# Self-grooming promotes social interaction in mice via chemosensory communication

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## AUTHOR CONTRIBUTIONS

Conceptualization, Y.-F.Z., D.W.W., and M.M.; Methodology, Y.-F.Z., E.J., J.P.B., and M.M.; Investigation, Y.-F.Z., E.J., and J.P.B.; Formal Analysis, Data Curation, and Visualization, all authors; Writing-Original Draft, Y.-F.Z., D.W.W., and M.M; Writing-Review & Editing, all authors; Supervision and Funding Acquisition, D.W.W. and M.M.

## ACKNOWLEDGEMETNS

We thank Drs. Steven Simmons and Amelia Eisch for their help in setting up the sound recording system. This work was supported by the National Institutes of Health R01NS117061, R01DA049545, and R01DA049449 to M.M. and D.W.W., R01DC006213 to M.M., R21DC019193 to J.P.B., and F31MH124372 to E.J.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

**Abstract**

Self-grooming is a stereotyped behavior displayed by nearly all animals. Among other established functions, self-grooming is implicated in social communication in some animals. However, whether self-grooming specifically influences behaviors of nearby individuals has not been directly tested, partly due to the technical challenge of inducing self-grooming in a reliable and temporally controllable manner. We recently found that optogenetic activation of dopamine D3 receptor expressing neurons in the ventral striatal islands of Calleja robustly induces orofacial grooming in mice. Using this optogenetic manipulation, here we demonstrate that observer mice display social preference for grooming over non-grooming mice regardless of biological sex. Moreover, grooming-induced social attraction depends on volatile chemosensory cues broadcasted from grooming mice. Collectively, our study establishes self-grooming as a means of promoting social interaction among mice via volatile cues, suggesting an additional benefit for animals to allocate a significant amount of time to this behavior.

## Introduction

Self-directed grooming is essential for hygiene maintenance, thermoregulation, de-arousal, and stress reduction, and not surprisingly, animals allocate significant time to this behavior [1, 2]. Although self-grooming is often conceptualized as a solitary or asocial behavior, it is implicated in social communication among conspecifics [3-7]. For instance, ground squirrels engage in self-grooming to deescalate agonistic encounters during territorial disputes [7], and female meadow voles appear to be more attracted to males who groom [3, 5]. These lines of evidence suggest that grooming broadcasts sensory cues that can influence behaviors among other nearby animals. However, direct evidence supporting this notion is still lacking, partly due to unpredictability of spontaneous grooming in experimental animals.

Recent advances in the understanding of neurobiological control of self-grooming make it possible to induce this behavior in laboratory mice via optogenetic manipulations of neuronal activity of specific cell types [8-10]. Notably, the islands of Calleja (IC), clusters of densely-packed granule cells situated mostly in the olfactory tubercle (OT; also called tubular striatum [11]), contribute to a ventral striatal circuit that is involved in grooming control. The IC granule cells are characterized by expression of the dopamine D3 receptor, and optogenetic activation of these neurons reliably induces orofacial grooming (i.e., Phase I to III nose-face-head grooming without Phase IV body licking [12, 13]) in a temporally controllable manner [10].

Using this optogenetic manipulation, we can induce orofacial grooming in mice with temporal precision and directly address previously unanswered questions: 1) do self-grooming mice attract conspecifics more than non-grooming counterparts, 2) whether such attraction shows sexual dimorphism, and 3) what sensory channel conveys the grooming signal from sender to receiver. Our results demonstrate that grooming mice attract conspecifics more than non-grooming counterparts regardless of biological sex, and that such attraction is mediated by orofacial secretions from grooming mice and requires functional main olfactory epithelia of recipient mice. Overall, this study establishes self-grooming as a means of promoting social interaction via chemosensory communication.

## Results

### Induction of self-grooming in mice in a temporally controllable manner

To investigate the potential role of self-grooming in social behaviors, we took advantage of an experimental approach which induces grooming with both reliability and temporal precision [10]. The IC in the ventral striatum (mainly in the OT) are clusters of densely-packed, granule cells expressing the dopamine D3 receptor (**Fig. 1A, B, and Supplemental Video 1**). Optogenetic activation of these D3 neurons via an optical fiber implanted in the OT of double transgenic D3-Cre/ChR2-EYFP mice robustly induces orofacial grooming (**Fig. 1B, C, and Supplemental Video 2; [10]**). Since most OT/IC D3-Cre/ChR2-EYFP neurons fired at a maximum rate of 20 Hz upon current injection and faithfully followed 20 Hz blue light stimulation in brain slices (**Fig. 1B; [10]**), we used 20 Hz stimulation for all behavioral experiments. A three-chamber apparatus was used to assess social preference (**Fig. 1D)**. Two D3-Cre/ChR2-EYFP mice (same-sex littermates) with an optical fiber implanted in the OT were placed in each of the two side chambers under a cup (M1 and M2 in **Fig. 1D**). These two mice served as the grooming and non-grooming counterparts, stimulated by blue and green light, respectively, while each observer mouse ($M_{ob}$; unoperated) was placed in the center chamber and the time it spent in each of the side chambers was recorded during 10 min (**Fig. 1D)**. To prevent exhaustion of firing and/or neurotransmitter release of D3-Cre/ChR2-EYFP neurons, the light alternated between ON (10 s) and OFF (50 s) during the 10 min test. Quantification of grooming time during light stimulation revealed reliable grooming elicited by blue light, but not by green light during the entire session **(Fig. 1E** and **Supplemental Video 3)**.

### Self-grooming mice attract nearby individuals regardless of biological sex

To examine the potential role of self-grooming in social settings, we performed the initial experiments with the grooming and non-grooming mice under customized cups (see Methods for details), allowing emission of several possibly salient sensory cues (visual, auditory, and olfactory) **(Fig. 2A1)**. The observer mice spent significantly more time investigating the

grooming mouse than the non-grooming mouse, reflected by the total duration of stay (**Fig. 2A2**), total investigation time, and number of investigation bouts (**Supplemental Fig. S1A**). Such difference was not observed under the baseline condition when no light stimulation was applied to induce grooming (**Fig. 2A2 and Supplemental Fig. S1A**). To test whether grooming mice attract conspecifics via visual communication, we covered the cups by paper towels with numerous tiny holes (**Supplemental Video 3**). Observer mice showed similar attraction to grooming mice even in absence of visual cues from the grooming/non-grooming mice (**Fig. 2B1-B4, C1-C3 and Supplemental Fig. S1B-E**), suggesting that visual cues are dispensable for grooming-induced social preference. We also examined whether grooming mice make audible calls and/or ultrasonic vocalizations by recording sounds up to 96 kHz. Grooming mice did not produce robust vocalizations, even though we were able to record spontaneous audible calls and sounds associated with grooming (**Supplemental Fig. S2 and Audio1, 2**). The sounds associated with some grooming strokes appeared to result from physical contacts of the optical fiber tether and the recording chamber, rather than grooming *per se*. We did not further investigate whether these sounds could attract other mice, given the necessity and sufficiency of chemosensory communication in grooming-induced social attraction as we uncovered by the following experiments.

Across the animal kingdom, many displays of communication are presented uniquely by each sex. Therefore, we next investigated whether grooming-induced social attraction is sexually dimorphic. With female grooming/non-grooming counterparts, the observer mice (both male and female) spent more time investigating the grooming mouse (**Fig. 2B1-B3 and Supplemental Fig. S1B, C**). Comparable findings were observed with male grooming/non-grooming pairs (**Fig. 2C1-C3 and Supplemental Fig. S1D, E**). These data indicate that self-grooming by one mouse attracts other mice regardless of their biological sex.

Additional control experiments were conducted to ensure that the observed social preference was indeed attributed to orofacial grooming. First, the observer mice did not show side preference under the baseline condition (no light stimulation to induce grooming). Second, when light-induced orofacial grooming was prevented by a collar, attraction towards

the grooming mice was eliminated (**Fig. 2B2, B3, C2, C3, and Supplemental Video 4**). Third, when we switched which mouse received the blue and green light, or when the chamber assignment was switched, the observer mice always showed preference towards the grooming mice (**Fig. 2B4**). Fourth, the time that the observer mice spent in the side of grooming mice was independent of the testing order (**Supplemental Fig. S3**), suggesting that self-grooming was effective in attracting conspecifics throughout the entire session. Furthermore, we quantified the self-grooming behavior conducted by the observer mice when they were near the grooming versus non-grooming mice and did not find a significant difference (**Supplemental Fig. S4)**. These findings indicate that grooming in one mouse does not promote self-grooming in other mice but instead that it specifically attracts conspecifics.

**Social preference towards grooming mice depends on chemosensory communication**

Since olfactory cues are pivotal for social communication in mice, we sought to directly test the role of the olfactory system in orofacial grooming-induced social attraction by rendering the observer mice hyposmic. We intraperitoneally injected saline (as control) or methimazole in the observer mice and performed behavioral tests four days later (**Fig. 3A1, B1**). This treatment reduces perception of volatile odors [14], which we hypothesize may mediate social preference for grooming mice through the perforated cups. Methimazole treatment ablated the main olfactory epithelium but left the vomeronasal epithelium intact **(Supplemental Fig. S5)**, and the same dose (75 mg/kg) did not cause a significant change in locomotion in our previous study [15]. As expected, saline-treated observer mice (both male and female) spent more time investigating the grooming than the non-grooming mice (female), while methimazole-treated mice did not show such preference (**Fig. 3A2, A3**). Similar results were obtained with male grooming/non-grooming counterparts (**Fig. 3B2, B3**). Furthermore, compared to the saline controls, methimazole-treated observer mice significantly decreased both the total time and numbers of bouts investigating grooming/non-grooming mice (**Supplemental Fig. S1F**). These findings demonstrate that self-grooming emits volatile cues that are received and responded to by the observer mice.

**Orofacial secretions from grooming mice attract conspecifics**

To provide direct evidence that self-grooming induces social attraction via orofacial chemical cues, we used mineral oil-moistened cotton swabs to sample the orofacial region (mouth, nose, cheek, and area surrounding eyes) of female grooming and non-grooming mice, immediately after light stimulations **(Fig. 4A1)**. The cotton swabs (each in a petri dish) were then placed under the cups to replace the grooming/non-grooming mice in the previous experiments. Both male and female observer mice showed preference for the cotton swab from the grooming mice over the non-grooming mice, in contrast to a comparison condition in which observer mice did not show preference for orofacial secretions of the same mice before they received light stimulations (baseline) **(Fig. 4A2 and A3)**. Similar findings were observed when the cotton swab experiments were conducted using orofacial secretions from male grooming/non-grooming mice **(Fig. 4B1-B3)**. These results indicate that orofacial secretions from grooming mice are broadcasted during grooming and are sufficient to attract conspecifics.

## Discussion

In the present study, using an optogenetic approach to induce orofacial grooming in a reliable and controllable manner, we demonstrate in mice that self-grooming promotes social attraction and that, regardless of biological sex, mice are attracted to grooming mice. This effect is predominantly mediated by orofacial secretions emitted as volatiles during self-grooming and perceived via the recipient's main olfactory system. This work complements observational studies among other types of animals in the field and extends those observations by providing insights into a causal sensory channel involved in intra-specific communication via self-grooming.

Self-grooming allows an animal to emit rich sensory cues which potentially affect the behaviors of nearby recipients. Grooming without doubt is visually observable and thus a likely channel whereby it is perceived by an observer would be visual. In contrast, in the

present experiments, we uncovered in mice that visual and even auditory cues are dispensable for grooming-induced social attraction because the effect persists without visual cues (**Fig. 2**) and robust audible calls are not emitted during grooming (**Supplemental Fig. S2**). Compared to visual and auditory signals, chemosensory cues offer the unique ethological advantage of communication over time and space. In fact, rodents heavily rely on chemosensory cues for their social behaviors (e.g., [16-20]). Through self-grooming, individuals can further the volatility of their bodily scents, potentially attracting conspecifics [3-6]. Rodents use multiple chemosensory organs including the main olfactory epithelium and vomeronasal organ to detect social cues [16-20]. While the vomeronasal organ is involved in communication via pheromones which are largely nonvolatile, the main olfactory epithelium receives and supports the processing of mainly volatile odors [21, 22]. Consistent with this notion, methimazole treatment, which ablates the main olfactory epithelium but not the vomeronasal organ in the recipients (**Supplemental Fig. S5**), abolishes their social preference for grooming mice (**Fig. 3**). Taken together, these findings strongly support that the main olfactory system of the recipient plays a key role in orofacial grooming-induced social attraction.

This finding is not without precedent. It has been proposed that grooming animals may emit volatile cues to communicate their sex, identity, and reproductive status and thereby attract nearby opposite-sex conspecifics [3-6]. However, to what extent self-grooming serves as a sexually dimorphic communication signal remains to be addressed. Male voles spend more time self-grooming when they are exposed to odors of females, and are more responsive to grooming females [3, 5, 6]. However, the frequency and duration of self-grooming of male voles do not predict mating success [23]. Here we demonstrate that grooming mice attract conspecifics regardless of sex (**Fig. 2**), suggesting that, in mice, self-grooming provides broadly-appealing social communicative signals. The discrepancies between these studies may mainly result from two reasons. First, secretions released from orofacial glands (e.g., salivary and Harderian glands) and their effects on the recipients may vary in different species. Even in the same species, the secretions may vary under different contexts, hormonal statuses, and experimental conditions. For example, in Mongolian gerbils, Harderian secretions

released during self-grooming are enhanced when the animals are exposed to cold temperatures [24]. Second, in rodents, a complete grooming bout consists of a syntactic chain that progresses sequentially from nose-face-head (phase I-III) grooming to body licking (phase IV) [12, 13]. Bodily secretions induced by distinct grooming phases probably vary, which may lead to different behavioral effects in the recipients even in the same species. The optogenetic approach used in our study induces orofacial grooming but not body licking [10]. Body licking involves licking of the genital area, which is correlated with sexual behavior in rats [25], probably due to grooming-induced release of sex-specific materials from genital glands.

We found that cotton swabs rubbed against the orofacial parts from grooming mice are sufficient to attract the recipient mice (**Fig. 4**), suggesting that self-grooming may lead to release of compounds from specifically the orofacial glands. Consistent with this finding, grooming-induced social attraction was absent when direct contacts of the forepaws with orofacial parts were prevented by a collar (**Fig. 2**). Orofacial grooming releases and spreads compounds from various glands such as salivary and Harderian [26-28]. These compounds can serve as social communicative signals in rodents, including mouse [29], Mongolian gerbil [30-32], and golden hamster [33]. Our study reveals that orofacial grooming attracts other mice regardless of sex, but it does not rule out the possibility that orofacial grooming also releases sex-specific signals. In fact, the Harderian gland in golden hamster exhibits pronounced sexual dimorphism in histology and products, and males are more attracted to fresh smears of Harderian gland from females than males [34]. In addition, Harderian gland secretions from male Mongolian gerbils contribute to the proceptive behaviors of estrous females [30]. Future studies are warranted to identify the specific types or combinations of orofacial secretions involved in grooming-induced general social attraction versus sexual attraction.

## Methods

### Animals

The transgenic D3-Cre line (STOCK Tg(Drd3-cre)KI198Gsat/Mmucd, RRID:MMRRC_031741-UCD) was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis, an NIH-funded strain repository, and was donated to the MMRRC by Nathaniel Heintz, Ph.D., The Rockefeller University, GENSAT and Charles Gerfen, Ph.D., National Institutes of Health, National Institute of Mental Health. The D3-Cre line was crossed with the Cre-dependent channelrhodopsin 2 (ChR2) line (JAX Stock No: 024109 or Ai32 line: Rosa26-CAG-LSL-ChR2(H134R)-EYFP-WPRE) [35] to generate D3-Cre/ChR2-EYFP mice. Mice were maintained in temperature- and humidity-controlled animal facilities under a 12 h light/dark cycle with food and water available *ad libitum*. Both male and female mice (2-3 months old) were used. Mice were group-housed until the surgery of intra-cranial implantation and singly-housed afterwards. All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

**Stereotaxic surgery and optical fiber implantation**

Mice were anesthetized with isoflurane (~3% in oxygen) and secured in a stereotaxic system (Model 940, David Kopf Instruments). Isoflurane levels were maintained at 1.5-2% during the surgery. Body temperature was monitored and maintained at 37 °C with a heating pad connected to a temperature control system (TC-1000, CWE Inc.). Local anesthetic (bupivacaine, 2 mg/kg, s.c.) was applied before skin incision and hole drilling on the dorsal skull. In order to target the IC in the OT, which is a large structure, two sets of coordinates from bregma were used: anteroposterior (AP) 1.2 (or 1.54) mm; mediolateral (ML) ±1.1 (or 1.15) mm; dorsoventral (DV), -5.5 (or -5.0) mm. The results were combined since we did not observe significant differences. A cannula (CFMC14L10-Fiber Optic Cannula, Ø2.5 mm Ceramic Ferrule, Ø400 µm Core, 0.39 NA; Thorlabs, Newton, NJ), customized to 6 mm length, was placed in the OT at the same coordinates as described above and fixed on the skull with dental cement. D3-Cre/ChR2-EYFP mice were returned to home cage for recovery for one week before behavioral tests. Only D3-Cre/ChR2-EYPF mice showed robust

grooming behavior upon blue light stimulation were used as grooming/non-grooming pairs in behavioral tests. Optical fiber placements near the IC were verified *post-mortem* for all grooming/non-grooming pairs.

### *In vivo* optical stimulation and behavioral assays

Behavioral tests were performed during the light cycle (9:00 am - 12:00 pm). Mice were acclimated to the testing room at least 1 h before the tests. In experiments using collars, mice were habituated with wearing the collar 2 h per day for 3 consecutive days. Before each test, mice were briefly anesthetized via isoflurane and a flexible optic tether was coupled to the implanted fiber stud with a mating sleeve (Thorlabs Inc.).

A pair of grooming/non-grooming mice (same-sex littermates) with optical fiber implanted in the OT were placed in each of the two side chambers in a three-chamber apparatus (30 cm × 15 cm × 20 cm) and covered under plastic cups. The wall of the cups had parallel vertical cuts (~10 mm in width with ~15 mm between two cuts) so that the observer mice could visualize the grooming/non-grooming mice. In experiments without visual cues, the cups were wrapped in paper towels punched with numerous tiny holes (~1 mm in diameter with ~5 mm between two holes) for ventilation. The observer mice (either male or female at the same age) were placed in the middle compartment of the three-chamber apparatus at the beginning of each test. The locations of blue and green light stimulated mice were counterbalanced across different tests to avoid any side bias. Each observer mouse was subjected to two tests with an interval of 24 h, in which the grooming/non-grooming pairs received no light (baseline) or blue/green light stimulation (experimental condition). For light stimulations, one mouse was optically stimulated with blue laser (473 nm) and the other one with green laser (532 nm) using same parameters (10-15 mW; 20 Hz with 10 ms pulses). For each observer mouse, the light stimulation was delivered for 10 mins in a protocol of 10 s ON and 50 s OFF. In experiments where self-grooming was blocked, light-stimulated mice were wearing a collar to prevent their forepaws from touching orofacial parts. In a subset of experiments, 24 h after the initial test, the location as well as the light pairing were switched for the grooming/non-grooming mice to exclude potential biases of the side and mouse. For

methimazole treatment, observer mice were intraperitoneally injected with either saline (as control) or methimazole (75 mg/kg), and behavioral tests were conducted four days post injection. The behavioral tests were video-taped via a webcam (30 frames/sec) and analyzed post hoc using the Any-Maze software by experimenters who were blinded to the experimental conditions.

**Collection of orofacial secretions from grooming and non-grooming mice**

Mice were first subjected to either blue or green light stimulations as aforementioned for 10 min. Secretions were immediately collected from the orofacial region (including the mouth, nose, cheek, and area surrounding the eye) using Q-tip cotton swabs that were moistened by mineral oil. For each mouse, a cotton swab was swiped against the orofacial region a total of 15-20 times. The cotton swabs were then placed in petri dishes (diameter: 6 cm), covered by the two cups in the two side compartments of the three-chamber apparatus. Observer mice were then placed at the center of the middle chamber at the beginning of each test, and their activities were videotaped for 10 min. The placement of the petri dish was counterbalanced between different tests to avoid potential side bias.

**Audio Recording**

Audio recording of blue laser induced self-grooming was performed in a sound-attenuated chamber. Within this chamber, a condenser ultrasound microphone (CM16/CMPA, Avisoft Bioacoustics) was fixed above a clean cage. Mice were habituated to the sound attenuated chamber and underwent 4 laser stimulation trials using parameters described above (20 Hz 10 ms pulses for 10 s) with a 5 min interval between stimulations. Acoustic data were acquired at 192 kHz to capture potential audible and ultrasonic sounds related to self-grooming. Data were acquired, visualized, and quantified using Raven Pro v1.4 software (the Cornell Lab of Ornithology).

***Ex vivo* electrophysiological recordings**

Whole-cell patch-clamp recordings were performed as we described previously [10]. Mice

were deeply anesthetized with ketamine-xylazine (200 and 20 mg/kg body weight, respectively) and decapitated. The brain was dissected out and immediately placed in ice-cold cutting solution containing (in mM) 92 N-Methyl D-glucamine, 2.5 KCl, 1.2 $NaH_2PO_4$, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Sodium L-ascorbate, 2 Thiourea, 3 Sodium Pyruvate, 10 $MgSO_4$, and 0.5 $CaCl_2$; osmolality ~300 mOsm and pH ~7.3, bubbled with 95% $O_2$-5% $CO_2$. Coronal sections (250 µm thick) containing the OT were cut using a Leica VT 1200S vibratome. Brain slices were incubated in oxygenated artificial cerebrospinal fluid (ACSF in mM: 124 NaCl, 3 KCl, 1.3 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 1.25 NaH2$PO_4$, 5.5 glucose, and 4.47 sucrose; osmolality ~305 mOsm and pH ~7.3, bubbled with 95% O2-5% $CO_2$) for ~30 min at 31ºC and at least 30 minutes at room temperature before use. For recordings, slices were transferred to a recording chamber and continuously perfused with oxygenated ACSF. D3-Cre/ChR2-EYFP cells were visualized through a 40X water-immersion objective on an Olympus BX61WI upright microscope equipped with epifluorescence.

Whole-cell patch-clamp recordings were controlled by an EPC-10 amplifier combined with Pulse Software (HEKA Electronik) and analyzed using Igor Pro 6 (Wavematrics). Recording pipettes were made from borosilicate glass with a Flaming-Brown puller (P-97, Sutter Instruments; tip resistance 5-10 MΩ). The pipette solution contained (in mM) 120 K-gluconate, 10 NaCl, 1 $CaCl_2$, 10 EGTA, 10 HEPES, 5 Mg-ATP, 0.5 Na-GTP, and 10 phosphocreatine. Light stimulation was delivered through the same objective via pulses of blue laser (473 nm, FTEC2473-V65YF0, Blue Sky Research, Milpitas, USA) at 20 Hz with 10 ms pulse.

**Immunostaining and confocal imaging**

Mice were transcardially perfused with 4% paraformaldehyde (PFA) in fresh phosphate buffered saline (PBS). For *post-mortem* verification of optical fiber placement, brains were post fixed in 4% PFA overnight at 4 ºC, then transferred into PBS. Coronal slices at 100 µm thick were prepared using a Leica VT 1200S vibratome. The slices were treated with glycerol in PBS (volume ratio 1:1) for 30 min followed by glycerol in PBS (volume ratio 7:3) for 30 min before being mounted onto superfrost slides (Fisher Scientific) for imaging. For

immunostaining of the nasal tissues, the heads were post fixed 4% PFA overnight at 4 ºC, and then decalcified in 0.5 M EDTA (pH 8.0, ethylenediaminetetraacetic acid) for four days and infiltrated in a series of sucrose solutions before being embedded in OCT. The frozen tissues were cut into 20 µm coronal sections on a cryostat. After antigen retrieval in a 95 °C water bath for 10 min, the tissue sections were blocked for 30 min in 0.3% Triton X-100 in PBS with 3% bovine serum albumin, and then incubated at 4 °C overnight in the same solution with the primary rabbit anti-OMP (olfactory marker protein; 1:500, O7889 from Sigma). Immunofluorescence was achieved by reaction with the secondary antibody donkey anti-rabbit-488 (A21206 from Molecular Probes, Invitrogen) at 1:200 for one hour. Tissues were washed in 0.3% Triton X-100 in PBS and mounted in Vectashield (Vector Laboratories). Fluorescent images were taken under a SP5/Leica confocal microscope with LAS AF Lite software.

**Statistical analyses**

Graphs are created in GraphPad Prism and assembled in Adobe Photoshop. Normal distribution of datasets was verified via Shapiro-Wilk tests and parametric tests (Student *t* and ANOVA) were used accordingly.

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## Figure Legends

**Figure 1. Paradigm for monitoring social preference during reliable induction of self-grooming.** A. A coronal section across the ventral striatum showing *in situ* hybridization of Cre mRNA in a D3-Cre mouse. Image credit: Allen Institute (Allen Mouse Brain Connectivity Atlas [36]: http://connectivity.brain-map.org/transgenic/experiment/304166273). PC, piriform cortex. NAc, nucleus accumbens. OT, olfactory tubercle. IC, islands of Calleja. Scale bar = 1 mm. B. D3-Cre/ChR2-EYFP neurons are densely packed in the IC. Left, a representative image (coronal section) showing the IC and the optical fiber tract (upper), as well as firing of an IC D3-Cre/ChR2-EYFP neuron upon laser stimulation at 20 Hz (lower). Scale bar = 200 µm. Right, an enlarged image of the IC (dotted rectangle in left panel). Scale

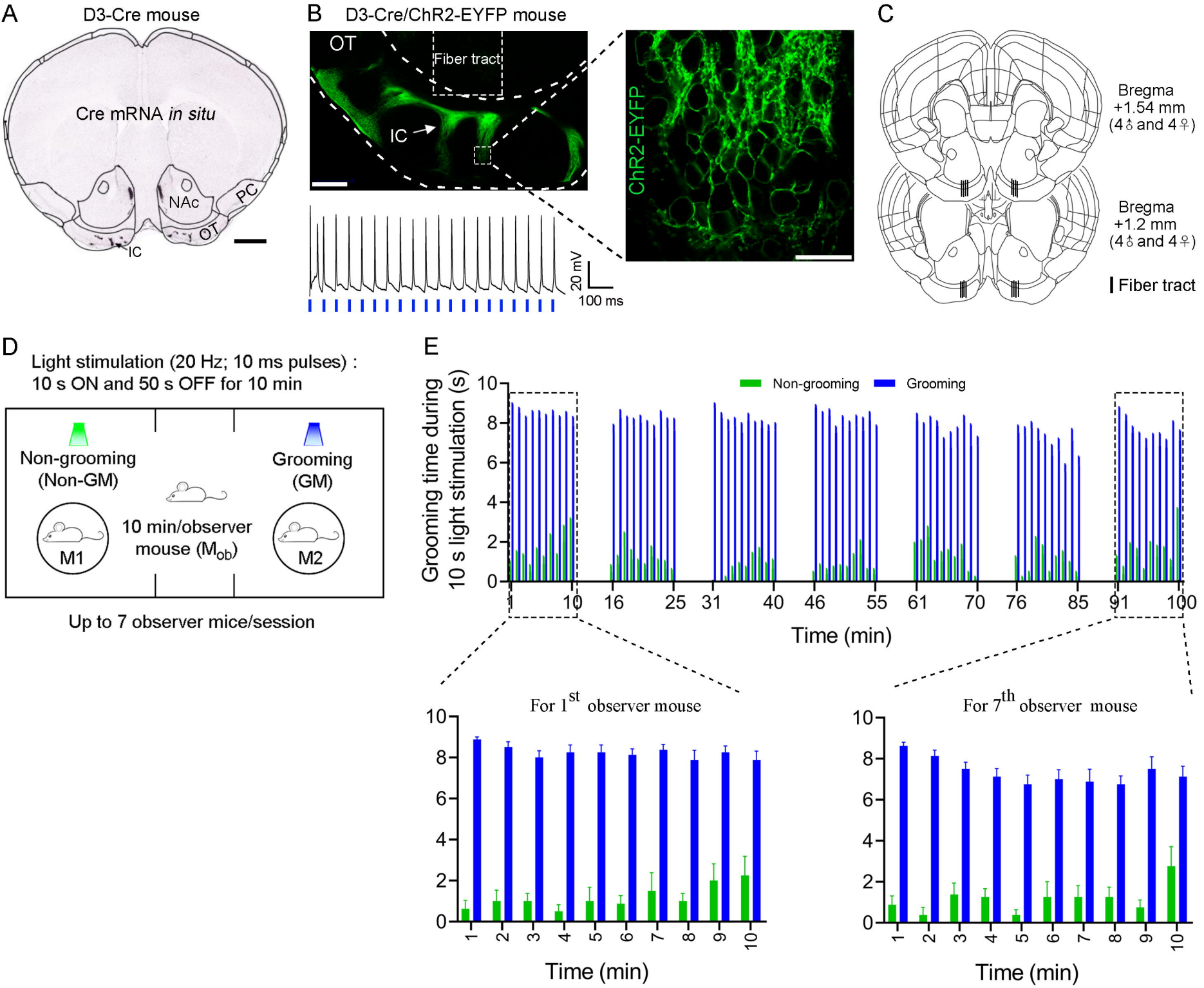
bar = 20 µm. C. Coronal brain panels showing optical fiber placements in D3-Cre/ChR2-EYFP mice. D. Schematic showing the behavioral strategy in the three-chamber apparatus. Blue light stimulation of OT D3-Cre/ChR2-EYFP neurons induced robust self-grooming (grooming, GM) while green light with the same stimulation parameters did not (non-grooming, Non-GM). The time spent by each observer mouse ($M_{ob}$) in either side during a 10 min test was quantified. E. Grooming time (mean ± SEM; n = 16 mice) during blue versus green light stimulation of OT D3-Cre/ChR2 neurons in an entire session (10 min for each observer mouse with a 5 min interval between two consecutive mice). Two-way Repeated Measurement ANOVA: $F_{light\ x\ time}$ (69, 966) = 1.923, p = 1.791E-05; $F_{light}$ (1, 14) = 1554.167, p < 0.01E-12; $F_{time}$ (69, 483) = 1.792, p = 0.071. Insets: robustness of blue light-induced grooming from the 1st to 7th 10 min (for the 1st to 7th observer mouse, respectively).

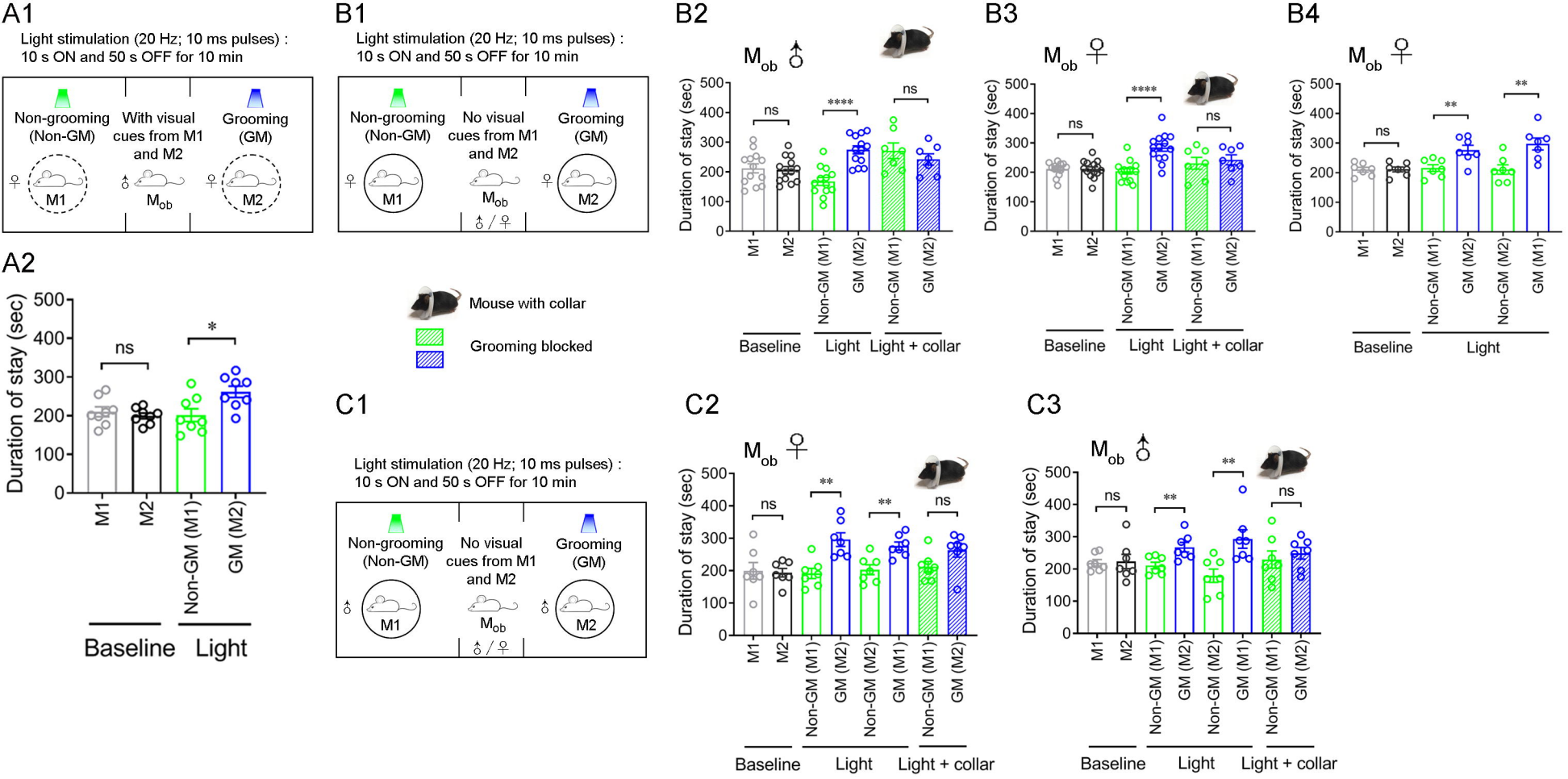
**Figure 2. Observer mice show social preference for grooming than non-grooming mice regardless of biological sex.** A. Observer mice (n = 8) spent more time in the side of a grooming than non-grooming mouse (with visual cues). A1, schematic showing the behavioral strategy. A2, total duration of stay in the grooming and non-grooming side under the baseline (no light stimulation) and light stimulation conditions. Baseline, t = 0.735 and p = 0.486; Light, t = 3.127 and p = 0.017. B. Both male and female observer mice were attracted to female grooming mice (without visual cues). B1, schematic showing the behavioral strategy. B2 and B3, total duration of stay of male (B2) and female (B3) observer mice in the grooming and non-grooming side under the baseline (no light stimulation) and light stimulation (with or without collar) conditions. Male observer mice: Baseline, t = 0.298 and p = 0.771; Light, t = 7.303 and p = 9.444E-06; Light + collar, t = 0.908 and p = 0.381. Female observer mice: Baseline, t =0.014 and p = 0.989; Light, t = 6.519 and p =1.944E-05; Light + collar, t = 0.443 and p = 0.665. B4, observer mice were always more attracted to the grooming mice. Grooming/non-grooming mice were swapped by switching the blue/green light stimulation. Baseline: t = 0.076 and p = 0.942; Light, t = 3.753 and p =0.009 (M2 grooming); t = 4.239 and p = 0.005 (M1 grooming). C. Both male and female observer mice were attracted to male grooming mice. C1, schematic showing the behavioral strategy. C2 and C3, total duration of stay of female (C2) and male (C3) observer mice in the grooming and non-grooming side under the baseline (no light stimulation) and light stimulation (with or without collar) conditions. Female observer mice: Baseline, t = 0.207 and p = 0.843; Light, t = 5.271 and p = 0.002 (M2 grooming); t = 4.754 and p = 0.003 (M1 grooming); Light + collar, t = 1.901 and p = 0.082. Male observer mice: Baseline, t = 0.167 and p = 0.873; Light, t = 3.815 and p = 0.009 (M2 grooming); t = 3.990 and p = 0.007 (M1 grooming); Light + collar, t = 0.603 and p = 0.558. n = 13 and 14 for male and female observer mice, respectively, in (B2, B3 without collar); n = 7 for both female and male observer mice in (B2, B3 with collar, B4, and C). All averaged data are expressed as mean ± SEM; Two-tailed, paired Student's t-tests: * p < 0.05, ** p < 0.01, **** p < 0.0001, and ns, not significant.

**Figure 3. Social preference towards grooming mice depends on volatile odor perception by observer mice.** A. Ablation of main olfactory epithelia of observer mice via methimazole

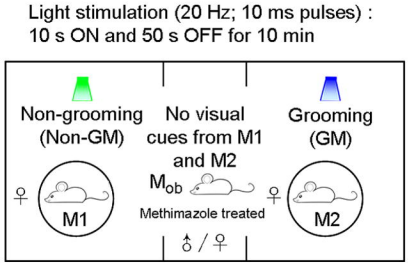
treatment eliminated their social preference for female grooming mice. A1, schematic showing the behavioral strategy. A2 and A3, total duration of stay of saline- or methimazole-treated male (A2) and female (A3) observer mice in the grooming and non-grooming side. Male observer mice: Saline, $t = 2.216$ and $p = 0.047$; Methimazole, $t = 0.117$ and $p = 0.909$; Female observer mice: Saline, $t = 4.563$ and $p = 6.51E-04$; Methimazole, $t = 0.765$ and $p = 0.473$ (M2 grooming); $t = 0.025$ and $p = 0.981$ (M1 grooming); B. Methimazole treatment in observer mice eliminated their social preference for male grooming mice. B1, schematic showing the behavioral strategy. B2 and B3, total duration of stay of saline- or methimazole-treated female (B2) and male (B3) observer mice in the grooming and non-grooming side. Female observer mice: Saline, $t = 3.571$ and $p = 0.012$; methimazole, $t = 0.669$ and $p = 0.529$; Male observer mice: Saline, $t = 3.478$ and $p = 4.56E-03$; methimazole, $t = 0.129$ and $p = 0.900$. $n = 7$ observer mice in each group. All averaged data are expressed as mean ± SEM; Two-tailed, paired Student's t-tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and ns, not significant.

**Figure 4. Observer mice spend more time investigating orofacial secretions from grooming mice.** A. Orofacial secretions from female grooming mice attracted both male and female mice. A1, schematic showing the behavioral strategy. A2 and A3, total duration of stay of male (A2) and female (A3) observer mice in chambers containing cotton swabs with orofacial secretions from female grooming or non-grooming mice. Male observer mice: Baseline (cotton swabs with orofacial secretions from the same mice without light stimulations), $t = 1.356$ and $p = 0.200$; Light, $t = 5.856$ and $p = 7.77E-05$; Female observer mice: Baseline, $t = 0.018$ and $p = 0.986$; Light, $t = 3.658$ and $p = 0.003$. B. Orofacial secretions from male grooming mice attracted both female and male mice. B1, schematic showing the behavioral strategy. B2 and B3, total duration of stay of female (B2) and male (B3) observer mice in chambers containing cotton swabs with orofacial secretions from male grooming or non-grooming mice. Female observer mice: Baseline, $t = 0.583$ and $p = 0.570$; Light, $t = 4.785$ and $p = 4.45E-04$; Male observer mice: Baseline, $t = 0.539$ and $p = 0.599$; Light, $t = 5.056$ and $p = 2.82E-04$. $n = 7$ observer mice in each group. All averaged data are expressed as mean ± SEM; Two-tailed, paired Student's t-tests: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns, not significant.

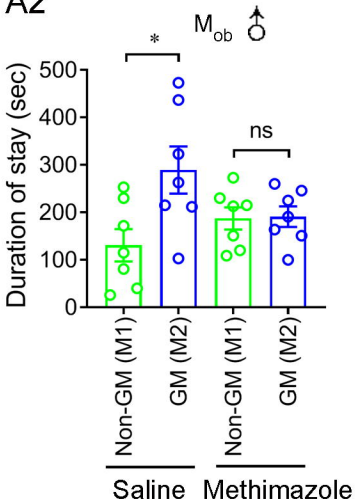

A
D3-Cre mouse
Cre mRNA in situ
NAc
PC
OT
IC
B
D3-Cre/ChR2-EYFP mouse
OT
Fiber tract
IC
ChR2-EYFP
20 mV
100 ms
C
Bregma
+1.54 mm
(4♂ and 4♀)
Bregma
+1.2 mm
(4♂ and 4♀)
Fiber tract
D
Light stimulation (20 Hz; 10 ms pulses) :
10 s ON and 50 s OFF for 10 min
Non-grooming
(Non-GM)
Grooming
(GM)
10 min/observer
mouse (Mob)
M1
M2
Up to 7 observer mice/session
E
Non-grooming
Grooming
Grooming time during
10 s light stimulation (s)
Time (min)
For 1st observer mouse
For 7th observer mouse
Time (min)
Time (min)


A1
Light stimulation (20 Hz; 10 ms pulses) :
10 s ON and 50 s OFF for 10 min
Non-grooming (Non-GM)
With visual cues from M1 and M2
Grooming (GM)
M1
Mob
M2
A2
Duration of stay (sec)
ns
*
M1
M2
Non-GM (M1)
GM (M2)
Baseline
Light
B1
Light stimulation (20 Hz; 10 ms pulses) :
10 s ON and 50 s OFF for 10 min
Non-grooming (Non-GM)
No visual cues from M1 and M2
Grooming (GM)
M1
Mob
M2
Mouse with collar
Grooming blocked
B2
Mob
Duration of stay (sec)
ns
****
ns
Non-GM (M1)
GM (M2)
Baseline
Light
Light + collar
B3
Mob
Duration of stay (sec)
ns
****
ns
Non-GM (M1)
GM (M2)
Baseline
Light
Light + collar
B4
Mob
Duration of stay (sec)
ns
**
**
Non-GM (M1)
GM (M2)
Non-GM (M2)
GM (M1)
Baseline
Light
C1
Light stimulation (20 Hz; 10 ms pulses) :
10 s ON and 50 s OFF for 10 min
Non-grooming (Non-GM)
No visual cues from M1 and M2
Grooming (GM)
M1
Mob
M2
C2
Mob
Duration of stay (sec)
ns
**
**
ns
Non-GM (M1)
GM (M2)
Non-GM (M2)
GM (M1)
Baseline
Light
Light + collar
C3
Mob
Duration of stay (sec)
ns
**
**
ns
Non-GM (M1)
GM (M2)
Non-GM (M2)
GM (M1)
Baseline
Light
Light + collar

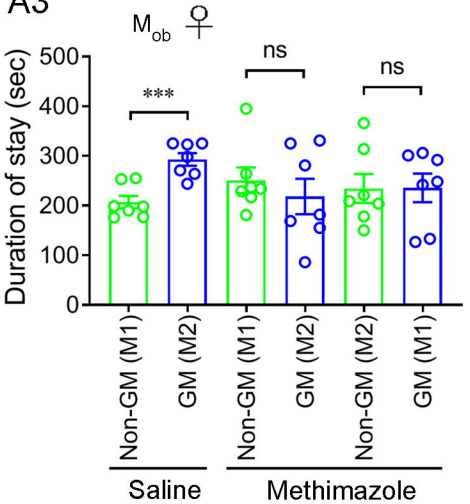
A1



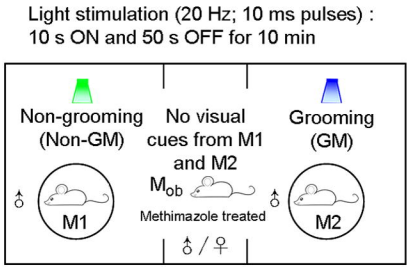

A2



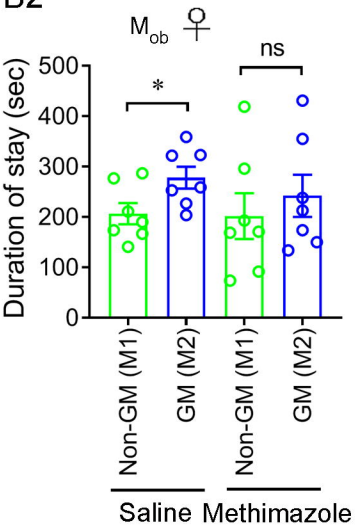

A3



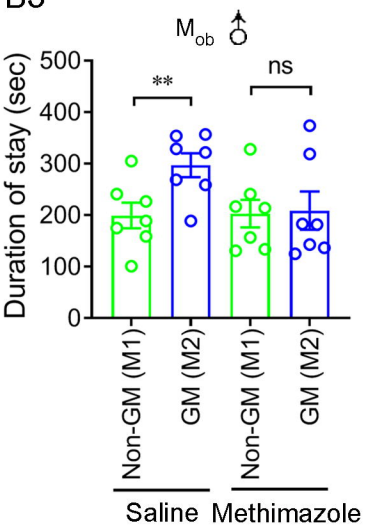

B1

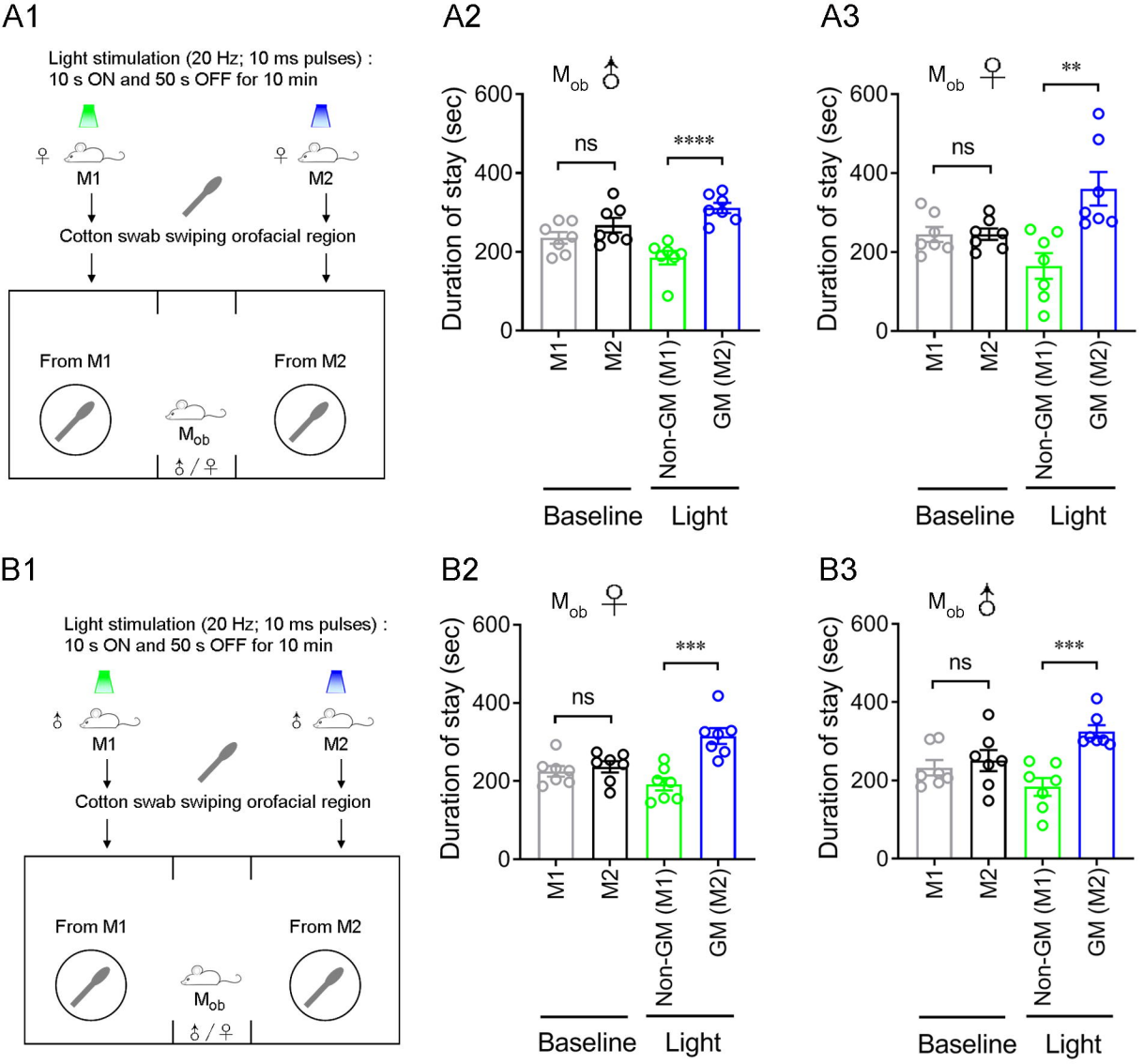



B2

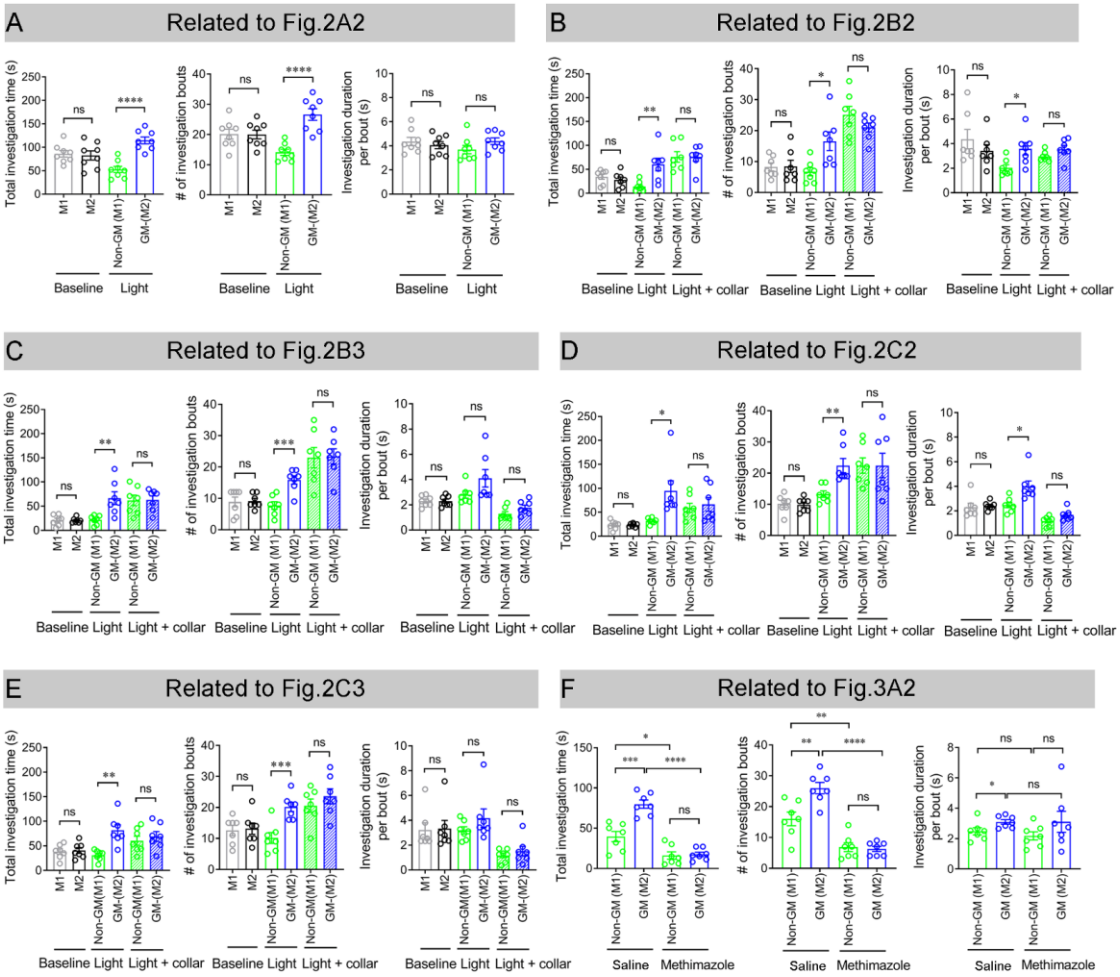



B3



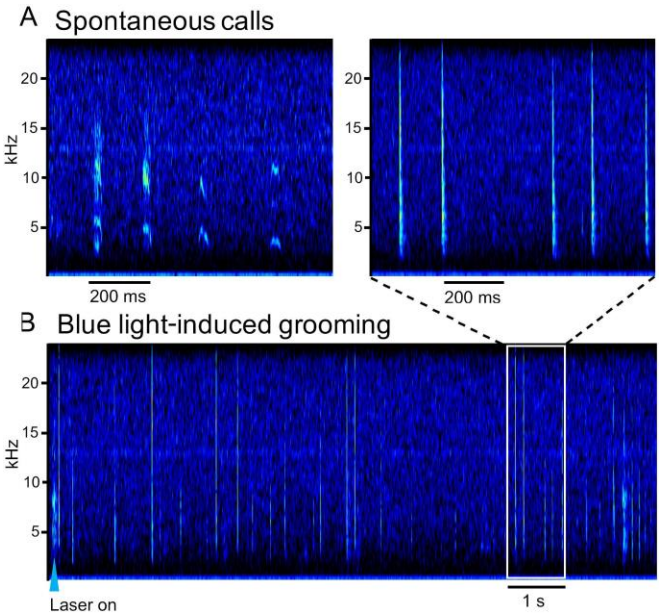

A1
Light stimulation (20 Hz; 10 ms pulses) :
10 s ON and 50 s OFF for 10 min
M1
M2
Cotton swab swiping orofacial region
From M1
From M2
Mob
A2
Mob
Duration of stay (sec)
600
400
200
0
ns
****
M1
M2
Non-GM (M1)
GM (M2)
Baseline
Light
A3
Mob
Duration of stay (sec)
ns
**
M1
M2
Non-GM (M1)
GM (M2)
Baseline
Light
B1
Light stimulation (20 Hz; 10 ms pulses) :
10 s ON and 50 s OFF for 10 min
M1
M2
Cotton swab swiping orofacial region
From M1
From M2
Mob
B2
Mob
Duration of stay (sec)
ns
***
M1
M2
Non-GM (M1)
GM (M2)
Baseline
Light
B3
Mob
Duration of stay (sec)
ns
***
M1
M2
Non-GM (M1)
GM (M2)
Baseline
Light

## Supplemental Figures

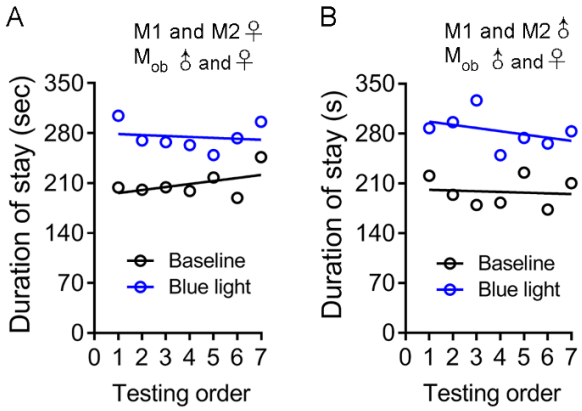



**Supplemental Figure S1. Observer mice spend more time investigating grooming than non-grooming mice.** A. Grooming and non-grooming mice in uncovered cups (with visual cues). Observer mice (n=8). Investigation time: baseline, t = 0.292 and p = 0.774; Light, t = 6.271 and p = 2.053E-05; Investigation bouts: baseline, t = 0.057 and p = 0.955; Light, t = 5.706 and p = 5.428E-05; Investigation duration per bout: baseline, t = 0.678 and p = 0.509; Light, t = 1.590 and p = 0.134. B-E. Grooming and non-grooming mice in covered cups (without visual cues). B. Male observer mice (n = 7) and female grooming/non-grooming mice. Investigation time: baseline, t = 0.822 and p = 0.427; Light, t = 3.347 and p = 0.006; Light + collar: t = 0.010 and p = 0.992; Investigation bouts: baseline, t = 0.061 and p = 0.953; Light, t = 3.021 and p = 0.011; Light + collar: t = 1.362 and p = 0.198; Investigation duration per bout: baseline, t = 1.018 and p = 0.329; Light, t = 2.751 and p = 0.018; Light + collar: t = 1.671 and p = 0.121. C. Female observer mice (n = 7) and female grooming/non-grooming mice. Investigation time: baseline, t = 0.062 and p = 0.952; Light, t = 3.264 and p = 0.007; Light + collar: t = 0.083 and p = 0.935; Investigation bouts: baseline, t = 0.161 and p = 0.875; Light, t = 4.588 and p = 6.235E-04; Light + collar: t = 0.106 and p = 0.917; Investigation duration per bout: baseline, t = 0.022 and p = 0.982; Light, t = 1.740 and p = 0.107; Light + collar: t = 0.185 and p = 0.856. D. Female observer mice (n = 7) and male

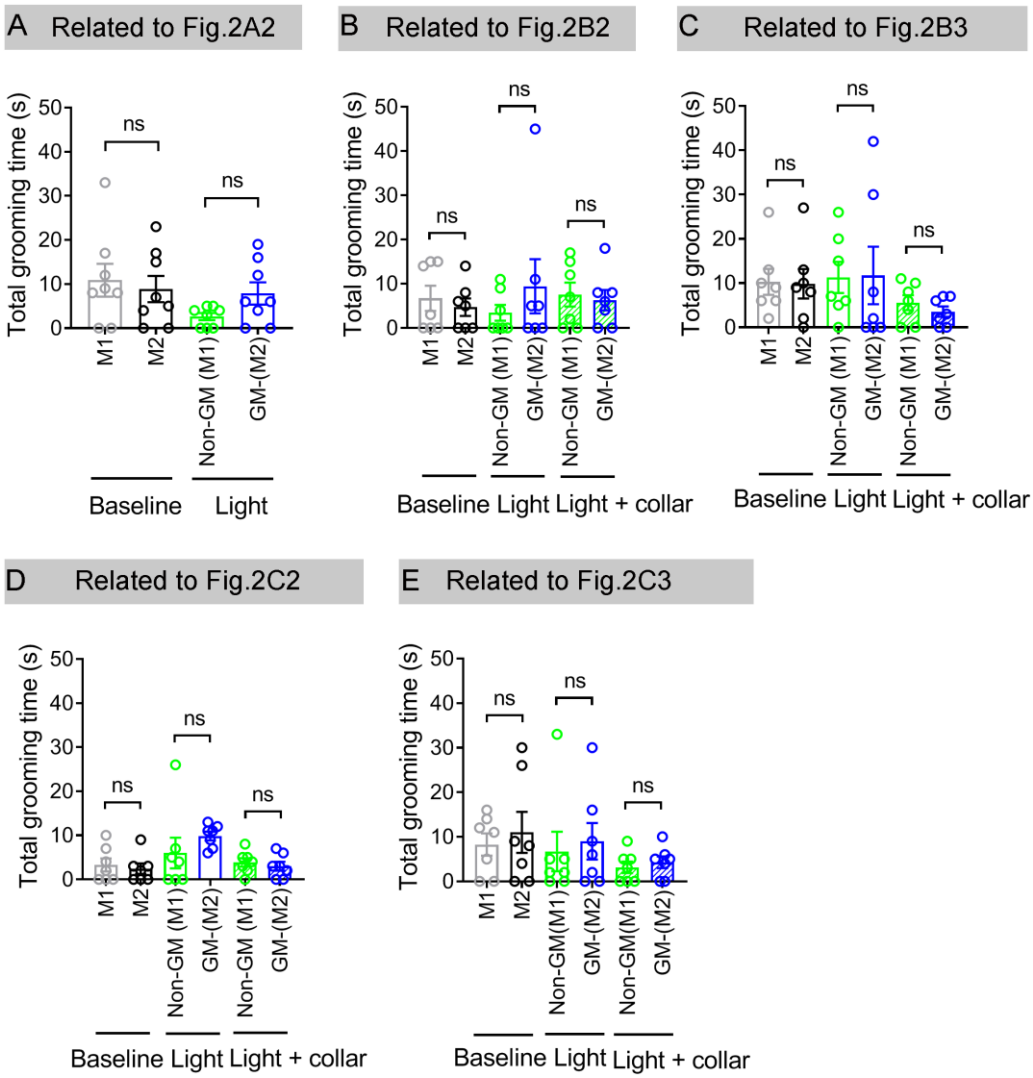
grooming/non-grooming mice. Investigation time: baseline, t = 2.981E-08 and p = 1.000; Light, t = 2.903 and p = 0.013; Light + collar: t = 0.425 and p = 0.678; Investigation bouts: baseline, t = 0.210 and p = 0.837; Light, t = 3.667 and p = 3.227E-03; Light + collar: t = 2.981E-08 and p = 1.000; Investigation duration per bout: baseline, t = 0.336 and p = 0.743; Light, t = 3.043 and p = 0.010; Light + collar: t = 1.085 and p = 0.299. E. Male observer mice (n = 7) and male grooming/non-grooming mice. Investigation time: baseline, t = 0.268 and p = 0.793; Light, t = 4.264 and p = 0.0011; Light + collar: t = 0.636 and p = 0.537; Investigation bouts: baseline, t = 0.232 and p = 0.820; Light, t = 4.372 and p = 9.087E-04; Light + collar: t = 0.923 and p = 0.374; Investigation duration per bout: baseline, t = 0.128 and p = 0.900; Light, t = 1.235 and p = 0.240; Light + collar: t = 0.008 and p = 0.994. F. Saline- or methimazole-treated male observer mice (n = 7) to non-grooming/grooming female mice. Investigation time: saline, t = 4.919 and p = 3.541E-04; Methimazole, t = 0.270 and p = 0.792; Investigation bouts: saline, t = 3.474 and p = 3.597E-03; Methimazole, t = 0.343 and p = 0.737; Investigation duration per bout: saline, t = 2.255 and p = 0.043; Methimazole, t = 1.247 and p = 0.236. All averaged data are expressed as mean ± SEM. Two-tailed, paired Student's t-tests: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, and ns, not significant.



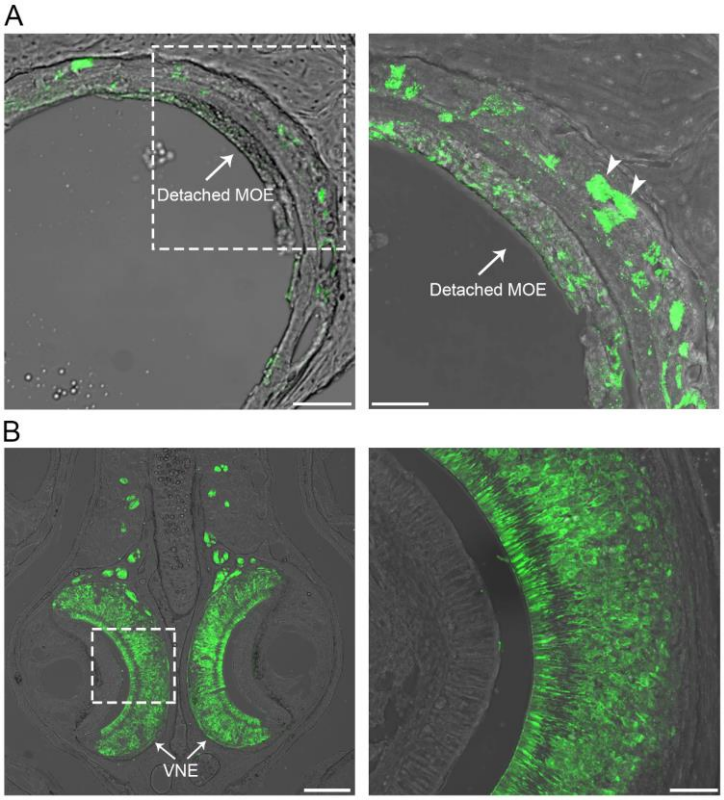

**Supplemental Figure S2. Mice do not emit discernable vocalizations during self-grooming.** Sonograms of spontaneous audible calls (A) and sounds associated with grooming strokes (B). Inset, an enlarged view of the rectangle area in (B). Whereas spontaneous calls, as expected, were restricted within frequency bands, self-grooming was associated with broad-band acoustic events which corresponded in time to movement of the fiber optic tether. Similar results were obtained from six mice recorded.




**Supplemental Figure S3. The testing order of observer mice does not impact the time spent in the side of grooming mice.** The duration of stay in the side of grooming mice is plotted against the test sequence of observer mice. A, female D3-Cre/ChR2-EYFP mice with blue light stimulation in the OT or without (baseline). B, male D3-Cre/ChR2-EYFP mice with blue light stimulation in the OT or without (baseline). Each data point is an average of four observer mice (two male and two female). Pearson's linear regression analysis was performed to determine potential correlation between the duration of stay and the test sequence of observer mice. In A, r = 0.492 and -0.151, p = 0.262 and 0.747 for baseline and blue light, respectively. In B, r = -0.103 and -0.400, p = 0.826 and 0.374 for baseline and blue light, respectively.




**Supplemental Figure S4. Observer mice show similar durations of self-grooming near grooming or non-grooming mice.** A. Grooming and non-grooming mice in uncovered cups (with visual cues). Observer mice (n = 8). Baseline, t = 0.417 and p = 0.683; Light, t = 1.993 and p = 0.066. B-E. Grooming and non-grooming mice in covered cups (without visual cues). B. Male observer mice (n = 7) and female grooming/non-grooming mice. Baseline, t = 0.577 and p = 0.575; Light, t = 0.940 and p = 0.366; Light + collar: t = 0.360 and p = 0.725. C. Female observer mice (n = 7) and female grooming/non-grooming mice. Baseline, t = 0.097 and p = 0.925; Light, t = 0.058 and p = 0.955; Light + collar: t = 0.973 and p = 0.350. D. Female observer mice (n = 7) and male grooming/non-grooming mice. Baseline, t = 0.446 and p = 0.664; Light, t = 1.061 and p = 0.309; Light + collar: t = 0.610 and p = 0.553; E. Male observer mice (n = 7) and male grooming/non-grooming mice. Baseline, t = 0.518 and p = 0.614; Light, t = 0.377 and p = 0.713; Light + collar: t = 0.617 and p = 0.549. All averaged data are expressed as mean ± SEM. Two-tailed, paired Student's t-tests. ns, not significant.




**Supplemental Figure S5. Methimazole treatment ablates the main olfactory epithelium but leaves the vomeronasal epithelium intact.** Four days post methimazole treatment, coronal sections of the nose were processed for immunoreactivity to the olfactory marker protein (OMP), which labels mature olfactory sensory neurons. A. Left, a representative image showing the detached main olfactory epithelium (MOE) without obvious OMP+ olfactory sensory neurons (OSNs). Right, an enlarge view of the dotted rectangle area in the left panel. Arrowheads denote OSN axon bundles. Scale bars = 100 (left) and 50 µm (right). B. Left, a representative image (coronal section) showing the intact vomeronasal epithelium with abundant OMP+ sensory neurons. Right, an enlarge view of the dotted rectangle area in the left panel. Scale bars = 200 (left) and 50 µm (right). MOE, main olfactory epithelium; VNE, vomeronasal epithelium. Similar results were obtained from 3 mice.

## Supplemental Videos and Audios

**Video 1.** Z-stack confocal images showing densely packed D3-Cre/ChR2-EYFP neurons in an island of Calleja in the OT.

**Video 2.** Blue light (but not green light) stimulation of OT D3-Cre/ChR2-EYFP neurons induces orofacial grooming.

**Video 3.** An observer mouse spends more time investigating a grooming than a non-grooming mouse in a three-chamber apparatus.

**Video 4.** Orofacial grooming induced by blue light stimulation of OT D3-Cre/ChR2-EYFP neurons is prevented by a collar.

**Audio 1.** Spontaneous audible calls recorded from a D3-Cre/ChR2-EYFP mouse.

**Audio 2.** Sounds recorded during blue light stimulation-induced grooming in a D3-Cre/ChR2-EYFP mouse. These sounds likely result from physical contacts of the tethered wire and the recording chamber, rather than grooming *per se*.