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Single or Repeated Ablation of Mouse Olfactory Epithelium by Methimazole

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[Abstract] Odor-detecting olfactory sensory neurons residing in the nasal olfactory epithelium (OE) are the only neurons in direct contact with the external environment. As a result, these neurons are subjected to chemical, physical, and infectious insults, which may be the underlying reason why neurogenesis occurs in the OE of adult mammals. This feature makes the OE a useful model for studying neurogenesis and neuronal differentiation, with the possibility for systemic as well as local administration of various compounds and infectious agents that may interfere with these cellular processes. Several different chemical compounds have been shown to cause toxic injury to the OE, which can be used for OE ablation. We, and others, have found that the systemic administration of the hyperthyroid drug, methimazole, reliably causes olfactotoxicity as a side effect. Here, we outline an OE lesioning protocol for single or repeated ablation by methimazole. A single methimazole administration can be used to study neuroepithelial regeneration and stem cell activation, while repeated ablation and regeneration of OE enable the study of tissue stem cell exhaustion and generation of tissue metaplasia.

Keywords: Olfactory epithelium, Methimazole, Lesion, Regeneration, Tissue stem cell, Metaplasia

[Background] New neurons are generated throughout life in the OE of mammals including humans (Hahn *et al.*, 2005) and mice (Graziadei *et al.*, 1979b; Kondo *et al.*, 2010). Continuous cell generation is primarily achieved by globose progenitor cells (Jang *et al.*, 2014). Progenitor cells also have the potential to reconstitute all OE cell types after injury, with horizontal basal cells having the greatest tissue stem cell potential (Leung *et al.*, 2007; Schnittke *et al.*, 2015; Gadye *et al.*, 2017).

There exist different methods to specifically injure the OE in laboratory rodents, which in turn allows the study of the regenerative process including stem cell activation, neurogenesis, and neuronal differentiation. Methods include removal of the olfactory bulb (OB) (Schwob *et al.*, 1992), olfactory nerve axotomy (Graziadei *et al.*, 1979a; Suzuki and Takeda, 1991), intranasal administration of zinc sulphate (Matulionis, 1975), inhalation of methyl bromide gas (Hurtt *et al.*, 1988), and intraperitoneal (i.p.) injection of olfactotoxic chemicals such as methimazole, colchicine, and dichlobenil (Genter *et al.*, 1995; Suzuki *et al.*,1998; Bergman and Brittebo, 1999). All these methods induce the destruction of one or several OE cell types followed by detachment of the OE and subsequent regeneration. The mechanism of action differs depending on the method; thus, there are important differences regarding which cell types are affected and how. Noteworthy, viruses such as SARS-Cov-2 can also specifically damage OE cells (Zhang *et al.*, 2020).

Methimazole (1-methyl-2-mercaptoimidazole, also called thiamazole) is used to treat hyperthyroidism

(Astwood *et al.*, 1945) and is known to cause a loss of the sense of smell (olfaction) as a side effect of treatment (Cooper, 1999). The OE is a pseudostratified epithelium in which all mature cells and horizontal basal cells are in contact with the basal lamina. In response to i.p. methimazole, several cell types in the OE, such as Bowman's gland cells, sustentacular cells, and olfactory sensory neurons, display swollen organelles within four hours, which is followed by detachment of cells from the basal lamina (Bergström *et al.*, 2003). After methimazole injury, the OE completely regenerates in approximately one month, and the first olfactory sensory neurons begin to make synaptic contacts with the OB at approximately 1-2 weeks (Suzukawa *et al.*, 2011). Methimazole has been shown to affect young and aged mice differently, on postnatal day 10 compared with 18 months old (Suzukawa *et al.*, 2011).

In rats, i.p. methimazole doses from 25 mg/kg to 300 mg/kg have been evaluated for OE toxicity (Genter *et al.*, 1995). We conducted a pilot study to elucidate the lowest dose suitable for reproducible OE ablation in mice (Håglin *et al.*, 2020). Single injection of either 75 mg/kg or 100 mg/kg resulted in detachment of the entire OE, whereas 50 mg/kg caused incomplete lesioning since there were residual patches of OE cells that appeared relatively unaffected. These results are in accordance with those of previous studies showing that lower doses of i.p. methimazole are insufficient to obtain complete OE lesions in mice (Brittebo, 1995) or rats (Genter *et al.*, 1995). Since 75 mg/kg and 100 mg/kg yielded similar results, we used the lower dose for further studies.

In our hands, methimazole more reliably causes OE ablation than does dichlobenil, and in practice, methimazole is easier to use than zinc sulphate or methyl bromide gas. Our protocol is the first to describe repeated OE ablation–regeneration cycles. By repeating the injury up to three times, we found a gradual decrease in the regenerative potential of the OE, which is accompanied by the progressive accumulation of two different types of metaplasia (Håglin *et al.*, 2020). Thus, repeated ablation–regeneration cycles of the mouse OE following methimazole treatment may be used, for example, to study: i) the exhaustion of the regenerative capacity of neurogenic tissue stem cells, which may mimic aging; ii) the robustness of the re-establishment of a tissue niche when subjected to repeated injury; iii) the plasticity of target neurons in the OB when challenged to re-establish synapses several times over; iv) the role of OE barrier function in protecting against a nasal route of infection as well as uptake of chemical substances to the brain; and v) the roles of different normal and disease variants of genes in these processes since there are many genetically modified mice available for study.

Materials and Reagents

1. 50 ml plastic, conical-base centrifuge tubes with screw cap (Sarstedt, catalog number: 62.547.254)
2. 2 ml sterile plastic microcentrifuge tubes with snap cap (Eppendorf 022363433, Fisher Scientific, catalog number: 05-402-11)
3. Micropipettes with appropriate tips

4. 0.5-1 ml insulin syringe (for example Beckton-Dickinson, catalog number: 329651) with 27G × 13 mm needle (BD, catalog number: 300635) or 0.5 ml syringe with attached 29G × 13 mm needle (BD, catalog number: 323001)
5. 100 ml and 250 ml glass bottles (VWR, catalog numbers: 10754-814 and 10754-816)
6. Permanent marker (please use one that withstands ethanol and freezing, such as the black Science-Marker VWR, catalog number: 76276-060)
7. Peel-A-way® embedding molds (Sigma-Aldrich, catalog number: E6032-1CS)
8. Superfrost Plus microscope slides (Thermo Fisher Scientific, catalog number: 12727307)
9. Cover slips no. 1 1/2 (Sigma-Aldrich, catalog number: CLS2980224-1000EA)
10. Boxes for microscope slides (VWR, catalog number: 82024-584)
11. Mice of the strain, genotype, age, and sex that you wish to analyze. See Procedure A for the protocol for C57BL/6 mice (Taconic, Denmark)
12. Methimazole (Sigma-Aldrich, catalog number: M8506), keep the container closed in a dry and well-ventilated area. Store at 4 °C
13. Sterile physiological saline 9 mg/ml (*e.g.*, Estericlean, catalog number: 7053249369080)
14. Paraformaldehyde (VWR, catalog number: 28794.295). Store the powder at 4 °C
15. Sucrose (VWR, catalog number: 27480.294)
16. 10× phosphate-buffered saline (PBS), pH 7.4 (*e.g.*, PanReac Applichem, catalog number: A0965,9010). Dissolve and store at room temperature
17. 5 M NaOH (VWR, catalog number: 31625.293)
18. OCT cryomount embedding medium (Sakura FineTek, catalog number: 45830)
19. Dry ice
20. Glycerol (VWR, catalog number: BDH1172-1LP)
21. Hoechst 33258 (Sigma-Aldrich, catalog number: ab228550). Prepare a 1000× stock solution of 0.5% (weight/volume) in water. Store the aliquots at –20 °C
22. Primary antibodies used to identify cell types in Figures 1 and 3 are:
    a. Anti-cytokeratin 5 for horizontal basal cells (rabbit; dilution 1:300, BioLegend, catalog number: 905501)
    b. Anti-IgGFc-binding protein (FcγBP) for respiratory secretory cells (rabbit, dilution 1:200, Novus Biologicals, catalog number: NBP1-90462)
    c. Anti-antigen Ki-67 (Ki-67) for cells in the cell cycle (rabbit, 1:500, Merck, catalog number: AB9076)
    d. Anti-olfactory marker protein (OMP) for mature olfactory sensory neurons (goat; dilution 1:1,000, Wako Chemicals, catalog number: 544-10001)
    e. Anti-retinalaldehyde dehydrogenase type 1/2 (RALDH1/2) for supporting and respiratory cells (mouse; dilution 1:1,000, Santa Cruz Biotechnology, catalog number: SC-166362)
    f. Anti-stathmin-1 (STMN1) for immature olfactory sensory neurons (rabbit; dilution 1:500, Abcam, catalog number: ab24445)

Equipment

1. Dissection instruments
   a. Scissors around 15 cm (Fine Science Tools, catalog number: 14001-14) and 10 cm (Fine Science Tools, catalog number: 14370-22) for the removal of fur and cutting through bone, respectively (expensive scissors will be destroyed)
   b. A smaller pair of fine scissors for trimming soft tissue (preferably blunt tip, Fine Science Tools, catalog number: 14108-09)
   c. A few forceps, such as the fine pointed ones that are used to remove the teeth (Fine Science Tools, catalog number: 11231-30), blunt ones (Fine Science Tools, catalog number: 11000-12), and ones with teeth (Fine Science Tools, catalog number: 11028-15). Use blunt plastic forceps to check for decalcification (Fine Science Tools, catalog number: 11700-03)
2. RDO Rapid Decalcifier (ApexEngineering directly www.rdodecal.com/contact/ or ESBE Scientific, catalog number: APE-RDOL4L)
3. Precision laboratory scale and analytical balance (for chemicals)
4. Precision laboratory scale (for mice)
5. Rocking platform or orbital shaker
6. Magnetic stirrer
7. Microwave oven
8. Cryostat with a knife for hard tissue
9. Inverted fluorescence microscope with camera and ×4, ×10, ×20 and ×40 objectives. A filter for fluorescence in blue (Hoechst 33258 is excited by light at 350 nm and emits at around 455 nm) is required at the least
10. –80°C freezer
11. Optional: Vacuum chamber or vacuum oven

Procedure

A. Pilot experiment to determine the methimazole dose required (recommended)
   1. We strongly recommend determining the specific methimazole dose required before setting up a larger experimental study in order to ascertain that reproducible ablation of the OE can be achieved in mice of the age and specific strain you wish to study. Note that it is unknown whether methimazole is toxic to the OE in mice younger than 10 days old (Suzukawa *et al.*, 2011). It is known that several enzymes in the OE are important for the metabolism of methimazole; therefore mouse strain differences will account for differences in the required dose (Xie *et al.*, 2011). In the planning stage, decide which methamizole doses you wish to evaluate. We evaluated 50 mg/kg, 75 mg/kg, and 100 mg/kg for adult 2-5 month-old male and female C57BL/6 mice weighing 40-60 g (Håglin *et al.*, 2020). Note that RTECS (Registry of Toxic Effects of Chemical Substances) lists the i.p. LD_{50} as 500 mg/kg for mice. It is recommended that at least

3 animals are analyzed per dose.

2. Prepare methimazole under aseptic conditions in sterile physiological saline on the same day as the injections. Methimazole powder will dissolve completely without visible precipitate. For example, if an injection of 75 mg/kg is planned, prepare 75 mg methimazole powder in 2 ml sterile physiological saline. Inject 2 µl/g or dilute further in physiological saline if you are more comfortable with injecting a larger volume. The maximum volume for i.p. injection in mice is 10 ml/kg (Morton *et al.*, 2001).
3. Be sure that you can identify each mouse, the dose the mouse received, and the tissue sample taken from that mouse, throughout the experiment. Weigh each mouse to determine the individual dose and make notes for each mouse.
4. When performing i.p. injections, it is imperative to be aware of the relatively frequent rate of unsuccessful injections due to problems with technique (Steward *et al.*, 1968). Useful instruction videos are available (Machholz *et al.*, 2012; Newcastle University, 2021). Practicing i.p. injections on recently euthanized mice with food coloring diluted in saline, followed by immediate examination of the color distribution in the peritoneal cavity can serve to reassure the performance of correct technique (Figure 1A).
5. Monitor the mice closely immediately following and the day after methimazole injections since a successful i.p. injection of methimazole normally results in some discomfort, which can be observed as sneezing and rubbing of the nose. Importantly, the mouse is not supposed to experience deterioration in its general condition.
6. Sacrifice the mice 2 days after i.p. injection and evaluate the effect of ablation by analyzing sections of tissue (see protocol C for histological analysis). The earliest effects of methimazole occur after 4 h (Brittebo, 1995; Bergström *et al.*, 2003), and the OE is not completely regenerated until one month post-treatment with methimazole (Suzukawa *et al.*, 2011).
7. Evaluate the degree of ablation in the different regions (called zones) of the OE and at several levels along the anterior–posterior axis of the nasal cavity (Figures 1C,D). Successful injection of an effective dose of methimazole results in the detachment of all cells, except for a monolayer of normally very flat horizontal basal cells lining the basal lamina. Varying amounts of detached tissue can often be observed in the nasal cavity 2 days after methimazole administration (Figure 1B).

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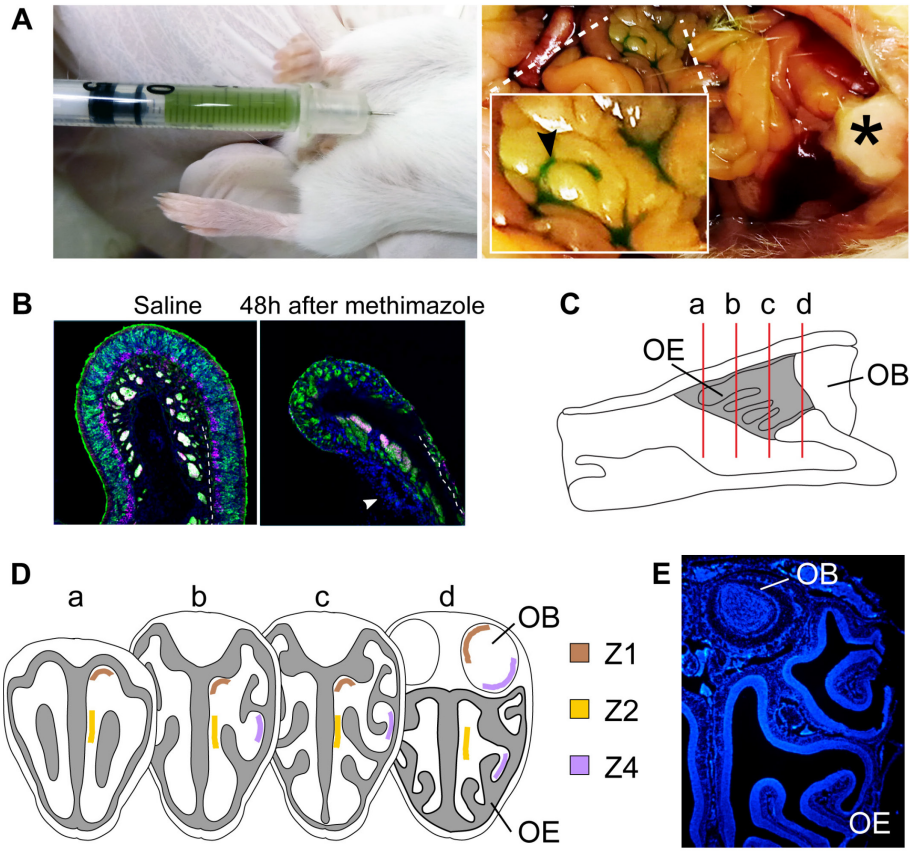

Figure 1. Example of methimazole ablation of the OE and a guide for analyzing different anatomical levels. A. Example of i.p. injection of a euthanized mouse with green food coloring. Open the abdomen immediately after injection since correct injection results in the rapid dilution and distribution of the color throughout the entire abdominal cavity. Note some green color outside of the intestines (arrowhead in insert). Incorrect injection into an abdominal organ is usually visible. Asterisk (*) indicates the sternum. B. Immunofluorescence analysis of a part of the OE in control (saline) mice and 48 h after a single dose of methimazole shows OE ablation after treatment. The dashed line shows the location of the basal lamina. Analysis using antibodies against marker proteins for immature (STMN1 in magenta) and mature (OMP in green) neurons shows that these cells are ablated simultaneously with all OE cells, except for horizontal basal progenitor cells (see Figure 3 for a close-up example). Note that detached cell debris (arrow) can be present in considerable amounts or absent from an individual OE section 48 h after methimazole treatment. C. Schematic illustration of the front part of a mouse head cut sagittally at the midline; a-d represent the approximate anatomical levels suggested for analysis of the OE throughout the anterior–posterior axis. D. Illustrations of the appearance of tissue sections taken from levels a-d in C. The OE is divided into several regions often termed zones. These zones have different characteristics; therefore, it is wise to assess the effect of methimazole at representative points in the OE and also in the corresponding region of the synaptic area for olfactory sensory neurons in the OB. Colored lines for zones 1 (Z1, brown), 2

(Z2, yellow), and 4 (Z4, purple) mark the representative locations. E. A micrograph of a section taken from level d, with the nuclei stained with Hoechst.

B. Methimazole injections for single or repeated ablation–regeneration cycles (main protocol)
   1. Firstly, make an experimental plan and decide which stage of regeneration is to be analyzed (proliferative phase, neuronal differentiation phase, or synaptogenesis). This will determine how long after ablation mice will be sacrificed. Analyze proliferation at 2-6 days, different phases of neuronal differentiation at 3-21 days, and synaptogenesis at 7-21 days (from the first synaptic contacts to virtually complete regeneration) after methimazole treatment. Next, consider whether you wish to study the OE after a single methimazole ablation, repeated injury, or both. The mature OE cells likely produce enzymes that metabolize methimazole into cytotoxic products. We waited 21 days between methimazole-induced ablations to allow for the regeneration of mature OE cells. Plan to include control mice in your experiment, which receive saline i.p. injection. You may also consider injecting bromodeoxyuridine to check for cells in S-phase at a specific time point after methimazole treatment (if so, see references An and Kang, 2013; Håglin *et al.*, 2020).
   2. Administer the first dose of methimazole as outlined in protocol A. Note that this protocol for repeated ablation–regeneration cycles was established for C57BL/6 mice that typically weigh 40-60 g and receive a 75 mg/kg i.p. dose of methimazole. If you have optimized the dose according to protocol A for the particular mouse line that you plan to analyze, use that dose instead.
   3. Optional: For a single ablation cycle, sacrifice the mice at the decided time point and prepare the tissue (protocol C).
   4. For analysis of the tissue after repeated methimazole-induced ablation, wait 21 days.
   5. Administer the second injection of methimazole at 75 mg/kg i.p. (or your optimized dose).
   6. Optional: For two ablation cycles, sacrifice the mice at the decided time point and prepare the tissue (protocol C).
   7. Wait 21 days post-injection.
   8. Administer the third injection of methimazole at 75 mg/kg i.p. (or your optimized dose). We evaluated up to three ablation–regeneration cycles, after which the OE was quite severely affected by large regions of metaplasia.
   9. For three ablation cycles, sacrifice the mice at the decided time point and prepare the tissue (protocol C).

C. Tissue preparation, fixation, and histological analysis (support protocol)
   1. This is a generic protocol for generating OE tissue sections for quick analysis when optimizing the dose required for complete ablation of the OE, as addressed by protocol A. This generic protocol is also useful for analysis of the OE and OB using many different antibodies. However, you may need to tailor the protocol or use another protocol, depending on the analyses you plan

to perform on the tissue since certain antibodies recognize antigen epitopes that are not accessible after paraformaldehyde fixation of tissue. Moreover, for superior standard histological examination after staining, with hematoxylin–eosin for example, it is recommended that you embed tissue in paraffin before sectioning.

2. Prepare 4% (weight/volume) paraformaldehyde in PBS (4% PFA) on the same day. Note that PFA is assigned hazard statements H350 (may cause cancer) and H317 (may cause allergic skin reaction); therefore, take precautions by wearing gloves and protective goggles. Work in a fume hood when handling the powder and solutions. For 100 ml: Weigh 4 g PFA and transfer to a glass bottle containing a magnet and place on a magnetic stirrer. Warm 90 ml distilled water in a microwave oven to almost boiling, and pour the hot water into the glass bottle while stirring. Add 5-10 µl 5 M NaOH to facilitate dissolution of the PFA. Once dissolved, add 10 ml 10× PBS. Plan for at least 40 ml 4% PFA per mouse.
   Also, prepare the same volume of 1× PBS and 20% sucrose in 1× PBS. These solutions should be cold when used (*e.g.*, keep on ice).
3. Plan to sacrifice and collect tissue from an experimental mouse and its control in parallel to avoid possible experimental confounding factors. For each mouse, label one 50-ml tube with the animal's unique identification number (or equivalent) and place on ice.
4. Sacrifice mice in accordance with local regulations. One suggestion is cervical dislocation followed by immediate exsanguination. Be sure to receive training how to perform this procedure correctly.
5. Quickly dissect the OE and OB as shown in Figure 2. Tissues should not have time to dry. If you plan on analyzing markers with very rapid expression dynamics, such as protein phosphorylation, you may need to use a protocol for the fixation of tissues via heart perfusion. Note however, that as the OE is superficial in the nasal cavity, the fixation liquid reaches the cells in the OE quite fast once the tissue is submerged in 4% PFA, and particularly if the nasal cavity is cut open.
6. Allow fixation of the OE tissue for at least 3 h at 4 °C in 4% PFA (note that overnight fixation is needed for analysis using some antibodies). Use approximately 40 ml 4% PFA per tube. Very gentle agitation of the sample using a rocking platform is recommended.
7. Pour off the 4% PFA and rinse the tissue and tube with 1× PBS. Remove the excess PBS from the tube using a small piece of paper towel.
8. For mice younger than 2 weeks old, this step is optional. Tissue from mice older than 2.5-3 weeks needs to be decalcified to obtain intact tissue sections since OE tissue contains calcified bone in the nasal cavity turbinates. Cover the tissue with the RDO decalcifing solution until the bone in the tissue softens. Use blunt plastic (not metal) forceps from now on to monitor as the bone softens. The bone should be compliant to light pressure, and decalcification takes 30-60 min depending on the age of the mouse. Treat experimental and control tissue in parallel for the same length of time.
   Note: RDO decalcification significantly decreases the in situ hybridization signal. If in situ

hybridization analysis is to be employed, consider 4% PFA fixation overnight at 4 °C followed by decalcification with 0.215-0.5 M EDTA in 1× PBS at 4 °C instead (at least 1-2 nights, check bone softness with forceps) (Ryan et al., 1991; Ishii et al., 2004).

9. Pour off the RDO and rinse the tissue and tube with 1× PBS. Remove the excess PBS from the tube with a small piece of paper towel.
10. Cryoprotect the tissue by incubating overnight to 24 h in 20% (weight/volume) sucrose in 1× PBS at 4 °C. Very gentle agitation is recommended. Use 40 ml per sample.
11. Residual sucrose on the surface of the tissue may result in difficulty in retrieving the tissue sections since they may separate from the cryomount embedding medium. Pour off the sucrose and quickly remove the excess by rinsing the tissue and tube with a small volume of 1× PBS; this will avoid the formation of a sucrose film around the tissue once frozen. Remove the excess PBS from the tissue with a small piece of paper towel but do not allow the tissue to dry.
12. Place the tissue in a labeled cryomold with the nose oriented upward. Indicate the orientation of the nose on the mold to allow you to later mount the cryoembedded tissue in the right orientation in the cryostat. Fill the mold with cryomount embedding medium so that it completely covers the tissue.
13. Optional: Orient the nose upwards and place the mold under a vacuum of up to 500 mbar for 10-20 min at room temperature. If there is air trapped in the nasal cavities, this will slowly surface (note bubbles). Removing air makes tissue sectioning less difficult.
14. Place the mold on dry ice and use forceps or pipette tips to hold the tissue in the right orientation while it freezes. Leave to freeze completely and move the mold directly to –80°C for storage.
15. Coronal OE tissue sections are commonly used for analysis. Plan how you should arrange the sections on microscope slides and analyze different levels along the anterior–posterior axis of the nasal cavity (Figure 1D). Cut 10-12 µm thick sections, at a relatively cold temperature, *e.g.* around –20°C. Start sectioning from the nose end of the tissue. Collect a section approximately every 10-20 sections to assess whether you are reaching the region of OE and/or OB and when to collect sections more frequently for your specific experiment.
    Note: It is harder to collect intact sections from the nasal cavity than from soft tissue since the nasal region has a combination of tissues possessing very different hardness and elasticity. If it is very difficult to obtain intact sections, consider decalcifying for longer than 30 min.
16. Store slides of tissue sections at –20°C or –80°C until use.
17. Thaw slides at room temperature and let dry for about 15 min.
18. Perform a quick evaluation of the degree of ablation stain, *e.g.*, using fluorescent nuclear stain Hoechst at a concentration of 0.0005% (weight/volume) for 3-5 min, followed by tap water for 3-5 min. Mount with glycerol or the mounting media of choice and cover slip.

    Examine the tissue sections under a fluorescence microscope with a filter for emission in blue (Figure 1E).

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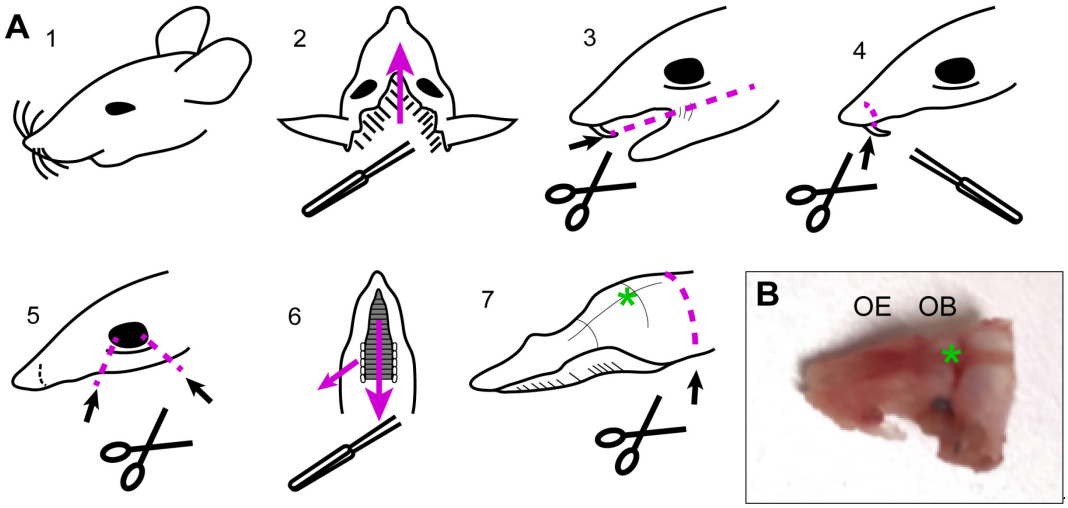

Figure 2. Dissection of nasal and OB tissue. A. Schematic illustration of the dissection procedure. Be light-handed and do not squeeze with the hand holding the tissue, while dissecting with the other hand. Be quick (practice dissection before performing the planned experiment). 1 = Head; 2 = Peel off the skin using blunt dissection (*i.e.*, fingers) and forceps; 3 = Cut off the lower jaw; 4 = Carefully cut open the bone around the front teeth; and remove teeth by careful detachment using repeated gentle sideways movements with forceps; 5 = Cut off the Os zygomaticum bone and remove the eyes and any soft tissue still on the facial bones; 6 = Peel off the soft palate (in grey) with forceps. Remove teeth by holding them and bending carefully outward using forceps; 7 = Cut off the skull so that it can stand with “nose pointing upwards” in the cryoembedding mold. Be sure that you know where the OB is so that you do not cut it away if you plan to analyze the OB (if not, it can be removed). B. Image of dissected nasal tissue. Marks where the OE is within the nasal cavity and the position of the olfactory bulbs in the OB. A green asterisk (*) marks the corresponding region in the schematic illustration in A7 and B.

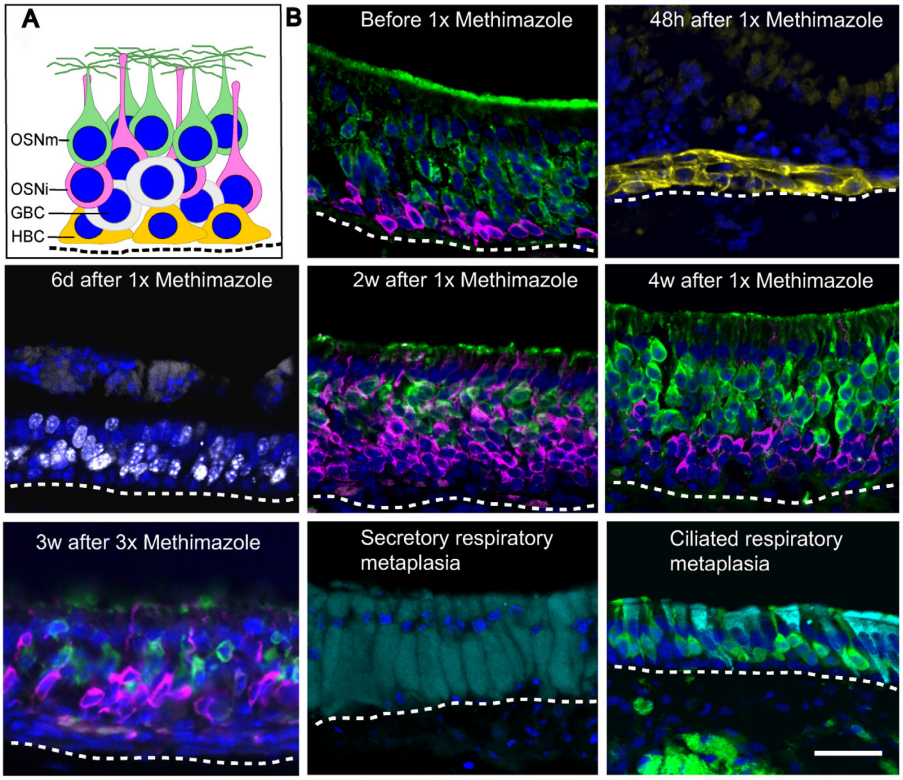

Figure 3. Example of cell types in the OE after one methimazole treatment or repeated OE ablation–regeneration cycles. A. Schematic representation of the neural lineage in the OE (sustentacular, microvillar, and Bowmann's gland cells are not illustrated). Mature olfactory sensory neurons (OSNm, positive for OMP, in green), immature olfactory sensory neurons (OSNi, positive for STMN1, in magenta), globose basal progenitor cells (GBC, positive for Ki-67, in white), and horizontal basal progenitor cells (HBC, positive for cytokeratin 5, in yellow). B. Immunofluorescence analysis of the normal OE before methimazole treatment (mature neurons in green, immature neurons in magenta). 48 h after methimazole treatment, only activated horizontal basal cells that have acquired a rounded morphology remain (yellow). 6 days post-treatment with methimazole, the most prominent cell type is the dividing globose progenitor (white). The difference in the appearance of the OE between 2 and 4 weeks after a single dose of methimazole is that more immature neurons (magenta) have differentiated into mature neurons (green) by 4 weeks. Repeated ablations by methimazole treatment every 21 days results in the OE no longer regenerating efficiently. Moreover, after repeated ablations, there is a gradual increase in secretory (positive for FcγBP, in green) and ciliated respiratory metaplastic patches (positive for RALDH1/2, in green) in the OE (Håglin *et al.*, 2020). The dashed line marks the location of the basal lamina. All sections were stained with the nuclear stain Hoechst, which is in blue. Scale bar, 25 μm.

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Technical-natural science faculty at Umeå University. The protocol is adapted from Håglin *et al*. (2020).

Competing interests

The authors declare no competing financial interests.

Ethics

The experiments leading to the development of the protocol were approved by the Local Ethics Committee for Animal Research at the Court of Appeal for the upper northern area of Norrland, Umeå, Sweden (ID A15-2015 for period 4/24/2015 to 4/24/2020).

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