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## Endothelin impacts on olfactory processing in rats

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**Abbreviations:** OM, olfactory mucosa; OSN, olfactory sensory neurons; SCs, sustentacular cells; ET-1, endothelin; CBX, carbenoxolone;

## **ABSTRACT**

In the olfactory epithelium, olfactory sensitive neurons and their axons are surrounded by glia-like cells called sustentacular cells, which maintain both the structural and ionic integrity of the olfactory mucosa. We have previously found that endothelin-1 (ET-1) can uncouple sustentacular cell gap junctions *in vitro* similarly as carbenoxolone, a known gap junction uncoupling agent. The role of gap junctions in odorant transduction remains controversial and we explored here if ET-1 naturally produced by the olfactory mucosa could impact odorant detection. Using calcium imaging on olfactory mucosa explant, we first confirmed that ET-1 uncouples gap junctions in an olfactory mucosa preparation preserving the tissue integrity. We next measured the olfactory epithelium responses to odorant stimulation using electro-olfactogram recordings. While the amplitude of the response was not modified by application of ET-1 and carbenoxolone, its repolarizing phase was slower after both treatments. We finally examined the behavioral performances of rat pups in an orientation test based on maternal odor recognition after intranasal instillations of ET-1 or carbenoxolone. While rat pups performances were decreased after ET-1 treatment, it was unchanged after carbenoxolone treatment. Overall, our results indicate that ET-1 modulates olfactory responses at least partly through gap junction uncoupling.

## 1. INTRODUCTION

Neuronal activity regulation was long thought to occur only in central structures, but it is now well established that it exists also in peripheral sensory neurons [1]. Some of these regulations clearly involve glial cells of sensory organs [2]. The first step of odorant detection takes place in the olfactory mucosa (OM). In this neuroepithelium, olfactory sensory neurons (OSNs) are surrounded by glial-like sustentacular cells (SCs) forming a structural barrier. SCs also provide metabolic and trophic support to neurons and participate in the maintenance of ionic gradients in OSNs [3]. Furthermore, part of the paracrine modulations of the OM implicates the SCs [4]. SCs express connexins 43 and 45, and are coupled by gap junctions [5, 6]. Whereas the role of these gap junctions between SCs remains to be fully described [7], the importance of gap junction communication has been broadly characterized in the central nervous system. Among others, they enable intercellular spread of electrical current, calcium wave propagation, and transmission of second messengers [8, 9]. Functions unrelated to intercellular communication such as cellular development or synaptic strength are now also well established for connexins [10, 11].

Endothelin (ET), one of the most potent vasoconstrictor peptides [12], is also known to inhibit gap junctional communication very efficiently especially in astrocytes [13]. Three peptides of 21 amino acids named ET-1 to ET-3 are characterized, ET-1 being the most widely distributed [14]. These peptides are secreted as pro-peptides (Big-ETs), which are matured locally by peptidase endothelin-converting enzymes (ECEs) and then act autocrinally and paracrinally on two different G protein-coupled receptors, named ET<sub>A</sub> and ET<sub>B</sub>. The peptides along with their receptors and their converting enzymes are classically presented as the endothelin system. Based on primary cell cultures of the OM, we previously reported that ET-1 acutely triggers robust Ca<sup>2+</sup> transients in most cells. Using pharmacology, we observed that OSNs preferentially express ET<sub>B</sub> receptors while most non-neuronal cells preferentially express ET<sub>A</sub> receptors. The non-neuronal cells include mainly olfactory ensheathing cells (OECs) and SCs [15]. In another study, we found that ET-1 acts as a neuroprotective

peptide for the olfactory cells [16]. While we have found that ET-1 could effectively uncouple gap junction communication, the protective effect did not appear to pass through gap junctions [17]. Although ET-1 has been shown to mediate a rapid and reversible morphological plasticity of the OECs the possible role of ET-1 in SCs remains unknown. Therefore it is likely that ET-1 acts indirectly on the peripheral odor signal generated in the OM by uncoupling gap junction in SCs.

In the present study, we have focused on a potential effect of ET-1 on olfactory processing of young rats as our previous studies were performed on 10 day old pups [17, 19]. We first confirmed *ex-vivo* with OM explants that ET-1, like a known gap junction uncoupling agent carbenoxolone (CBX), inhibits gap junctional permeability, limiting intercellular  $Ca^{2+}$  wave propagation. Then, using electro-olfactogram (EOG) recordings on hemi head, we found that ET-1 and CBX slowed down the repolarizing kinetics of the olfactory responses, without impacting their amplitudes. Finally, we tested the behavioral performances of rats in an odor-based orientation test. We confirmed the importance of ET-1 in olfactory processing but suggest that this effect does not rely solely on gap junction uncoupling as CBX application did not affect pup behavior.

## **2. 2. Material and methods**

### **2.1. Animals**

Wistar male rat pups (*Rattus norvegicus*) were obtained from multiparous females, and housed in our local animal care facilities (Unité Expérimentale Animalerie Rongeurs (UEAR), Jouy-en-Josas, France) in 12 h light, 12 h dark cycles. Mothers were fed water and standard chow *ad libitum* and bedding was changed weekly. All animal experiments were conducted in accordance with the European Communities Council Directive 2010/63/EU on the protection of animals used for scientific purposes, and approved by our French Local Ethics Committee (Comethea, CNREEA # 45; validated protocol #12-058). All efforts were made to minimize the number and suffering of the rats used. PC and NM hold the authorization for performing experiments in animals, including the animals

experiments conducted in the present study, provided by Préfecture des Yvelines (France), according to French and European laws (agreements #78-154).

## **2.2 Calcium imaging on OM explants**

OM explants were prepared from Wistar male rat pups (post-natal day 10 to 13: P10-13) killed by decapitation. For the OM explants, upper skull bone, lower jaw, teeth and palate were removed and the explant placed in ice-cold artificial cerebrospinal fluid (ACSF) solution containing (mM): 124 NaCl, 5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 2 MgSO<sub>4</sub>, 2 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub> and 10 Glucose, saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.4, ~300 mOsmol/L). A paramedial sagittal section of the nose allowed harvesting of the medial septum that separates the two nasal cavities. OM attached to both sides of the septum was submerged in oxygenated ACSF until use.

Calcium-sensitive dye Oregon green (5 mM stock solution in DMSO) was mixed 1:1 with 20% Pluronic F-127 (Molecular Probes) and diluted in oxygenated ACSF to a loading solution of 20 μM. OM slices or OM explants attached to the septal wall were kept in the loading solution for 30–40 min, followed by a 10-min washing out period in oxygenated ACSF. OM explants were transferred, one at a time, to a home-made recording chamber and anchored by a customized net with the mucus layer facing up for explants. The recording chamber was gravity perfused continuously with oxygenated ACSF at a rate of ~2.5 ml/min, and placed under a x20 long-range water-immersion objective (XLUMPLFL20XW, NA, 0.95) of an upright microscope (BX51WI, Olympus, Rungis, France), equipped with differential interference contrast and an IR video camera (placed after a x40 magnification changer).

Fluorescence measurements were performed using a dedicated Xcellence RT real time live cell imaging system (Olympus, Soft Imaging Solutions-OSIS, Münster, Germany) consisting of a computer-controlled fast illumination MT20 system with a 150W Mercury–Xenon mixed gas short arc burner, and an cooled C9100-13 EM-CCD camera (Hamamatsu Photonics, Massy, France). Fluorescence images were collected at 535nm at 1 Hz, with short exposure times (10-100 ms) to minimize bleaching.

To evaluate gap junctions coupling between SCs, we generated electrical extracellular stimulation on OM explants. The electrical stimulation was delivered by a bipolar theta glass pipette filled with 1M NaCl solution (500mV; 500ms duration). The pipette was placed ~10  $\mu\text{m}$  upon the tissue. A first stimulation was performed as a reference of signal propagation. It was followed by 30s of bath perfusion with either ACSF (n=22), ET-1 at  $10^{-7}\text{M}$  (n=32), CBX at  $10^{-4}\text{M}$  (n=20) or ATP at  $10^{-4}\text{M}$  (n=20). This duration was long enough to fully exchange the bathing medium with the stimulating solution. This bathing medium change was followed by 9.5 min of perfusion with ACSF allowing the recovery of the stimulated cells before applying a second identical electrical stimulation. The signal propagation was again compared between the same two ROIs. We measured the impact of the treatment on the gap junction communication as the percentage of propagation between second and first stimulation. Results are presented as mean  $\pm$  standard error of the mean (SEM). For all offline analysis of calcium transients, images were background subtracted and changes in fluorescence were calculated relative to the averaged baseline (from 30s before stimulation) and expressed as  $\% \Delta F/F$  ( $[(F - F_{baseline}) / F_{baseline}] \times 100$ ) using Xcellence RT software.

### **2.3 Electro-olfactogram (EOG) recording**

EOG recordings were made from the olfactory mucosa of rat pups (P10-13) in an opened nasal cavity configuration as described earlier [20]. The hemi-head was placed in a recording chamber under an upright Olympus SZ51 stereo microscope (Olympus, Rungis, France) equipped with a low magnification objective (0.8 to 4x) and two MX-160 micromanipulators (Siskiyou, Inc., Grants Pass, OR, USA). The odor stimulation device was modified from Scott and Brierley [21]. The hemi-head was kept under a constant flow of humidified filtered air (~1000 ml/min) delivered through a 9 mm glass tube. This tube was positioned 2 cm from the epithelial surface. Odor stimulations were performed by blowing air puffs (200 ms, 200 ml/min) through an exchangeable Pasteur pipette enclosed in the glass tube containing a filter paper impregnated with 20  $\mu\text{L}$  of odorant (Sigma Aldrich, Saint-Quentin Fallavier, France). Recordings were made with glass micropipettes of 4–5M $\Omega$ . Simultaneous EOG

recordings of the responses evoked by isoamyl acetate (1:10 in mineral oil) were recorded from endoturbinates II<sub>b</sub> and III. The recording electrodes were placed in the center of these endoturbinates. This position gave robust, reproducible and long-lasting EOG recordings. Stimulation with only mineral oil always produced signals of less than 1 mV amplitude.

EOG responses were recorded until their amplitudes stabilized, (i.e. at least 3 successive responses displaying the same amplitude), and the last response was taken as the reference. One endoturbinate was treated by local application of a saline solution, while the other endoturbinate received the tested molecule ( $10^{-3}$ M CBX (n=5) or  $10^{-6}$ M ET-1 (n=11)) through constant diffusion from a glass micropipette of  $\sim 10$   $\mu$ M diameter. The ionic composition of the saline solution was chosen to match as much as possible the mucus composition according to previous studies. Control and treated endoturbinates (II<sub>b</sub> and III) were systematically alternated from one rat hemi-head to the other. Responses were recorded on both endoturbinates simultaneously every 5min, for 20min following local treatment (**Fig. 2B**). Analysis were performed off-line using Clampfit 9.2 (Axon Instruments). Peak amplitude, rise time (from 10% to 90%), as well as fast (from 100% to 80%) and slow (from 40% to 10%) decay slopes of EOG responses were measured. Since the EOG response kinetics highly correlate with the amplitude, the rise time and the two decay slopes were normalized to the corresponding response peak amplitude prior to statistical analysis. The amplitude and kinetics of the olfactory signals recorded following the drug applications were normalized to those of the control responses prior to this treatment. Results are presented as mean  $\pm$  standard error of the mean (SEM).

## **2.4 Odorant driven orientation behavior**

In order to evaluate the impact of gap junction communication on olfactory performances of rat pups (P10-12), we monitored their orientation toward nest odorants in a behavioral test as described previously [22]. Tests were conducted between 10:00 and 11:00 a.m. on 12 litters of 10 to 12 Wistar rats (139 animals in total including male and female). For each litter, pups were separated from their

mother 45 minutes prior to testing and marked for video-tracking purposes using low-odor markers (UniPosca, Mitsubishi Pencil Co. Ltd, Worcester, UK). Spots were made at the center of the head and at the center of the body (**Fig. 3A**). Prior to the test, pups from the same litter received 10  $\mu$ L per nostril of the tested drug ( $10^{-4}$ M CBX (n=27 from 6 litters) or  $10^{-7}$ M ET-1 (n=27 from 6 litters)) or saline as a control (n=28 or 29 respectively).

10 min after the nasal instillation, pups were introduced individually at the center of a closed custom-made box (LxWxH: 29.7 x 18.7 x 5.5cm<sup>3</sup>), with a continuous airflow incoming from two opposite compartments expelled by a small fan at the base of the box ( $\sim 30\text{L}\cdot\text{min}^{-1}$ ; i.e.  $\sim 10$  box volume $\cdot\text{min}^{-1}$ ). One compartment contained sawdust collected from the home cage while the other contained only fresh sawdust. Videotaping was performed at 15 frames per second for 1min or until the animal tried to enter one of the compartments indicating a choice between the two odor sources. Pups treated with CBX or ET-1 were run alternatively in the test, while keeping exactly the same odor sources.

Videos were analyzed tracking both spots (head and body) using Kinovea freeware (<http://www.kinovea.org/>). We used the coordinates to calculate two unit vectors, one represented by the body dot to head dot (animal orientation vector) and one by the body dot to mother litter compartment (perfect orientation vector). For each frame, we calculated the difference of the scalar product of those vectors. A value of 2 indicates that both are aligned and of the same direction. In that case, the animal is perfectly orientated toward its mother smell while a scalar of -2 indicates the animal is perfectly orientated toward the fresh sawdust. A value of zero indicates no choice. If the animal chooses a compartment before the 1 min testing limit, the scalar value was assigned to 2 or -2 for the remaining frames according to the chosen compartment. The differences in product scalar values are then averaged for all frames to calculate a score of odor based orientation for each pups. The score was normalized by the mean of the group treated with saline. We also monitored their average velocity and pups that did not move were excluded from the analysis (n=13/7/8 for saline,

ET-1 and CBX treatment respectively). The pups not moving represented approximately 20 % of all animal tested (28 among 139). Results are presented as mean  $\pm$  standard error of the mean (SEM).

## 2.5 Statistical analysis

Data were statistically analyzed using GraphPad Prism software (GraphPad software Inc., CA, USA). We used one-way ANOVA for calcium imaging, two-way ANOVA for EOG analysis, and a Mann-Whitney test for behavior. When ANOVA indicated a significant effect, post-hoc test were used to determine individual differences between responses. We used Newman-Keuls multiple comparison of means test for one-way ANOVA, and Bonferroni multiple comparison of means for two-way ANOVA. A probability value of  $P < 0.05$  was used as an indication of significant differences.

## 3. RESULTS

### 3.1 ET-1 induces gap junction uncoupling of sustentacular cells *ex vivo*

Previously, using a primary culture of OM cells, we have demonstrated that SCs are sensitive to ET-1 [15] and that ET-1 uncouples their gap junctions [17]. In order to explore the biological relevance of this phenomenon in a preserved cellular environment of SCs, we used calcium imaging in OM explants to measure the impact of ET-1 treatment on the propagation of a calcium wave initiated by an electrical impulse (**Fig. 1A**). The propagation was evaluated by comparing the level of fluorescence induced near the stimulating electrode with the background fluorescence measured on a distant annular region. This relative propagation was analyzed on the same region, before and after application of either a saline solution (ACSF) or various pharmacological treatments. Results are presented as the relative propagation after treatment compared to the first stimulation used as a reference (**Fig.1B**). A one-way ANOVA indicated significant differences among groups ( $P < 0.0001$ ). On average, signal propagation was reduced after the first electrical stimulation for saline solution-treated cells (61% of remaining fluorescence), and post-hoc Newman-Keuls multiple comparison of

the means indicated that this decrease was significantly enhanced after treatment with ET-1 and CBX (28.1 and 39.4% respectively) but not ATP (65.5%).

### 3.2 ET-1 and CBX application on the OE modulates odorant detection kinetics

In order to evaluate the impact of ET-1 and CBX on odorant detection, we performed electro-olfactogram recordings (EOG) in an opened nasal cavity configuration (**Fig. 2A**). EOG signals arise from the summation of local depolarizations and repolarizations of OSNs during olfactory transduction [23]. Pilot experiments had shown that ET-1 application potentially modulates the kinetics of odorant responses of the OM for high concentration of odorants. We thus focused on the responses to isoamyl acetate diluted 1:10 in mineral oil. Simultaneous EOG recordings of the responses evoked by odorant stimulations were performed from the neighboring endoturbinates IIb and III for 20 min following treatment with either saline,  $10^{-6}$ M ET-1, or  $10^{-3}$ M CBX. Signals were normalized to the amplitude of the response prior to local treatment (**Fig. 2B**).

EOG signals are characterized by a fast decrease in amplitude reflecting the summation of olfactory transduction of OSNs near the recording electrode (**Fig. 2C**). Over time, the normalized EOG signal amplitude did not differ significantly between saline and treatment with ET-1 ( $F_{(1,80)}=0.04$ ;  $P=0.847$ ) or CBX ( $F_{(1,32)}=1.43$ ;  $P=0.266$ ) (**Fig. 2D**). After a few seconds following the odor stimulation, the signal returned to its initial value, reflecting the repolarization of OSNs. This repolarization is composed of a fast and slow part and only the latter appeared to be impacted by ET-1 and CBX treatment (Decay slope; **Fig. 2C**). The evolution over time of the slow component of the repolarizing phase was indeed significantly different from the saline treatment for both ET-1 ( $F_{(1,80)}=8.81$ ;  $P=0.018$ ) and CBX ( $F_{(1,32)}=16.51$ ;  $P=0.0036$ ) (**Fig. 2D**). These results indicate that ET-1 and CBX treatments delay the return of OSNs to the basal state of activity after odorant processing.

### 3.3 Odorant driven orientation behavior is impaired by ET-1 treatment but not by CBX

As the kinetic of OSN responses to an odorant was modulated by ET-1 and CBX, we next evaluated whether the same treatments would impact behavioral indices of olfactory detection. We used an orientation test for pups based on their innate attraction to maternal odors. At 12 days of age, rat pups are still blind and thus rely mainly on olfactory cues for orientation. In this test, we calculated the difference between two scalar products of unit vectors to score the orientation of the pup during the test (**Fig. 3A**). The difference in scalar product ranges from positive to negative values, respectively indicating a direct or opposite orientation of the pup toward the maternal odor source (**Fig. 3B**). We measured an orientation score based on the mean of the difference in scalar product for each frames. A Mann-Whitney test indicated that ET-1 treatment significantly lowered the orientation score of the pups compared to saline ( $P=0.005$ ) while the CBX treatment did not ( $P=0.78$ ; **Fig. 3C**). The velocity was not statistically different between groups ( $2.87\pm 0.44$  vs  $3.06\pm 0.55$  cm/s for saline and ET-1 group respectively;  $3.03\pm 0.35$  vs  $2.99\pm 0.37$  cm/s for saline and CBX group respectively).

## 4. Discussion

We have previously established that ET receptors were highly expressed in the OM [17]. We have also found that ET-1 could uncouple gap junction communication in OM primary culture cells, with no clear link to its anti-apoptotic effect [16]. In the present study, we investigated the impact of gap junction uncoupling by ET-1 on odorant detection.

In order to confirm the gap junction uncoupling effect of ET-1 on SCs in a preserved preparation, we first performed *ex vivo* calcium imaging experiments on explant of OM. In this preparation, all the apical cell bodies that can be visualized for imaging are SCs. We observed that both ET-1 and CBX significantly reduced the calcium wave propagation initiated locally by electrical stimulation. This observation is consistent with our previous study showing that ET-1 and CBX reduced gap junction coupling between SCs in primary culture. We next examined whether local application of ET-1 and

CBX on the surface of the OM would impact odorant detection. This detection is initiated in the OSN cilia where odorants binding to olfactory receptors induces changes in the activation state of those GPCRs, leading to modulation of the membrane potential [24]. EOG allows the recording of the summation of responses to odorant stimulation for thousands of OSNs [23]. We observed that ET-1 and CBX did not impact the amplitude of the odorant-induced EOG signal but slowed its recovery. The EOG amplitude signal is related to the olfactory receptor potential and the kinetic of the repolarization could be linked to the ionic composition of the mucus as shown before [25]. Indeed, the olfactory signal transduction consists of an inward current of sodium and calcium which in turn opens chloride channel allowing a chloride efflux. All those ionic movements impact the local ionic concentration surrounding OSN cilia and their concentration has to be restored in order to return to the basal state of OSN activity. Alteration of mucus ionic composition is known to impact the EOG signal [26]. SCs may play a key role in maintaining the ionic gradient required to fulfill the olfactory transduction, and uncoupling their gap junction could thus impact the restoration of mucus composition. Gap junctions are also present in OSN including Cx57 [27], thus the observed effect could be linked to a direct action of ET-1 and CBX on OSNs. However this seems unlikely, as EOG reflects the olfactory transduction in OSN cilia where gap junctions are absent. This would also be consistent with another study performed on the salamander *Necturus maculosus*, indicating a role for the coupling of OSN in cellular dynamics rather than olfactory transduction [28]. Our result contrasts with a study in mice expressing a dominant negative form of Cx43 in OSNs [29]. In those mice, the response to odorants recorded by EOG and calcium imaging were reduced. Similarly the authors observed that CBX application induced a decrease in response to odorant stimulation with calcium imaging [7]. The main differences with our study may be the animal age (10 day old rats *versus* 8-16 weeks mice) and the protocol for OM preparation. We used aerial EOG while the previous results were obtained with the OM immersed in saline. Thus, our experimental design preserves the mucus originating from the OM as much as possible, which could explain the difference between studies. Indeed, as the mucus layer is still present around OSN cilia, so are most

of the detoxifying enzyme that may limit the action of ET-1 and CBX [30]. Thus an impact of gap junction uncoupling agent on the mucus ionic composition may be seen with an aerial preparation but overlooked with an immersed preparation.

We next examined how the observed change in odorant detection could affect the animal ability to smell. The kinetic study of ET-1 action on OSN repolarization after odor stimulation indicates that ET-1 was most effective 10 min after treatment. Such delay matches the time window used to explore the impact of ET-1 on calcium wave propagation induced by electrical stimulation on OM explants. We thus used the same treatment time to study the impact on ET-1 nasal instillation on olfactory performances. Using a two odorant choice test, we found that ET-1 but not CBX intranasal instillation reduced the olfactory performances of rat pups. As CBX and ET-1 OM application have similar effects on the gap junction communication and odorant transduction kinetic, this indicates that in our study, the slowed repolarization of OSNs has no major impact on the ability of an animal to smell. These results contrast with the work on OMP KO mice where OSN repolarization is impaired along with deficits in olfactory performances [31]. However, OMP has been also described to be essential for olfactory bulb organization [32]. The difference between ET-1 and CBX treatment on odor based behavior of pups remains thus to be explored. A simple explanation would be that the steroid-like structure of CBX may be more sensitive to degradation by detoxifying enzymes of the mucus and thus impact less the odorant detection than ET-1 *in vivo* [30]. Another explanation could be that ET-1 have targets additional to CBX on the OM. Endothelin has first been characterized for its vasoconstrictor effect [12] and one could expect to observe a decrease of olfactory performances due to reduced blood flow in the OM treated with ET-1. This hypothesis is unlikely as other vasoconstrictor messengers like epinephrine are known to improve the ability to smell [33] and  $\alpha$ -adrenergic receptor agonists are classically used as nasal decongestants without impairment of olfactory performances [34, 35]. A more plausible possibility is that ET-1 not only acts on SCs but also on OSNs. Indeed, gap junction uncoupling may act both on the receptor potential through action on SCs and afterward the calcium wave in the OSNs cell body. ET-1 could act on OSN through

modulation of Cx43 phosphorylation as described by [7]. ET-1 could also act more deeply in the OM as peptides can cross the OM and reach the central nervous system [36]. It could then act on the OECs surrounding the axon bundles of OSNs [17]. Gap junction uncoupling of OECs suppress the propagation of calcium wave between those cells [37] potentially modulating the transmission of the information from the OM to the olfactory bulb [38].

Further studies are required to investigate whether our results also apply to adult rats. Indeed, in 10 day old rats the nervous system is immature and GAP junction component expression is known to change according to the development stage [39]. Our study also leaves many questions unanswered. Under what physiological conditions are ET levels modulated in the OM? Would food odors be similarly affected as home cage odors? Further studies are required to explore those questions.

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**Conflict of interests**

The authors declare no conflict of interests.

**Author contributions**

NM designed and performed experiments, analyzed data and wrote the paper; BB and MLB designed performed experiments and analyzed the data, PC, OR and CM designed experiments. BB, PC, CM and OR edited the manuscript.

## REFERENCES

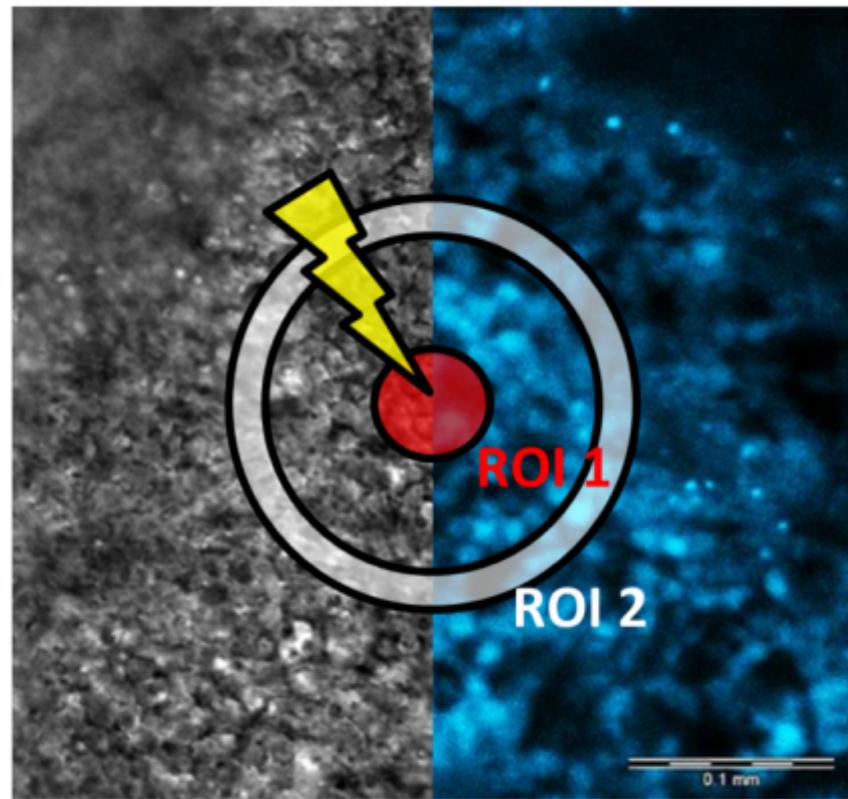
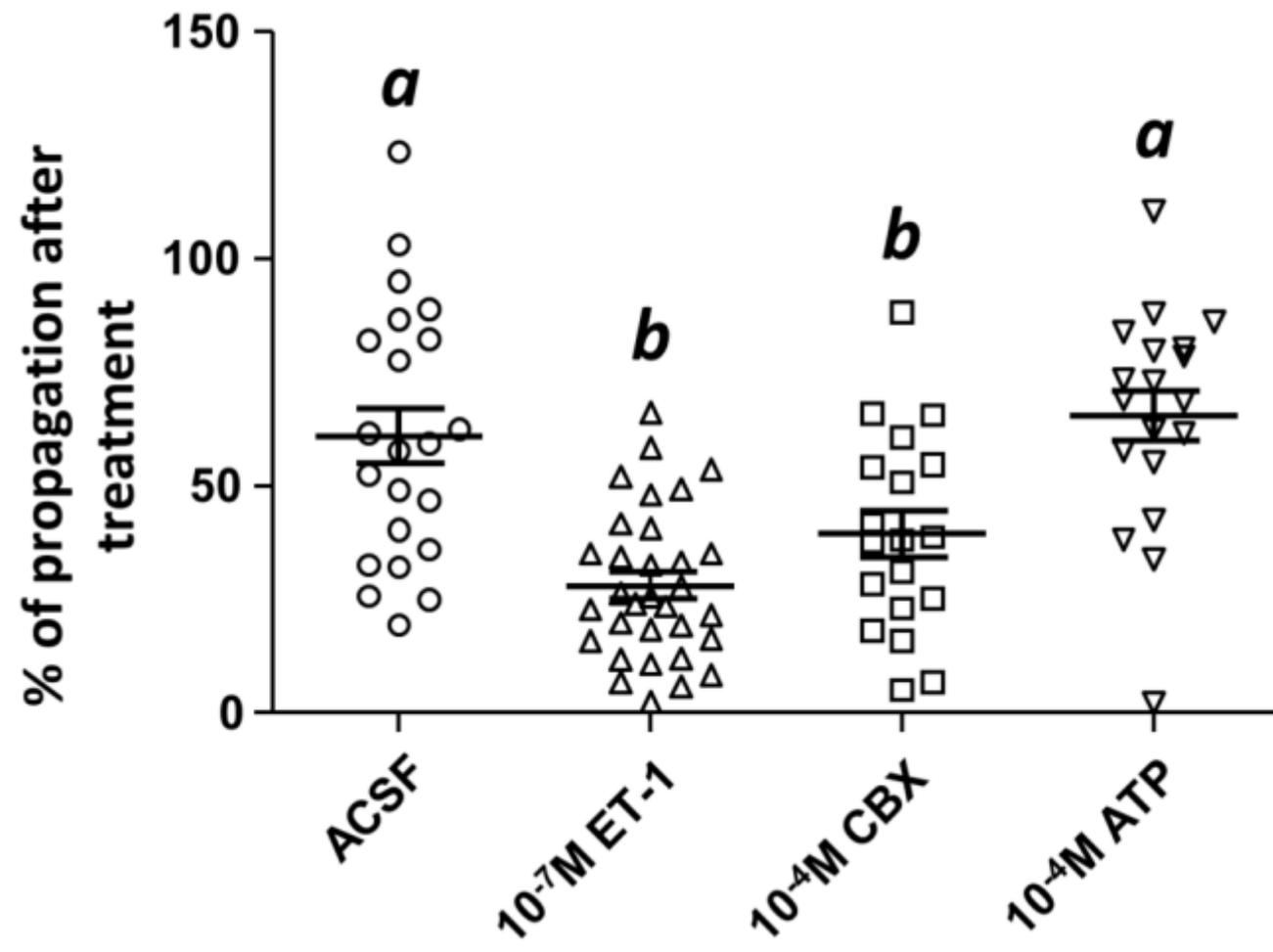
- [1] M.T. Lucero, Peripheral modulation of smell: fact or fiction?, *Seminars in cell & developmental biology* 24(1) (2013) 58-70.
- [2] A.K. Pack, L.J. Pawson, Neuroglial modulation in peripheral sensory systems, *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 16(4) (2010) 342-8.
- [3] M.L. Getchell, T.V. Getchell, Fine structural aspects of secretion and extrinsic innervation in the olfactory mucosa, *Microsc Res Tech* 23(2) (1992) 111-27.
- [4] R. Dooley, A. Mashukova, B. Toetter, H. Hatt, E.M. Neuhaus, Purinergic receptor antagonists inhibit odorant-mediated CREB phosphorylation in sustentacular cells of mouse olfactory epithelium, *BMC Neurosci* 12 (2011) 86.
- [5] C. Zhang, D. Restrepo, Expression of connexin 45 in the olfactory system, *Brain Res* 929(1) (2002) 37-47.
- [6] F. Vogalis, C.C. Hegg, M.T. Lucero, Electrical coupling in sustentacular cells of the mouse olfactory epithelium, *J Neurophysiol* 94(2) (2005) 1001-12.
- [7] Y. Yu, C. Zhang, The role of connexin 43 in mediating odor response, *Eur J Cell Biol* 94(6) (2015) 267-75.
- [8] J.C. Herve, M. Derangeon, Gap-junction-mediated cell-to-cell communication, *Cell Tissue Res* 352(1) (2013) 21-31.
- [9] M.S. Nielsen, L.N. Axelsen, P.L. Sorgen, V. Verma, M. Delmar, N.H. Holstein-Rathlou, Gap junctions, *Comprehensive Physiology* 2(3) (2012) 1981-2035.
- [10] W.H. Evans, Cell communication across gap junctions: a historical perspective and current developments, *Biochemical Society transactions* 43(3) (2015) 450-9.
- [11] U. Pannasch, D. Freche, G. Dallerac, G. Ghezali, C. Escartin, P. Ezan, M. Cohen-Salmon, K. Benchenane, V. Abudara, A. Dufour, J.H. Lubke, N. Deglon, G. Knott, D. Holcman, N. Rouach, Connexin 30 sets synaptic strength by controlling astroglial synapse invasion, *Nat Neurosci* 17(4) (2014) 549-58.
- [12] M. Yanagisawa, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, T. Masaki, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, *Nature* 332(6163) (1988) 411-5.
- [13] F. Blomstrand, L. Venance, A.L. Siren, P. Ezan, E. Hanse, J. Glowinski, H. Ehrenreich, C. Giaume, Endothelins regulate astrocyte gap junctions in rat hippocampal slices, *Eur J Neurosci* 19(4) (2004) 1005-15.
- [14] A. Khodorova, J.P. Montmayeur, G. Strichartz, Endothelin receptors and pain, *J Pain* 10(1) (2009) 4-28.
- [15] E. Gouadon, N. Meunier, D. Grebert, D. Durieux, C. Baly, R. Salesse, M. Caillol, P. Congar, Endothelin evokes distinct calcium transients in neuronal and non-neuronal cells of rat olfactory mucosa primary cultures, *Neuroscience* 165(2) (2010) 584-600.
- [16] I. Laziz, A. Larbi, D. Grebert, M. Sautel, P. Congar, M.C. Lacroix, R. Salesse, N. Meunier, Endothelin as a neuroprotective factor in the olfactory epithelium, *Neuroscience* 172 (2011) 20-9.
- [17] M. Le Bourhis, S. Rimbaud, D. Grebert, P. Congar, N. Meunier, Endothelin uncouples gap junctions in sustentacular cells and olfactory ensheathing cells of the olfactory mucosa, *Eur J Neurosci* 40(6) (2014) 2878-87.
- [18] A.J. Vincent, A.K. West, M.I. Chuah, Morphological plasticity of olfactory ensheathing cells is regulated by cAMP and endothelin-1, *Glia* 41(4) (2003) 393-403.
- [19] A. Francois, V. Bombail, D. Jarriault, A. Acquistapace, D. Grebert, X. Grosmaître, N. Meunier, Daily oscillation of odorant detection in rat olfactory epithelium, *Eur J Neurosci* (2017).
- [20] A. Francois, D. Grebert, M. Rhimi, M. Mariadassou, L. Naudon, S. Rabot, N. Meunier, Olfactory epithelium changes in germfree mice, *Scientific reports* 6 (2016) 24687.

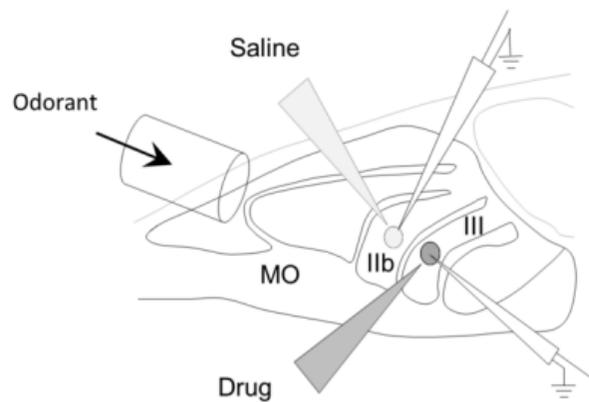
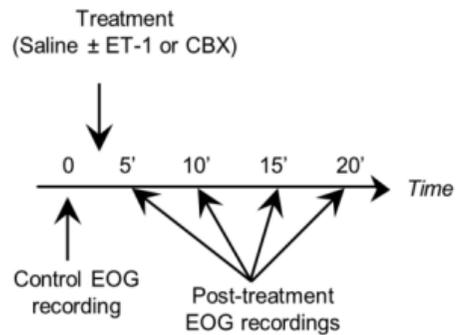
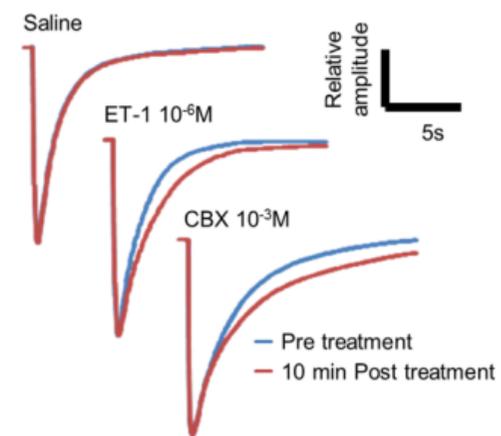
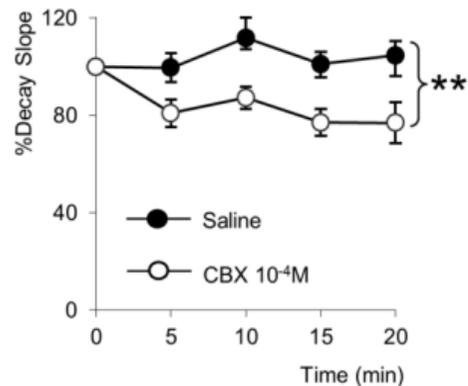
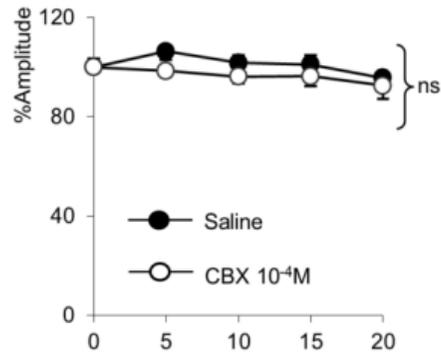
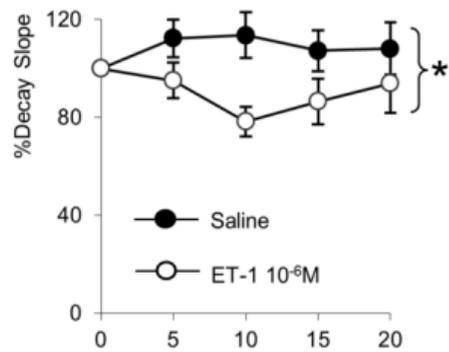
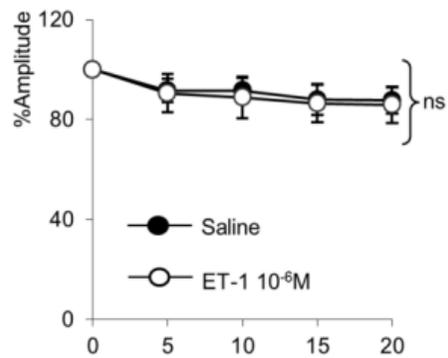
- [21] J.W. Scott, T. Brierley, A functional map in rat olfactory epithelium, *Chem Senses* 24(6) (1999) 679-90.
- [22] N. Meunier, B.L. Nielsen, Using scalar products to refine the interpretative value of an orientation choice test, *Chem Senses* 39(6) (2014) 551-60.
- [23] J.W. Scott, P.E. Scott-Johnson, The electroolfactogram: a review of its history and uses, *Microsc Res Tech* 58(3) (2002) 152-60.
- [24] M. Dibattista, S. Pifferi, A. Boccaccio, A. Menini, J. Reisert, The long tale of the calcium activated Cl<sup>-</sup> Channels in olfactory transduction, *Channels (Austin)* (2017) 0.
- [25] E.R. Griff, N.K. Kleene, S.J. Kleene, A selective PMCA inhibitor does not prolong the electroolfactogram in mouse, *PLoS One* 7(5) (2012) e37148.
- [26] S.F. Takagi, G.A. Wyse, H. Kitamura, K. Ito, The role of sodium and potassium ions in the generation of the electro-olfactogram, *J Gen Physiol* 51(4) (1968) 552-78.
- [27] C. Zhang, Expression of connexin 57 in the olfactory epithelium and olfactory bulb, *Neurosci Res* 71(3) (2011) 226-34.
- [28] R.J. Delay, V.E. Dionne, Coupling between sensory neurons in the olfactory epithelium, *Chem Senses* 28(9) (2003) 807-15.
- [29] C. Zhang, Gap junctions in olfactory neurons modulate olfactory sensitivity, *BMC Neurosci* 11 (2010) 108–123.
- [30] J.M. Heydel, A. Coelho, N. Thiebaud, A. Legendre, A.M. Le Bon, P. Faure, F. Neiers, Y. Artur, J. Golebiowski, L. Briand, Odorant-binding proteins and xenobiotic metabolizing enzymes: implications in olfactory perireceptor events, *Anat Rec (Hoboken)* 296(9) (2013) 1333-45.
- [31] J. Reisert, K.W. Yau, F.L. Margolis, Olfactory marker protein modulates the cAMP kinetics of the odour-induced response in cilia of mouse olfactory receptor neurons, *J Physiol* 585(Pt 3) (2007) 731-40.
- [32] D.F. Albeanu, A.C. Provost, P. Agarwal, E.R. Soucy, J.D. Zak, V.N. Murthy, Olfactory marker protein (OMP) regulates formation and refinement of the olfactory glomerular map, *Nat Commun* 9(1) (2018) 5073.
- [33] O. Escanilla, A. Arrellanos, A. Karnow, M. Ennis, C. Linster, Noradrenergic modulation of behavioral odor detection and discrimination thresholds in the olfactory bulb, *Eur J Neurosci* 32(3) (2010) 458-68.
- [34] D.A. Johnson, J.G. Hricik, The pharmacology of alpha-adrenergic decongestants, *Pharmacotherapy* 13(6 Pt 2) (1993) 110S-115S; discussion 143S-146S.
- [35] Y.G. Jung, S.Y. Ha, Y.G. Eun, M.G. Kim, Influence of intranasal epinephrine and lidocaine spray on olfactory function tests in healthy human subjects, *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 145(6) (2011) 946-50.
- [36] T.P. Crowe, M.H.W. Greenlee, A.G. Kanthasamy, W.H. Hsu, Mechanism of intranasal drug delivery directly to the brain, *Life sciences* (2017).
- [37] M. Stavermann, P. Meuth, M. Doengi, A. Thyssen, J.W. Deitmer, C. Lohr, Calcium-induced calcium release and gap junctions mediate large-scale calcium waves in olfactory ensheathing cells in situ, *Cell calcium* (2015).
- [38] A. Thyssen, D. Hirnet, H. Wolburg, G. Schmalzing, J.W. Deitmer, C. Lohr, Ectopic vesicular neurotransmitter release along sensory axons mediates neurovascular coupling via glial calcium signaling, *Proc Natl Acad Sci U S A* 107(34) (2010) 15258-63.
- [39] R. Dermietzel, O. Traub, T.K. Hwang, E. Beyer, M.V. Bennett, D.C. Spray, K. Willecke, Differential expression of three gap junction proteins in developing and mature brain tissues, *Proc Natl Acad Sci U S A* 86(24) (1989) 10148-52.

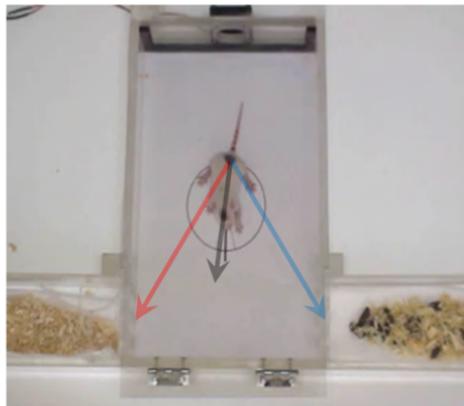
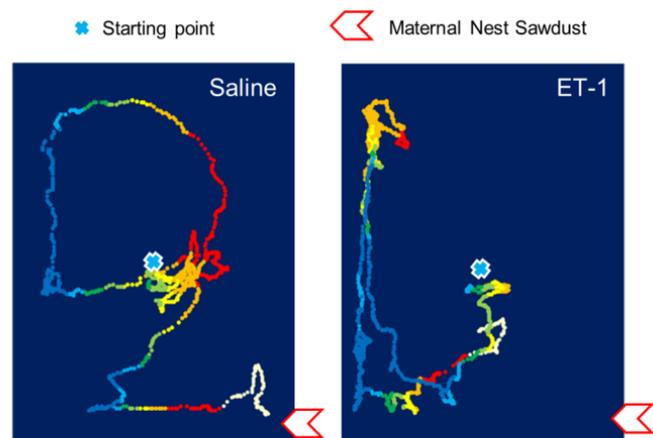
**Figure 1:** ET-1 application decreased the propagation of a calcium wave in an OM explant similarly as the gap junction uncoupling agent CBX. **(A)** DIC/IR (left) and epifluorescence (right) images of an explant loaded with the calcium dye Oregon Green. Region of interest (ROI) used to measure the propagation of a calcium wave in ROI 2 initiated by an electrical stimulation at the center of ROI 1. **(B)** Dot plot of the percentage of propagation between the first and second stimulation performed after various treatments: Control saline solution ACSF (n=22), ET-1 (n=32), CBX (n=20) or ATP (n=20). Results are presented as mean  $\pm$  standard error of the mean (SEM). Different letters indicate significant differences (One-way ANOVA; Newman-Keuls Multiple Comparison Test;  $P < 0.01$ ).

**Figure 2:** Kinetic of olfactory transduction is slowed down by ET-1 and CBX treatments. **(A)** Schematic diagram of the experimental preparation allowing the simultaneous recording of two OM areas treated with saline or a drug. EOG recordings were performed on endoturbinates IIb and III located centrally in the nasal cavity. **(B)** Time course of recordings and treatments. **(C)** Typical normalized EOG responses to isoamyl acetate before and after treatment. **(D)** Effects of treatment on the time course of normalized EOG amplitudes and slow component of the repolarizing phase (decay slope) in response to isoamyl acetate. Mean  $\pm$  SEM values are expressed as percentage of control EOG recordings prior to treatment (n=11 and 5 for ET-1 and CBX treatments, respectively). Two-way ANOVA (ns, non-significant; \*P<0.05; \*\*P<0.01).

**Figure 3:** Orientation based on olfactory cues is impaired by ET-1 but not CBX treatment. **(A)** Aerial view of the box used for olfactory performance evaluation. The rat pup is placed in the middle of the arena at the beginning of the test. Three vectors are used to score the orientation of the pup. The score is based on the difference between the scalar product of the pup orientation vector (gray) and the vector directed to the dam odor source (blue) or clean saw dust (red). **(B)** Examples of locomotor behavior for two pups during the 1-min trial, each dot represents a video frame and the color goes from dark blue (pup orientated toward cleaned saw dust) to white (pup orientated toward dam odor). **(C)** Dot plot representing the score of each pup relative to saline condition with mean  $\pm$  SEM (Saline/ET, n=29/27; Saline/CBX, n=28/27). Mann-Whitney (ns, non-significant; \*P<0.01).

**A****B**

**A****B****C****D**

**A****B****C**