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Ex vivo multisite electro-olfactogram recordings in rabbit neonates

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Key words: electro-olfactogram, *ex vivo* olfactory mucosa, olfactory receptor neurons, newborn rabbit, pheromone, learning

List of abbreviations

EOG: electro-olfactogram

MP: mammary pheromone

OM: olfactory mucosa

OR: olfactory receptor

ORN: olfactory receptor neurons

Abstract:

Electro-olfactogram (EOG) recording is a technique that has been used in the field of olfaction for many decades to measure the electrical activity generated by olfactory receptor neurons (ORNs) in response to odor stimuli. In the late 1980s, the development of *ex vivo* preparation facilitated EOG recordings in mammals. This method provides access to all olfactory turbinates, allowing for recordings lasting several hours. It has greatly contributed to our understanding of olfactory physiology and the functional links between olfaction, various behaviors and pathologies. Although many innovative techniques are available to study olfaction in the 21st century, EOG recordings can still provide valuable insights, particularly when combined with other approaches. For example, the relationship between peripheral olfaction and food intake can be studied, by manipulating orexigenic and anorexigenic agents, or by modifying the animals' diet. Moreover, the connections between olfaction and the intestinal and/or nasal microbiota composition, diabetes, or peripheral plasticity resulting from learning processes can also be examined from a peripheral perspective. The *ex vivo* multisite EOG recording technique has been recently proved to be particularly useful in newborn rabbits. This technique has demonstrated the high native sensitivity of ORNs to a signal naturally emitted by all rabbit mothers, the mammary pheromone, throughout the olfactory mucosa. Moreover, it has also been observed that ORNs are involved in increased behavioral reactivity due to pheromone-induced odor learning, as well as in differentially processing synthetic and analytical odor mixtures. The *ex vivo* multisite EOG recording This method is expected to remain a useful tool in future research, having in mind that it is both simple and cost-effective to implement.

1. Introduction

The first and crucial step in olfactory perception involves the interaction of odorant molecules with specialized olfactory receptors (ORs) expressed by olfactory receptor neurons (ORNs) located in the olfactory mucosa (OM) lying in the nasal cavities. Understanding how these neurons respond to different odors and how different neurochemicals can modulate for instance their responses, metabolic states and learning abilities of animals, provides crucial information about the initial stages of olfactory processing.

EOG recordings are ideal for studying odor sensitivity and spatial discrimination as they directly measure the electrical activity of olfactory sensory neurons in response to odor stimulation. Varying the stimulus concentration in EOGs provides an indication of population sensitivity and dynamic response (e.g. [1-5]). By using odorants either individually or in combination in mixture, EOGs provide insights into interactions between odorants at the population level during the early stages of olfactory processing [6, 7].

Historically, in terrestrial animals that sample the olfactory world in the air they breathe, the population-based electrophysiological recording technique or EOG was developed by Ottoson in 1954 [8] and proved to be the simplest technique for recording ORN responses to gas phase stimulation. It has been widely used *in vivo* in amphibians (locally anaesthetised and curarized), where access to the nasal cavity is quick and easy. In frogs [2, 9] and salamanders [10], the roof of the olfactory sac can be easily removed, providing access to the floor of the cavity lined by the ventral OM, i.e. the olfactory eminence.

In contrast, obtaining this type of *in vivo* recording in mammals requires dissections, which are delicate and time-consuming, to access the olfactory turbinates. As a result, *in vivo* recordings are rare and limited to a restricted area of the OM due to the difficulty of surgically accessing the olfactory turbinates. For example, in our rat recordings [2, 6, 11, 12], which aimed to capture the unit activity of ORNs and EOGs simultaneously, recordings were performed exclusively from endoturbinates II. Similarly, in experiments that coupled EOG and bulb recordings in rats, EOG recordings were obtained exclusively

from the dorsal recess. This area avoids opening of the nasal cavity and provides access to the OM through its dorsal surface [1, 13-15].

Fortunately, it was discovered in the late 1980s in rodents that EOGs could be recorded from *ex vivo* preparations of hemiheads, providing access to all olfactory turbinates. The *ex vivo* preparation has proven to be very useful in a wide range of issues, as the population of ORNs is capable of generating EOGs for significant periods of time. In their review about EOG, its history and uses, Scott and Scott-Johnson [16] emphasized that EOG can be maintained for several hours after the cessation of circulation, even though single-unit activity disappears after the animal's death. For our part, during recordings of ORN unit activity in rats following respiratory arrest, we observed the disappearance of unit activity after a period of erratic hyperactivity (unpublished personal data). Combined with the persistence of EOG, these observations confirm the original hypothesis of Adamek and co-workers [17]: EOG is a field potential that reflects a localized electrical phenomenon, named olfactory transduction, in the cilia of ORNs where ORs are expressed. In an *ex vivo* preparation, EOGs persist because they reflect the molecular cascades that accompany olfactory transduction, resulting in a slow and local change in potential. Thus, *ex vivo* EOGs can be recorded for several hours after the animal's death and show parameters (kinetics, shape and amplitude) that are identical in all respects to EOGs recorded *in vivo* (*see note 1*).

Edwards and co-workers [18] introduced the *ex vivo* preparation in rats and mice. Animals were killed by anaesthetic overdose or decapitation; the head was split in two down the middle and the septal cartilage delimiting the two nasal cavities was resected uncovering the olfactory turbinates. This preparation enables direct application of odorants to the OM, mimicking natural air odor sampling, similar to *in vivo* studies in frogs and salamanders. The *ex vivo* preparation has subsequently been used to study the spatial distribution of responses [19-23].

However, in the 21st century, with the advent of more innovative techniques, a legitimate question arises: can the technique of recording electro-olfactograms still provide new insights into olfaction?

The answer is yes. Although there are many innovative techniques available to study olfaction, EOG recordings can still provide valuable insights, especially when combined with other approaches. Below, we present a selection of recent studies demonstrating the relevance of recording EOG using *ex vivo* mammalian preparations, including parts of our own work in newborn rabbits. These studies cover a wide range of contemporary issues and show how this method can still contribute to a deeper understanding of olfaction today.

Olfactory function, is closely linked to nutritional processes, and is therefore modulated by metabolic states and associated neuropeptides [24, 25]. *Ex vivo* EOG recordings was used in rats to observe the direct effects of two anorexigenic agents, insulin and leptin, which target specific receptors in the OM. Once applied locally, insulin and leptin induced a reduction in EOG responses [26, 27]. In similar experiments, neuropeptide Y, a potent orexigenic peptide in the central nervous system, has been shown to increase EOGs [28]. More recently, the effects of different diets on olfactory function have been studied by combining behavioral testing, molecular biology and *ex vivo* EOG recordings [29]. The results showed alterations in molecular signaling and EOG amplitudes in newborn and weaned mice. However, the authors did not observe any impairment in odor-guided behavior. In contrast, Makhoulouf and colleagues [30] pinpointed that short-term exposure to high-fat diet had negative effects on EOG responses (measured *ex vivo*) and behavior. Regarding metabolic state and pathology, in mice, Kuboki and colleagues [31] demonstrated that diabetes reduces peripheral EOG and cFos responses in the olfactory bulb, as well as performance in odor-related tasks.

Other research has recently linked olfaction and gut and olfactory microbiomes. For instance, François and co-workers [32] demonstrated that the physiology of OM was modulated according to the gut microbiome type using a combination of molecular biology, histology, behavior, and *ex vivo* EOG recordings. Next, mice with different microbiota lineages were shown to have *ex vivo* EOG responses to monomolecular odorants that reflect different peripheral sensitivities, using similar techniques [33].

Elsewhere, the *ex-vivo* EOG recording technic has recently been used to investigate the effects of trigeminal stimulation on OM reactivity to odor stimuli [34]. The authors report a decrease in *ex vivo* EOGs when the trigeminal nerve is stimulated and shows that this regulation depends on specific cation-permeable ionic channels.

The OM undergoes intense plasticity (for a review, see [35]). First, olfactory receptor neurons (ORNs) are rhythmically renewed every 6-8 weeks [36]. Second, in addition to this programmed plasticity, individual's life experience, learning, and exposure to the naturally or experimentally changing environment can induce plasticity. For instance, in their study about the effects of deprivation (nostril occlusion) versus olfactory enrichment in mice, Fitzwater and Coppola [37] observed no change in *ex vivo* EOG responses after enrichment, while deprivation resulted in increased EOG responses. Their hypothesis of an increase in EOG response due to peripheral induction [38] by enrichment was thus invalidated under their conditions.

Our experimental model, the newborn rabbit, is initially blind and deaf and strongly dependent on the perception of a monomolecular pheromone emitted by all lactating rabbit females in their milk, the mammary pheromone (MP; 2-methylbut-6-enal) which allows the pups to rapidly find the nipples and suck [39-41]. Multisite *ex vivo* electro-olfactogram (EOG) recordings were used to demonstrate, for the first time, the native sensitivity of the OM to the MP. This highlights how the periphery of the main olfactory system adapts to a biologically significant signal, facilitated by the peripheral processing by olfactory receptor neurons (ORNs), which contributes to strong behavioral responsiveness in neonates [7].

The choice of recording at several sites made it possible to highlight any drastic differences in sensitivity or, on the contrary, a generalization of the OM reactivity to the tested stimuli. In the same model, we further investigate for the first time in neonates the induction phenomenon [38]. The newborn rabbit is relevant for such investigations since the MP, in addition to its activity as behavioral releaser, functions also as a promoter of very rapid (5 min) appetitive odor learning by single-trial associative conditioning

[41-44]. Thus, after simultaneous exposure to MP + a new and target odorant (or mixture of odorants) initially inactive in terms of behaviour, newborn rabbits strongly respond the day after to the conditioned odorant when presented alone, and as efficiently as to the MP by itself. Strikingly, this learning is accompanied by a concomitant significant peripheral increase in the EOG in response to the learned odorant [7]. Thus, in contrast to experimental conditions of Fitzwater and Coppola in adult mice [37], the plasticity induced in newborn rabbits by pheromone-induced odor learning leads to an increase in ORN responses to the conditioned stimulus, i.e., to peripheral induction. Furthermore, our experiments on newborn rabbits also revealed differential peripheral processing of binary mixtures that are perceived as configural and elemental by rabbit neonates (as in other mammals including humans) (e.g., [45-47]). Indeed, *ex vivo* EOG recordings highlighted synergistic vs suppressive interactions between the odorants that compose the two types of mixtures [48]. In this chapter, we detail below the methodology allowing to run *ex vivo* EOG recording in newborn rabbits.

In conclusion, it is worth noting that conducting experiments involving *ex vivo* EOG recordings can be a cost-effective alternative to more advanced and expensive neuroimaging techniques. This makes EOG recordings a practical choice in certain research contexts, especially in settings where resources may be limited.

2. Materials

2.1 Equipment and instruments

1. For euthanasia of the animal: Large 22.5 cm chrome saddler's scissors (Saddler's scissors, CAMARD N°8, chrome steel, blade length: 10 cm, screw length: 22 mm) or a guillotine designed for mice
2. Surgical instruments: Scalpel handle and blade for nasal bone incision. Arched tip forceps Dumont forceps n°5 (Fine Science Tools by Dumont, Switzerland) for gripping the skin and tissue. Fine Scissors: One straight pointed pair, one curved pointed pair. Ultra Fine Forceps-fenestrated pointe droite n°5 (ref 9980; Fine Science Tools by Moria, Switzerland). Micro-scissors for ophthalmic

surgery (ref 7865; Moria SA, Antony, France). Micro spatulae or iris spatulae (Fine Science Tools).

Small plate of glass or hard material to place the head on.

3. Optical fibres.
4. Headset magnifier and/or binocular loupe.
5. For the manufacture of electrodes: Tinned copper wire diameter: 1 mm. silver wire diameter: approx. 0.15-0.2 mm. Tin wire for solder. Bunsen burner. Female banana plugs.

2.2 Reagents and solutions

1. 0.09% NaCl saline solution.
2. HCl 0.1 N for electrode chlorination.
3. Transparent nail polish or clear lacquer (for electrodes).

2.3 Recordings

1. Three EOG electrodes: homemade silver wire electrodes ended with a silver ball plated with AgCl.
2. Three basic manual micro-descenders x-y-z.
3. Three amplifiers (WPI, DAM80) and their probes.
4. PC in oscilloscope mode.

2.4 Signal acquisition

Recording visualization and acquisition are made on-line using homemade Neurolabscope© software. Data analysis is made off-line through a python script designed by S. Garcia (CRNL, Lyon).

2.5 Olfactory stimuli and delivery

Pure odors or binary mixtures (see [7] for an exhaustive list of compounds) can be applied directly over the turbinate surface using a homemade olfactometer (Fig. 1). Stimuli consisted of 0.5 to 5-second odor pulses delivered at 300 ml/min through a large olfactometer nozzle (internal diameter: 7.5 mm) to avoid drying out the organic matter and to minimise the possibility of mechanical

stimulation. The design of this olfactometer was largely inspired by other stimulators designed for the same types of recordings [26; 28, 32, 49, 50]. However, our stimulator was especially designed to use lower flow rates than those found in the literature so that responses to low odorant concentrations can be visualised without being masked by the mechanical response of the tissue [50, 51].

Between stimulation periods, the preparation received 600 ml of continuously humidified clean air. During stimulation, 300 ml/min was diverted to pass through the tube containing the odorant. The odorants were diluted in the liquid phase. The liquid phase in which the odorant was diluted filled approximately 50% of the height of the tube, so that the airflow pushed the odorised gas saturating the top of the tube towards the sample.

3. Methods

3.1. *EOG electrodes (Fig. 2)*

1. Cut tinned copper rods about 5 cm long.
2. Solder these to the banana sockets using a soldering iron.
3. Solder a 5 mm piece of silver wire to the end of each tinned copper rod. Heat the silver wire with the flame of a Bunsen burner to form a silver ball (\varnothing approx. 200-500 μm ; impedance $\leq 100 \text{ k}\Omega$).
4. Under a magnifying glass, cut the ball to a flat surface. Cutting off the ball allows for a better chlorination and prevents the electrode from slipping on the turbinate.
5. Chlorinate the spheres thus cut from the electrodes by electrolysis in a bath of hydrochloric acid (0.1 N); the EOG electrode is the cathode and the anode is a graphite cylinder. Power the circuit with a 9V battery. Slow down the rate of electrolysis by placing a resistor between the battery and the cathode.
6. Apply a coat of clear varnish along the entire length of the electrode, except for the ball. Allow to dry.

3.2. *Animal breeding*

Under our conditions, adult female and male New Zealand rabbits (Charles River strain, France) were housed in individual cages at the CRNL breeding unit. The ambient air temperature was maintained at 21°C, with constant 12:12h light/dark cycle (light on at 7:00 am). Water and food were provided *ad libitum*. Two or three days before the day of parturition, a nest-box (L x P x H: 41.5 x 29.5 x 29.2 cm) containing wood shavings was attached to the cages of pregnant females to allow them to prepare their nest. The day of delivery/birth was considered as postnatal day 0 (d0). In this species, females leave the nest voluntarily immediately after the nursing that immediately follows parturition, and return only once per day for interacting with their litter to nurse during 3-5 min [52, 53]. In domestic conditions, this natural rhythm of visits, i.e. single and brief daily access to the nest, must be respected to optimize the welfare of females and welfare/survival of pups [53]. In our breeding unit, the nest was opened at 11:30 a.m. and closed at 1:00 p.m.

3.3. *Surgery*

All experiments were carried out in accordance with the ethical rules enforced by French law and have been approved by the Ethics Committee of the University of Lyon 1 (CEEA-55) and by the French Ministry of Higher Education and Research under no. 9745. EOG recordings were generally performed on animals between 2-4 days of age (*see Note 2*). On the day of experiment, each rabbit pup was brought to the experimental room in a polystyrene box containing wood shavings and maternal hair, then quickly decapitated. The head skin was removed and the skull cutted lengthwise with a scalpel along the medial suture to expose the nasal cavities (*see Note 3*). The result was two half heads (Fig. 3; *see Note 4*); the scalpel blade was naturally deflected by the septum. In the nasal cavity, one of the two hemi-heads retained the septal wall, which may or may not be covered by septal mucosa (Fig. 3A, it was not the case, the cartilage was bare). As a result, the other hemihead may either have turbinates I, II, III and IV directly exposed (as in Fig. 3B) or may be still closed by the septal mucosa corresponding to its side (*see Note 5*). To access the turbinates, the septal wall lined (or not) with the olfactory/respiratory mucosa must be chirurgically removed. This operation was performed using ophthalmic scissors. To that goal:

1. Insert vertically one of the two blades at the front of the nasal cavity (where there is no olfactory turbinate) and cut upwards along the dorsal nasal bone to the cribriform lamina.

2. Starting from the initial opening, make a cut along the bone separating the nasal cavity from the palate to the base of the cribriform lamina.
3. Grasp the front of the septal wall triangle with forceps, lift it and cut along the cribriform plate, freeing a large triangle corresponding to the inter-narrow septal wall. This will release the turbinates (*see Note 6*).

3.4. *Ex vivo EOG recording*

The two half-heads were fixed in a silicone chamber and placed under the nozzle of the olfactometer. The positioning of the preparation is such that the flow delivered by the stimulator sweeps the turbinates from the anterior to posterior parts, as expected from the anatomy of the nasal cavity [5]. The preparation is surrounded by cotton saturated with water to maintain a humid environment throughout each recording session; under these conditions, the turbinates maintained good aspect and reactivity for several hours. EOG recordings are made simultaneously from the three first turbinates (Fig. 4). For this purpose, each electrode is connected via the female banana plug to the male connector of the input probe of an amplifier (WPI, DAM80, amplifier, DC, gain 1000). This probe is attached to a simple manual x-y-z micromanipulator. The amplifier output signal is connected to a computer via an acquisition card (analogue-to-digital interface). The ground wires of the 3 amplifiers are connected to a calibrated voltage generator, which is connected to the ground circuit; grounding is ensured by a wire connected to the start of the generator, the end of which is a pellet (AgCl pellet, Phymep, Paris) placed in the brain matter of the recorded hemisphere. This generator sends a square wave of 1 or 10 mV to the circuit, allowing the calibration of each electrode and the calculation of the signal amplitude.

The fact that the electrode rods are flexible means that their shape can be adapted as closely as possible to the preparation. Once the three electrodes have been placed over the target area, they are gently lowered until they touch the mucus surface (i.e. make contact). It is important that the mucus is clear, fluid and abundant to ensure that the ciliary membranes of the neurons are in good condition and the molecular cascades involved in transduction are functioning properly, and to ensure good contact with the electrode. As the turbinates are elevated in relation to the nasal cavity, don't hesitate to moisten them

as often as necessary with 0.9% NaCl using a Pasteur pipette (*see Note 7*), without keeping them immersed during the recordings.

Recordings are made simultaneously from the three turbinates. In our experiments, the electrodes are placed in the distal part of the turbinates. However, since the precision of these locations cannot be based on any coordinate and the animals naturally show variations in morphology (size, inclination and proximity of the turbinates), this choice was only intended to standardise the measurements without deriving any chemotopic information from them, knowing that the existence of chemotopy has not yet been proven. The recordings are illustrated in Fig. 4, which shows three simultaneously recorded EOGs (raw signals visualised by Clampfit 5.1 software) from the three turbinates in response to 0.5-sec-stimulation of MP (2-methylbut-1-enal) at 10^{-5} g/l, i.e. 7×10^{-8} mol/l in gas phase.

4. Notes

1. The recordings require mucosal surfaces that are sufficiently moist to allow optimal maintenance of mucus, ensuring good contact with the electrode and the persistence of membrane biochemical functions.
2. The *ex vivo* EOG technique can be performed on adult rats and mice. In rabbits, it is certainly possible to extend this period to more advanced ages than the neonatal period, although the size of the rabbit undoubtedly limits the use of the dissection method described here. Nevertheless, dissection is feasible at least up to postnatal day 10. It's important to bear in mind that the older the animal, the shorter the survival time of the tissues and the more delicate their preservation at the correct humidity and with a fluid layer of mucus.
3. Insert vertically at the nasofrontal suture, then cut by lowering the blade towards the front of the nose. Then, turn the head 180° so that the tip of the scalpel can again be inserted flush into the slit of the suture, and make the same cutting movement but this time backwards.
4. It is advisable to keep the hemi-head with the olfactory turbinates protected in this way and to begin the dissection with the hemi-head, which immediately exposes the turbinates. It is also suitable to cover them with cotton soaked in 0.9% NaCl. If both cavities are open, cover one of them with absorbent cotton.
5. If part of the septal mucosa separates from the cartilage and covers part of the turbinates. In this case, it can be gently removed with forceps and ophthalmic scissors.
6. After application, allow the cavity to "drain" slightly so that the horns are not flooded and the trace of each electrode, visualised on the computer in Scope mode, stabilises.
7. After application, allow the cavity to "drain" slightly so that the horns are not flooded and the trace of each electrode, visualised on the computer in Scope mode, stabilises.

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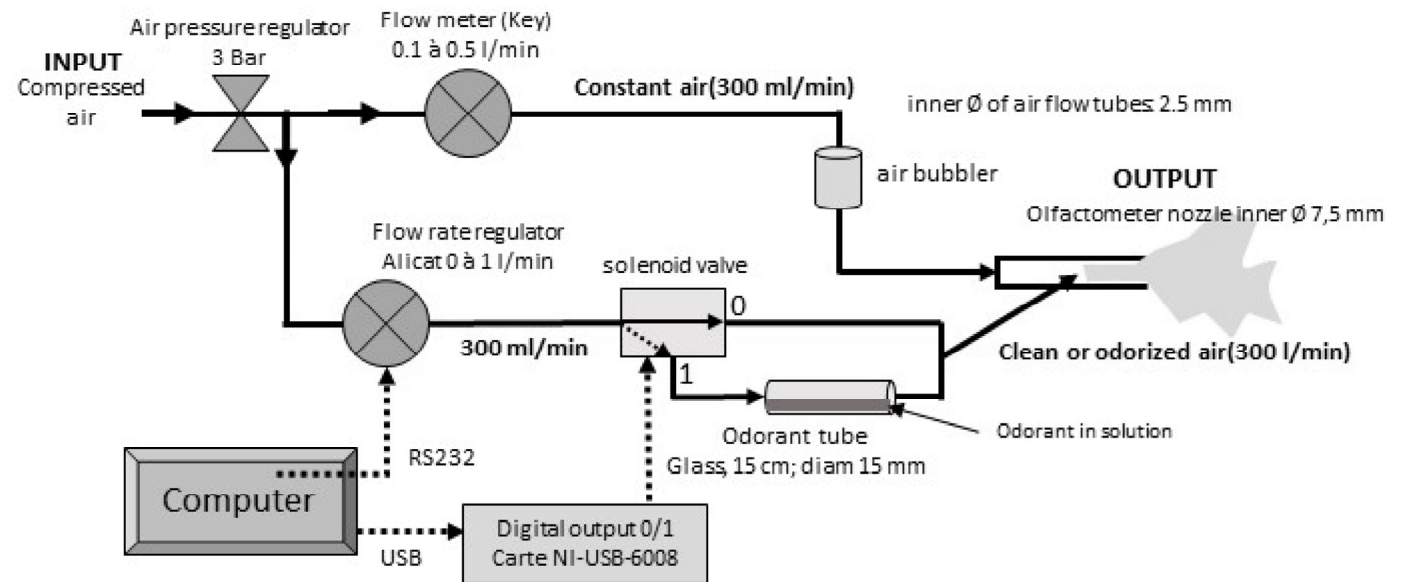
Figure captions

Figure 1. Lab-made Olfactometer: see the operating principle in the text. References for materials: KEY flow meter ref. FR2A12VBN (Radiospares supplier); Alicat Scientific mass flow controller, ref. MC-2SLPM-D/5M (System-C instrumentation supplier); Solenoid valve ref. 1331P015HO 61111 - 24VDC (supplier Contact Evolution France); Ni USB-6008 card (supplied by National Instrument); Control software developed in the laboratory under LabVIEW 21.0 (National Instrument)

Figure. 2: From the top to bottom: Photograph of the entire electrode, from its connector on the left to the ball on the right. Below, 3 photographs taken with a macroscope (Zeiss Axio zoom. V16) at 27.5x magnification of the metal electrodes. They show the terminal part of the electrodes (corresponding to the dotted white frame), including the soldering between the tinned copper rod and the agent wire, at the end of which a ball has been formed. From top to bottom: Electrode with ball intact, electrode with ball cut off, electrode with ball cut off and electrode with ball cut off chlorinated. The lacquer was applied to the latter, avoiding the end. The lighting can change the apparent diameter of the silver wire at the end, but it always has the same diameter (diameter: 0.15-0.2 mm).

Figure 3. Photo of left hemisphere after dissection. **A:** The nasal cavity is covered by septal cartilage (roughly demarcated by a white dotted line). The island with a remarkable density of blood capillaries at the front of the nose (also demarcated by a white dotted line) indicates the location of the vomeronasal organ (VNO). Beneath the cribriform lamina is the septal olfactory region, which is covered by olfactory mucosa on both sides of the cartilage. This area can also be identified by a high density of capillaries (not visible in this picture) and by the greater thickness of the cartilage protecting the turbinates. **B:** After resection of the septal cartilage, the olfactory turbinates appear. Note that here the olfactory bulb (OB) has been removed and its location is shown in white. The brightness of the tissues is an indication that the specimen is in good condition.

Figure 4. Recordings' configuration. A: The three EOGs are recorded simultaneously on 3 turbinates numbered from front to back as shown in the photo of the preparation. B: example of raw recording (visualized with clampfit 10.5, Axon instrument): EOG responses to a 0.5 s stimulation of MP at 10^{-5} g/ml (i.e. estimated in gas phase $\approx 7 \cdot 10^{-7}$ Mol/l). The black horizontal line at the bottom of the tracings indicates application the stimulation application. The EOGs obtained show a classic course with rapid initial kinetics up to a maximum and then, slower repolarization concomitant with cessation of stimulation with or without crossing the initial baseline. The response latency is relatively long: this is due to the low stimulation rate combined with the large diameter of the olfactometer nose, which together ensure that the odorant is diffused in a non-mechanically stimulating way.





Simultaneous recordings on 3 turbinates

