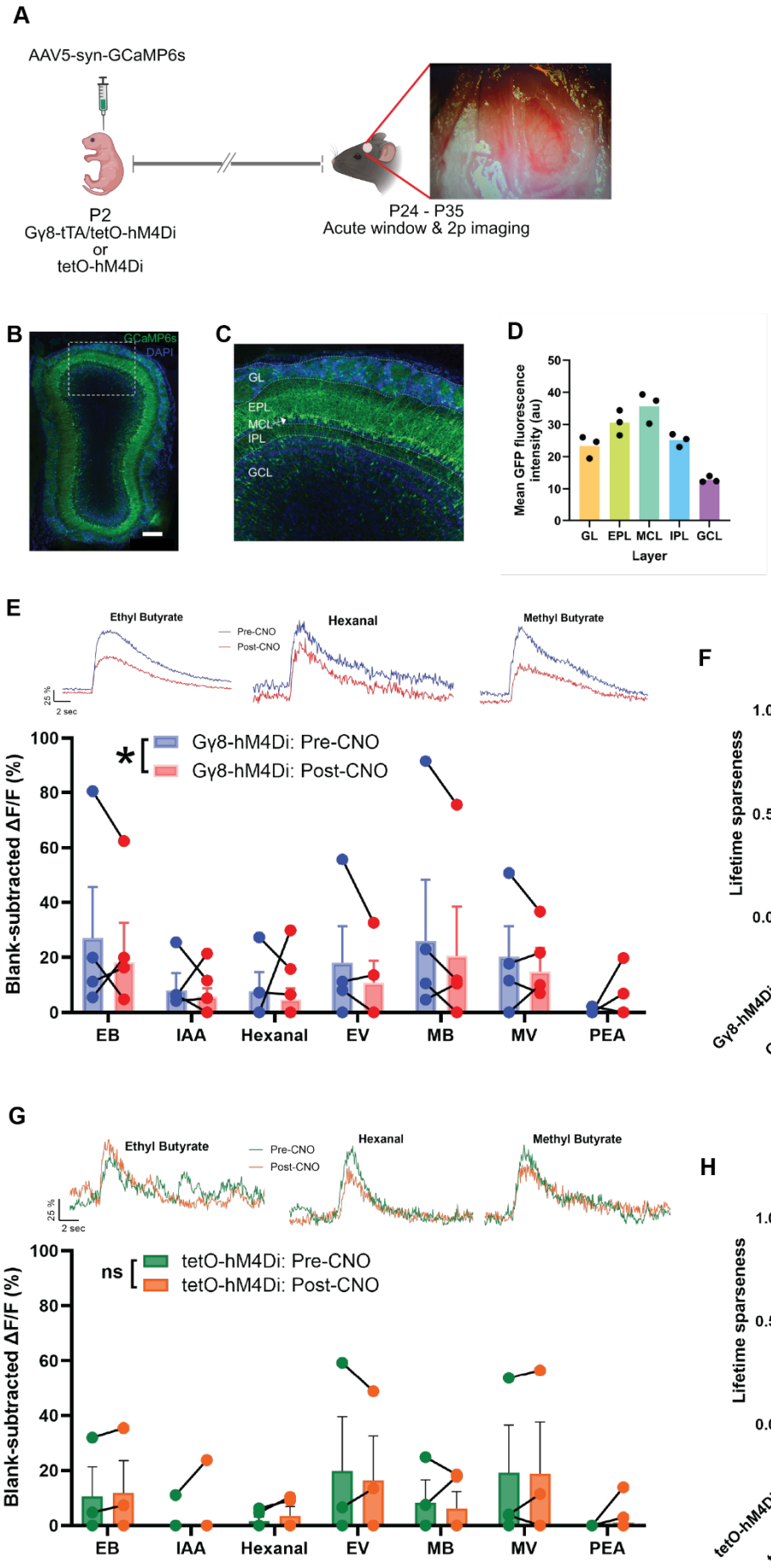
A
AAV5-syn-GCaMP6s
P2
Gγ8-tTA/tetO-hM4Di
or
tetO-hM4Di
P24 - P35
Acute window & 2p imaging
B
GCaMP6s
DAPI
C
GL
EPL
MCL
IPL
GCL
D
Mean GFP fluorescence intensity (au)
GL EPL MCL IPL GCL
Layer
E
Ethyl Butyrate
Hexanal
Methyl Butyrate
Pre-CNO
Post-CNO
25 %
2 sec
Gγ8-hM4Di: Pre-CNO
Gγ8-hM4Di: Post-CNO
Blank-subtracted ΔF/F (%)
EB IAA Hexanal EV MB MV PEA
F
ns
Lifetime sparseness
Gγ8-hM4Di: Pre-CNO
Gγ8-hM4Di: Post-CNO
G
Ethyl Butyrate
Hexanal
Methyl Butyrate
Pre-CNO
Post-CNO
25 %
2 sec
ns
tetO-hM4Di: Pre-CNO
tetO-hM4Di: Post-CNO
Blank-subtracted ΔF/F (%)
EB IAA Hexanal EV MB MV PEA
H
ns
Lifetime sparseness
tetO-hM4Di: Pre-CNO
tetO-hM4Di: Post-CNO

Figure 3. Acute silencing of immature OSN input dampens odor-evoked responses of postsynaptic cells across the OB. A. Neonatal injection strategy to introduce GCaMP6s into the OB for acute imaging. Gγ8-hM4Di (n = 4) and tetO-hM4Di (n = 4) mice received a 1 μL injection of AAV5-syn-GCaMP6s into the right OB at postnatal day 2 (P2). **B.** Neonatal injection of AAV5-syn-GCaMP6s results in GCaMP6s expression that is stronger in the MCL and EPL. Stitched 10x magnification image of coronal OB showing GCaMP expression throughout the entire OB. Scale bar = 250 μm. **C.** Inset of dorsal OB showing GCaMP6s expression is stronger in the MCL and EPL. IPL = internal plexiform layer, GCL = granule cell layer. **D.** Mean GFP fluorescence intensity of GCaMP6s expression throughout the OB following anti-GFP staining. **E.** Acute silencing of immature OSNs via CNO injection decreases mean odor-evoked response amplitude in Gγ8-hM4Di mice (2-way ANOVA. Effect of CNO administration: $P = 0.014$, $F_{1,21} = 7.27$. Effect of odors: $P = 0.74$, $F_{1,21} = 0.58$. Interaction between odors and CNO administration: $P = 0.78$, $F_{1,21} = 0.53$. $n = 4$ mice). Odors used in panel: ethyl butyrate [EB], isoamyl acetate [IAA], hexanal [HEX], ethyl valerate [EV], methyl butyrate [MB], methyl valerate [MV], and phenethylamine [PEA]. Traces above graphs (from left to right) are example traces of EB-, HEX-, and MB-evoked responses before and after CNO administration. Bars: mean with standard deviation. Symbols: individual mice. **F.** Lifetime sparseness of glomerular odorant responses is not affected by CNO administration in Gγ8-hM4Di mice (paired t-test. $P = 0.44$, $t(13) = 0.80$, n = 16 responding glomeruli from 4 mice). Symbols: values for individual glomeruli. **G.** CNO administration does not affect odor-evoked response amplitude in tetO-hM4Di control mice (2-way ANOVA. Effect of CNO: $P = 0.096$, $F_{1,21} = 3.04$. $n = 4$ mice. Effect of odors: $P = 0.80$, $F_{6,21} = 0.50$. Interaction between odors and CNO administration: $P = 0.91$, $F_{6,21} = 0.34$. $n = 4$ mice). Odors, traces and symbols as in E. **H.** CNO does not affect lifetime sparseness of odor-evoked responses in tetO-hM4Di mice (paired t-test. $P = 0.34$, $t(10) = 1.00$. $n = 12$ responding glomeruli from 4 mice). Symbols: values for individual glomeruli.

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