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ESTRADIOL RAPIDLY MODULATES ODOR RESPONSES IN MOUSE VOMERONASAL SENSORY NEURONS

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Abstract

In rodents, many social behaviors are driven by the sense of smell. The vomeronasal organ (VNO), part of the accessory olfactory system mediates many of these chemically driven behaviors. The VNO is heavily vascularized, and is readily accessible to circulating peptide or steroid hormones. Potentially, this allows circulating hormones to alter behavior through modulating the output of the primary sensory neurons in the VNO, the vomeronasal sensory neurons (VSNs). Based on this, we hypothesized that steroid hormones, in particular 17 β -estradiol, would modulate activity of VSNs. In this paper, we show that the estrogen receptors, GPR30 and ER α , were present in VSNs and that estradiol may be synthesized locally in the VNO. Our results also showed that 17 β -estradiol decreased responses of isolated VSNs to dilute urine, a potent natural stimulus, with respect to current amplitudes and depolarization. Further, 17 β -estradiol increased the latency of the first action potential (AP) and the AP amplitude. Additionally, calcium responses to sulfated steroids (present in the low molecular weight fraction of urine) that act as ligands for apical vomeronasal receptors were decreased by 17 β -estradiol. In conclusion, we show that estradiol modulates odorant responses mediated by VSNs and hence paves the way for future studies to better understand the mechanisms by which odorant mediated behavior is altered by endocrine status of the animal.

Keywords

estrogen; vomeronasal organ; GRP30; ER α ; aromatase; estrogen production

INTRODUCTION

In rodents, the olfactory system comprises four main areas located in the nasal cavity: (1) the main olfactory epithelium, (2) the vomeronasal organ (VNO), (3) the septal organ, and (4) the Gruenberg Ganglia. While all of the olfactory neurons in these areas respond to chemical cues, the vomeronasal sensory neurons (VSNs) respond to numerous socially relevant chemical cues found in bodily secretions such as sweat or urine that represents complex mixtures of low molecular weight compounds such as sulfated steroids (Nodari et

al., 2008) and higher molecular weight peptides (Leinders-Zufall et al., 2004) and proteins (Halpern and Martinez-Marcos, 2003). Structurally, the VNO is a paired tubular organ located anteriorly in the nasal cavity adjacent to the septum. The lumen of this tubular structure is ensheathed by epithelia. The VSNs line the medial portion of the epithelium. Chemical cues are drawn into the lumen of the VNO through a duct where they can access microvilli on the dendritic knobs of VSNs (Meredith and O'Connell, 1979; Meredith et al., 1980; Ben-Shaul et al., 2010). Odors/pheromones bind to specific receptors, V1Rs located in the apical portion of sensory epithelium of the VNO and V2Rs expressed in VSNs located in the basal portion of the sensory epithelium. When bound, these G-protein-coupled receptors activate phospholipase C (PLC), releasing diacylglycerol (DAG) that activates TRPC2 (Transient Receptor Potential Channel type 2), a non-selective cation channel (Lucas et al., 2003). Additionally, DAG may be converted to arachidonic acid that can activate calcium-permeable channels (Zhang et al., 2010). Further amplification of these cation currents is facilitated by calcium-activated chloride channels (Yang and Delay, 2010; Kim et al., 2011; Dibattista et al., 2012). Signals from VSNs are relayed into the accessory olfactory bulb which is connected to the medial amygdala and other portions of the limbic system, such as the hypothalamus and the bed nucleus of stria terminalis thereby illustrating that olfactory cues dictate certain behaviors (Halpern and Martinez-Marcos, 2003).

Functionally, surgical removal of the VNO leads to behavioral deficits such as a decreased aggression and ultrasonic vocalizations in males (Wysocki and Lepri, 1991). Additionally, in female mice, normal pregnancy termination due to exposure to strange males (Bruce effect) is eliminated. Moreover, TRPC2^{-/-} male mice show a decrease in aggression and an increase in male-male mounting (Leypold et al., 2002; Stowers et al., 2002). It has also been reported that TRPC2^{-/-} females show increases in mounting both males and females and, interestingly, ultrasonic vocalizations toward both sexes (Kimchi et al., 2007). For these reasons, the VNO appears to be a crucial peripheral sensory structure that has a strong role in determining behavioral responses to chemosensory stimuli.

Behavior is often influenced by the endocrine status of the animal. For example, female mice are more interested in male mice in the middle of their estrous cycle. Since the VNO is highly vascularized, any hormone released in the blood will reach the VSNs quickly and could affect their chemosensory responses. However, responses to specific chemosensory stimuli could alter the endocrine status of an individual mouse i.e. male mice show a surge in testosterone when exposed to females (Wysocki et al., 1983). Thus, it appears that the VNO and the endocrine state of the animal are linked and each is influenced by the other. As a result, there has been considerable interest in better understanding how the steroid hormone, estradiol, modulates behavior through the VNO. Studies have shown that male mice show a preference for urine with estradiol rather than urine alone (Achiraman et al., 2010). Further, a single injection of estradiol increased aggression in mice within 15 min, thereby suggesting a role for estradiol in rapid behavioral changes (Trainor et al., 2007, 2008). In rodents, such rapid effects of estradiol are facilitated via local production of estradiol in brain (Woolley, 2007). This non-circulatory source is evident by the presence of aromatase, an enzyme that converts testosterone to estradiol. Aromatase is present in peripheral sensory structures such as the taste epithelium and main olfactory epithelium

(Lupo et al., 1986; Toyoshima et al., 2007). Based on these findings, we hypothesized that estradiol can rapidly affect VSN signaling.

In this paper we show data that (a) VSNs express estrogen receptors GPR30 (G-protein-coupled receptor 30) and ER α (estrogen receptor α); (b) estradiol decreased responses induced by urine and select sulfated steroids; and (c) estradiol may be locally produced in the VNO. Our results supported our hypothesis that estradiol functions as a neuromodulator in the VNO decreasing responses to most applied stimuli rapidly.

EXPERIMENTAL PROCEDURES

Animals

Wild-type C57BL/6, BALB/c, B6 and OMP-GFP mice over 2 months of age were used for these experiments. For immunocytochemistry, OMP-GFP transgenic mice (a kind gift from Peter Mombaerts, Rockefeller University) (Potter et al., 2001) were used to positively identify VSNs. In these mice, green fluorescent protein (GFP) is expressed under the promoter for the olfactory marker protein (OMP), which is only present in mature olfactory neurons in all olfactory structures. VNOs from ovariectomized (OVX) B6 mice were a gift from Dr. Cori Teuscher, University of Vermont. All animals were maintained and euthanized in accordance with the University of Vermont Animal Care and Use Committee (IACUC) guidelines.

Preparation of isolated VSNs

VSNs were isolated from the mouse VNO according to a previously reported protocol (Zhang et al., 2008). In brief, the VNO was dissected from euthanized mice and cut into small pieces in divalent cation-free solution in mM: 140 NaCl, 10 HEPES, 10 Glucose, 5 KCl, pH 7.4) with 55 μ g/ml papain at RT and kept in the solution for 15 min. This was followed by trituration of the dissociated tissue using a wide-mouthed Pasteur pipette. Ringers (in mM: 138 NaCl, 10 HEPES, 10 Glucose, 5 KCl, 2 MgCl₂, 2 CaCl₂, pH 7.4) with 11 μ g/ml leupeptin was added to stop the activity of papain. After filtration through a 250- μ m nylon mesh, the cells were kept in Ringers for up to 4 h.

For immunocytochemistry, VSNs were isolated using a trypsin-collagenase-DNase protocol instead of the papain protocol for higher yield of VSNs and supporting cells (Maue and Dionne, 1987; Liman and Corey, 1996). Briefly, the tissue was isolated and cut into several small pieces in a mixture of trypsin (1 mg/ml) and collagenase from *Clostridium histolyticum* type 1A (1 mg/ml) in dissociation solution (in mM: 140NaCl, 10 HEPES, 10 Glucose, 5 KCl, 2 EGTA) for 5 min and gently rocked for 1 h at RT. The preparation was centrifuged at 4200 rpm for 1 min and the supernatant decanted. The pellet was resuspended in DNase 1 bovine pancreas (1 mg/ml) in Ringers solution and gently rocked for 15 min after which cells were ready to be used.

Solutions and chemicals—Sulfated steroid mix at a final concentration of 10 μ M was made using stocks of E1050 (1,3,5(10)-estratrien-3,17 diol disulfate, disodium salt), A7864 (5-androsten-3 β ,17 β diol disulfate, disodium salt), P8200 (4-Pregnean-3 β -ol-20-one sulfate sodium salt), Q1570 (4-pregnen-11 β ,21-diol-3,20-dione 21-sulfate sodium salt). Each of the

stocks was made as 10 mM and dissolved in methanol as per the manufacturer's instructions (Steraloids, MA, USA). 17 β Estradiol was dissolved in dimethyl sulfoxide (DMSO) and diluted in Ringers solution. Gramicidin was freshly dissolved in DMSO and diluted in intracellular solution just before use. Methanol at a concentration less than 0.1% and DMSO at a concentration less than 0.5% had no effect on VSNs.

All chemicals were purchased from Sigma unless stated otherwise. Other chemicals purchased included: papain and leupeptin were from USB, OH, USA. Trypsin was from GIBCO, NY, USA. Fura-2AM was from Invitrogen, CA, USA. Synthetic sulfated steroids were purchased from Steraloids, MA.

Mouse urine collection—Urine was pooled from approximately 30 female and male mice. The urine was filtered using a 0.2- μ m filter and stored in -80°C . For patch clamp experiments, urine was diluted in Ringers (1:100–500).

RT-PCR

Total RNA was extracted from the mouse VNO and brain according to the manufacturer's instructions (Qiagen RNeasy Mini Kit, Germany). The amount and quality of RNA were determined using a Nanodrop Spectrophotometer. Approximately 4- μ g total RNA (in 20 μ L) plus 1.25 μ M random hexamers (Invitrogen, NY, USA), and 200U Superscript III reverse transcriptase (Invitrogen) were used to synthesize cDNA. The negative control included RNA from the brain and VNO without any reverse transcriptase. Two microliters of cDNA was used as template for the following PCR reactions: 95 $^{\circ}\text{C}$ 5 min, 35 cycles of 95 $^{\circ}\text{C}$ 1 min, 58 $^{\circ}\text{C}$ 1 min, 72 $^{\circ}\text{C}$ 1 min, 72 $^{\circ}\text{C}$ 10 min. Primers for GPR30, ER α , ER β (estrogen receptor β) and aromatase were as follows (5' to 3'):

(GPR30) F-CCTCTGCTACTCCCTCATCG; R-ACTA TGTGGCCTGTCAAGGG (234 bp) (NM_029771); (ER α) F-ACCATTGACAAGAACCGGAG; R-CAGAATAGATCATGGGCGGT (307 bp) (NM_007956) (ESR1); (ER β) F-GAAGCTGGCTGACAAGGAAC; R-GTGTCA GCTTCCGGCTACTC (440 bp) (NM_010157) (ESR2); (Aromatase) F-CCTGACACCATGTCGGTCACT; R-GGGCTTAGGGAAGTACTCGAG (309 bp) (Karolczak et al., 1998).

Products were run on 1.3% agarose gels at 120 V, stained using Ethidium Bromide, and imaged using a digital camera. Products were cut out and gel digestion was performed using a gel extraction kit (E.Z.N.A, OmegaBio-Tek, GA, USA). Some PCR products were treated with the ExoSAP kit (Affymetrix, CA, USA) instead of using the gel extraction protocol. At the Vermont Cancer Center DNA Analysis facility, extracted DNA was sequenced using ABI Prism 3130X1 Genetic Analyzer and analyzed using Sequencher v4.8 software. Identity of consensus sequences (obtained from sequencing of both sense and antisense strands of the gene and confirmation of the exact sequence identity at each base) was determined using BLAST. Gel images were adjusted for brightness and contrast using Image J.

Immunohistochemistry—VNO tissues from BALB/c or OMP-GFP mice were fixed for an hour with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) in 0.1 M phosphate buffer. The VNO was cryoprotected by running through a graded sucrose

series (0.5, 1, 1.5 and 2 M). Tissues were sectioned at 20 μ m using a cryostat. Free-floating sections were processed for immunohistochemistry as described below.

Immunocytochemistry—Isolated VSNs from BALB/c or OMP–GFP mice were placed on concanavalin A-coated cover slips and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. After rinsing with phosphate buffer, cells were incubated with blocking solution for 1 h (8% normal goat serum + 0.6% Triton X-100 in phosphate-buffered saline (PBS)) and incubated in rabbit anti-GPR30 (1:2000 in blocking solution) or rabbit anti-ER α (1:100) overnight. Rabbit anti-GPR30 antibody (MBL, MA) recognizes the intracellular C terminus of mouse GPR30 (for Fig. 3A, B), while anti-ER α (ThermoFisher, MA, USA) recognizes the N terminus of mouse ER α . After washing, the cells were incubated with 1:500 (GPR30) and 1:1000 (ER α) diluted goat-anti rabbit Rhodamine Red X-628 (Invitrogen) for 1 h. Control cells were treated the same as experimental, but without incubation with the primary antibody. All immunocytochemistry images were taken with Deltavision deconvolution microscope system in UVM COBRE Imaging facility with a Rhodamine filter for secondary antibody labeling (excitation wavelength is 541–569 nm and emission wavelength is 580–654 nm) and a FITC filter for GFP labeling (excitation wavelength is 480–500 nm and emission wavelength is 509–547 nm) at 0.1 s exposure and deconvoluted with SoftWorx software (Applied Precision, WA, USA). Image J (NIH) was used for an analysis of images from immunohistochemistry and immunocytochemistry.

Western blot—VNO and control tissues were homogenized and sonicated in a lysis buffer containing RIPA and protease inhibitors on ice. Proteins were run on a gradient SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) (Precise Protein Gel 8–16%). The proteins were then electroblotted onto nitrocellulose membranes using a Mini-PROTEAN blotting apparatus (Biorad) overnight at constant voltage of 33 V overnight at 4 °C. After transfer, the nitrocellulose membrane was blocked with an Odyssey blocking buffer (Li-COR Biosciences) for 45 min on a rocker. The membrane was then washed with TBST (Tris-buffered Saline and Tween20) and incubated in anti-rabbit ER α antibody (1:100) made in blocking solution for 1.5 h at RT. This was followed by washes with TBST and incubation in goat anti-rabbit IRDyeCW800 (Li-COR) (1:10,000). Washes were done in TBST and imaged using an Odyssey imaging system. The membranes were then incubated with anti-rabbit pan α actin antibody (1:1000) (Cell Signaling) followed by washes and incubation with goat anti-rabbit IRDyeCW800. Uterus, brain and MCF7 breast cancer cells were used as positive controls. MCF7 cells were a gift from Dr. Gary Stein, University of Vermont.

Electrophysiology

Isolated VSNs were placed in a recording chamber and observed under a Nikon Eclipse TE 2000S inverted microscope. For perforated patch clamp recordings, gramicidin was used as a channel-forming agent since it allows Na⁺ and K⁺ but not Ca²⁺ or Cl[−] to move across the membrane. Electrodes with a resistance of 5–8 MOhm were filled with intracellular solution (in mM: 110 K-gluconate, 30 KCl, 10 NaCl, 10 HEPES, 1 EGTA, 1 MgCl₂, 0.023 CaCl₂, pH 7.4) at the tip and back filled with intracellular solution + 0.27 mg/ml gramicidin

prepared fresh daily. Borosilicate glass (o.d. = 1.5 mm, i.d. = 0.86 mm) was used to pull electrodes using a Flaming/Brown P-97 micropipette puller (Sutter Instruments, CA, USA). After giga-seal formation, 45–60 min were required for complete perforation which was evaluated by the appearance of peak voltage-gated inward currents within 10 ms of voltage stimuli (–90 mV to +90 mV with 20 mV increment). For current clamp experiments, cells were injected with a small amount of current (0.1–10 pA) to set the initial membrane potential at –60 mV. Electrophysiological recordings were made using a Molecular Devices Digidata 1322, Multiclamp 700B, and pClamp 8.2 software. Data were Bessel filtered at 4 kHz with a sampling interval of 10 kHz. Stimuli were delivered for 500 ms in voltage clamp and 2 s in current clamp using a Warner fast step SF 77B perfusion system under continuous bath flow. Data were analyzed using Clampfit (Molecular Devices, CA, USA) and Origin 7.5 (OriginLab, MA, USA) was used to prepare figures. The amplitude of the urine-induced inward current was measured by subtracting the peak response from the holding current at –80 mV.

Calcium imaging

Isolated VSNs were plated on concanavalin A (1 mg/ml)-coated cover slips fitted into a recording chamber. Cells were loaded with 8 μ M Fura 2-AM (Molecular Probes, Eugene, OR, USA) and 1% pluronic (F-127, Molecular Probes, Eugene, OR) in Ringers solution for 20 min at RT. Loaded cells were washed in Ringers solution for 10 min prior to recording. Calcium imaging was done using a Nikon Eclipse TE2000S microscope. A Xenon arc lamp (Ushio) was used as power source. Images were acquired at 5-s intervals using a high resolution CCD digital camera (ORCA-285, Hamamatsu). Simple PCI 6.0 software (Hamamatsu) was used to acquire images. Exposure time was 100 ms for both excitation wavelengths (340 and 380 nm) with emission at 510 nm. VSNs were identified based on morphology. Criterion for a response was any Ca^{2+} -transient that was 10% above baseline and reproducible. Calcium transients (expressed as F340/F380) were background subtracted before analysis and quantified with the following equation:

$$R \text{ (response)} = R \text{ (peak)} - R \text{ (baseline)}$$

The viability of cells that did not respond to sulfated steroid mix was determined from calcium responses to ATP (Vick and Delay, 2012). Stimuli were locally applied with the same perfusion setup used for electrophysiology.

Determination of estradiol in VNOs by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Sample preparation—Estradiol concentration in VNOs was determined as previously described (Anari et al., 2002; Nguyen et al., 2011). VNOs (5–10 mg) were homogenized and sonicated in 0.5 mL PBS (pH 7.2).

Four-hundred microliters of ethyl acetate was added to 150–200 μ L of the tissue homogenates, along with deuterated beta E₂ (17 β -estradiol-IS: 17 β -estradiol-16,16,17-d₃, Sigma) as internal standard. After mixing thoroughly and centrifugation, the upper layer (95%) was transferred to another centrifuge tube. The tissue homogenates were extracted

two more times with ethyl acetate. The organic layer of each homogenate (a total volume of ~1.2 mL) was dried down by Speedvac. Calibration standards consisted of a serial dilution of 17 β -estradiol (1500, 1000, 750, 500, 375, 250, 187.5, 125, 93.75, 62.5, 46.875, 31.25, 23.4375, 11.71875 pg in 500 μ L methanol) were freshly prepared before each analysis. One hundred-microliter calibration standards spiked with 17 β -estradiol-IS were dried. To the dried organic extracts and calibration standards 50 μ L of 100 mM sodium bicarbonate buffer (pH 10.5) was added followed by vortexing. Since estradiols have low ionization efficiencies in electrospray ionization (ESI), derivatization was done to introduce ionizable moieties and enhance the detection limits of ESI-MS analysis. Derivatization was carried out by adding 50 μ L of dansyl chloride solution (1 mg/mL in acetone). After thorough mixing, the mixtures were incubated at 60 °C for 10 min with occasional shaking and then stored at –80 °C until LC–MS analysis.

LC–MS/MS—LC–MS analyses were performed on the Finnigan LTQ linear ion trap mass spectrometer (ThermoFisher Scientific, CA, USA) coupled to a Prominence HPLC system (Shimadzu, Nakagyo-ku, Japan). Operating parameters were as follows: spray voltage at 4.5 kV, sheath gas at 10 units, and capillary temperature at 275 °C. Maximum Injection Time = 100 ms. Tuning of instrument parameters was performed at a flow rate of 50 μ L/min by introducing derivatized 17 β -estradiol (250 μ g/mL in 50% acetonitrile/0.1% formic acid) into the LC flow (48 μ L/min) using a syringe pump (2 μ L/min) with a T-connection. Organic extracted samples and calibration standards (0.56 – 36 μ g on column; spiked with IS) were chromatographed on a C18 reverse phase column (Hypersil Gold, 100 $\times$ 1 mm, particle size: 3 μ m, pore size: 17.5 nm; equipped with a 10 $\times$ 1 mm drop-in guard cartridge) at a flow rate of 50 μ L/min. The solvent systems were water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B). The LC gradient was as follows: 50% B for 5 min, 50–100% B in 4 min, followed by a hold at 100% B for 3 min and an immediate return to 50% B and a hold at 50% B until the end of the run (20 min). The salt containing effluent during the first 12 min of the run was diverted to waste using the divert/inject valve on the LTQ. The temperature of the autosampler was set at 5 °C. The dansyl chloride derivative specific transitions for estradiol (m/z 506.3 > m/z 171.1) and 17 β -estradiol-IS (m/z 509.3 > m/z 171.1) were monitored (Selected Reaction Monitoring). Isolation window was set as 1 Da and collision energy as 42%. Mass range of m/z 169– 173 was monitored. Samples and calibration standards were analyzed over two consecutive days in three technical triplicates. Peak areas were manually integrated using the XCalibur program (ThermoFisher Scientific, CA), and quantification was carried out by comparing the normalized peak elution areas [Area (E₂)/Area (17 β E₂-IS)] against the calibration curve (Gaussian: 5; Enable Baseline subtraction: Polynomial order: 2; Below curve (%): 10; Tolerance: 0.01). Assay accuracies were determined by analyzing 0.75, 1.125, 1.5, 3, 6 and 12 pg of 17 β -estradiol spiked with 17 β -estradiol-IS in PBS treated in the same manner as samples undergoing ethyl acetate extraction.

Statistical analysis

For electrophysiology experiments, each isolated VSN was usually from a different animal; however more cells per animal were recorded for calcium imaging. Graph pad and Origin 7.5 were used for statistical tests and making graphs. Normality of data was tested using

D'Agostino–Pearson test. A repeated measures analysis of variance (ANOVA) or two-tailed t-test was used for statistical tests. Data are presented as Mean $\pm$ SEM.

RESULTS

Receptors for estrogen were present in VSNs

17 β -estradiol elicits physiological responses by binding to either of the two classical receptors, ER α and ER β , or the recently characterized G-protein-coupled estrogen receptor, GPR30. To determine if any of these three estrogen receptors were present in the VNO, we first examined mRNA expression using RT-PCR. Our results showed the presence of ER α and GPR30 but not ER β in the VNO (Fig. 1A). The bands were sent to the Vermont Cancer Center DNA Analysis facility and they confirmed the identity of these PCR products by sequencing and BLAST.

The ER α antibody was relatively new and did not have a control peptide so as an additional control, western blot experiments were done with the ER α antibody. Bands were seen as expected at 67 kDa that is the full length ER α receptor. Two additional bands were seen around 80 kDa and 50 kDa, probably splice variants. These three bands were seen in VNO from both male and female mice (Fig. 1B).

To determine if VSNs expressed these two estrogen receptors, we used immunohistochemistry on coronal sections of VNO (Figs. 2A and 3A) and immunocytochemistry on isolated VSNs (Figs. 2B and 3B, C). Shown are the results from experiments done on tissues from OMP–GFP mice, so that all the mature VSNs could be identified by GFP expression and from BALB/c mice to rule out any cellular changes in the OMP–GFP mice. Our results showed that the expression patterns of ER α and GPR30 were different.

ER α —In order to determine the expression of ER α , we used an antibody that targeted the N terminus. ER α was found throughout the epithelium and under the basal lamina, in what appeared to be axon bundles (Fig. 2A). The VSNs appeared to be labeled in the dendritic region, but it was difficult to be certain since the ER α label overwhelmed the GFP fluorescence. In order to confirm that the VSNs were labeled, we examined isolated cells. Most of the isolated VSNs showed faint labeling in the soma while the dendrites and dendritic knobs were strongly labeled (Fig. 2B; $n = 35/39$; $\sim 89\%$).

GPR30—To determine the expression of GPR30, we used an antibody that targeted the C terminus of GPR30. Since sections from tissues with OMP–GRP mice were visually difficult to separate the GPR label (see Fig. 2A) from the Rhodamine-tagged antibody, we used VNOs from BALB/c. We observed widespread labeling of GPR30 in the tissue sections (Fig. 3A), which was still difficult to unambiguously identify the cell types. Controls leaving out the primary antibody showed little to no labeling. By using immunocytochemistry, we were able to resolve GPR30 labeling in the somato-dendritic region, plasma membrane, dendrite, and knob of VSNs (approximately 90% of the cells labeled; Fig. 3B). No differences between males and females were observed in the expression patterns of both

receptors with any of the antibodies. In Fig. 3C we show microvilli from an isolated VSN being labeled for GPR30 in the experimental but not in the control.

17 β -Estradiol altered urine-induced responses of VSNs

17 β -estradiol alone had little to no effect of the membrane potential of VSNs (Fig. 4A). Urine is a natural stimulus for VSNs, so we hypothesized that 17 β -estradiol could modulate urine-elicited responses. To test this hypothesis, we used perforated patch clamp with isolated VSNs. Since the resting potential of the VSNs varied, we injected current to set the membrane potential near -60 mV at the start of the experiments. When we applied dilute urine (1:500) for 2 s, VSNs typically depolarized and fired action potentials (AP). Multiple cells ($n \sim 10$) were tested of which 50–70% showed modulation of the urine responses by estradiol and the modulation was repeatable after wash. As seen in Fig. 4A, the pattern of the depolarization and APs varied from cell to cell. In response to urine, cell 1 showed a moderate depolarization and had a uniform firing pattern. When applied with urine, 17 β -estradiol (100 pM) greatly reduced the depolarization, delayed the onset of firing, and decreased the firing frequency to one spike. This effect was recoverable upon removal of 17 β -estradiol. Cell 2 showed a large depolarization but only fired APs initially in response to urine. Similar to cell 1, 17 β -estradiol (100 pM) greatly decreased the depolarization to urine and delayed the onset of firing. However, 17 β -estradiol also increased the AP amplitude in cell 2. This effect was recoverable upon removal of 17 β -estradiol. In response to urine, cell 3 showed a depolarization and non-uniform firing pattern to urine. Application of urine and 17 β -estradiol (100 pM) elicited a decrease in the depolarization compared to urine alone, and the firing pattern appeared to be more uniform. After wash, the urine response partially recovered to that observed before 17 β -estradiol application.

17 β -Estradiol decreased degree of urine-induced depolarization—Depolarization was measured by taking the average of the distance from baseline to base of each spike as shown by the dashed line in Fig. 4B. The average depolarization in response to urine was 22.3 ± 3.5 mV, while 17 β -estradiol decreased the urine-induced depolarization to an average of 11.3 ± 1.8 mV. Upon washout of 17 β -estradiol, there was recovery of the urine-induced depolarization to 22.3 ± 3.7 mV. Our results showed that 17 β -estradiol decreased the depolarization elicited by urine in most cells by approximately 40% ($n = 6/8$, p value < 0.05 , repeated measures ANOVA, Bonferroni post hoc; Fig. 5A).

17 β -Estradiol increased latency of first spike in response to urine—Urine responses are mediated by G-protein-coupled receptors. Thus, there is always a small delay in the response after application of dilute urine. Latency was measured as the time from beginning of stimulus application to the first AP (Fig. 4B). Latency for first AP in urine was 489.4 ± 36.1 ms. 17 β -estradiol increased the latency to 883.3 ± 67.3 ms. Our results showed that 17 β -estradiol triggered a latency of the first spike in response to urine in most cells tested, almost doubling the amount of time to first spike ($n = 6/10$ cells, p value < 0.05 , repeated measures ANOVA, Bonferroni test post hoc; Fig. 5B).

Overall, 17 β -estradiol increased the amplitude of urine-induced APs in isolated VSNs ($n = 5/7$). The mean amplitude of spikes in urine was 66.9 ± 4.8 mV. 17 β -estradiol increased the

mean amplitude to 80.7 ± 4.4 mV, and there was recovery to 60.3 ± 5.7 mV upon removal of 17β -estradiol.

17β -Estradiol did not change frequency of firing in response to urine—Since a change in firing frequency is another mechanism by which synaptic output can be enhanced in neurons, we measured the interspike interval (ISI). Our results showed that 17β -estradiol did not have any effect on the firing frequency of VSNs. Mean ISI elicited by urine alone (151 ± 47.4 ms) was not different from the mean ISI elicited by urine with 17β -estradiol (151.2 ± 39.9 ms).

17β -Estradiol decreased urine-induced inward currents

Given that 17β -estradiol altered urine-induced APs in VSNs, it seemed plausible that 17β -estradiol was altering the chemosensory second messenger pathway or affecting the voltage-gated channels mediating the APs. To test if 17β -estradiol (1 nM) was altering currents directly activated by the chemosensory transduction pathway, we tested the effects of 17β -estradiol on the urine-induced currents. VSNs respond to urine with an inward current in approximately 50% of cells tested. Fig. 6A shows a sample of a typical response to urine (1:500) applied for 500 ms when the VSN was voltage clamped at -80 mV. This representative VSN responded to urine with approximately 45-pA inward current. 1nM 17β -estradiol decreased this inward current to approximately 15 pA which recovered with wash. The mean urine-induced current was 50.9 ± 10.1 pA. 17β -estradiol decreased the urine-induced currents to -24.5 ± 5.4 pA. With washout of 17β -estradiol, recovery was close to baseline (49.2 ± 9.3 pA). Our results showed that 17β -estradiol significantly decreased urine-induced inward currents in most cells ($n = 19/27$, $p < 0.0001$, repeated measures ANOVA test, Bonferroni post hoc; Fig. 6B). To determine if the effect of estradiol was different between males and females we compared the ratio of urine responses with estradiol to the corresponding urine response. Two-tailed t-test was used to determine if sex had any effect on urine-induced currents with 17β -estradiol. Our results showed that sex did not have any significant effect on 17β -estradiol-mediated effects on urine responses (Fig. 6C). Interestingly, urine-induced responses were larger for females than males ($p < 0.05$, two-tailed t-test; Fig. 6C inset).

VSNs responded to sulfated steroids

One problem with using urine (a mix from 30+ animals) is that it is a complex mixture that contains some trace amounts of 17β -estradiol. Therefore, we decided to test sulfated steroids (SS), a major chemosensory component of urine. We hypothesized that 17β -estradiol would modulate the responses of VSNs to a SS mix in which we selected those SS that gave the highest response rate in a previous report by Meeks et al. (2010) (A7864, E1050, P8200 and Q1570; androstenedione, estradiol, progesterone and corticosteroid). For these experiments, we used a ratiometric dye, Fura2-AM, as the Ca^{2+} indicator. Of the isolated VSNs only a little over 50 % ($n = 27/50$) of the SS responses. Of these, 17β -estradiol modulated the response in ~40% of the SS responses. The SS mix (10 μM of each) was applied for 15 s to isolated VSNs to elicit Ca^{2+} transients. Fig. 7A shows responses of a sample cell that was stimulated three times with SS mix followed by two applications of SS mix with 17β -estradiol (100 pM) with 4-min washes in between stimulus applications. We also tested 17β -

estradiol by itself and the vehicle neither of which elicited any Ca^{2+} transients in the cell. We also varied the order of the stimulus application to determine if 17β -estradiol had any effect if applied first and never observed a response to 17β -estradiol. However, the cell responded to ATP by eliciting a smaller Ca^{2+} response. To account for the decrease in Ca^{2+} amplitude after each stimulus (as seen in Fig. 7A), most likely a result of receptor desensitization, we plotted a predicted mean by regression of the first and second peaks in response to SS. We observed that the decrease in amplitude of peaks due to 17β -estradiol was larger than that in the predicted reduction attributable to desensitization. Overall, our results showed that 100 pM estradiol significantly decreased Ca^{2+} transients in about half of the cells that responded to the SS mix. This is shown as the ratio of SS (predicted) or SS with estradiol to the corresponding SS response ($n = 6/14$ cells, $p < 0.05$, Two-tailed t -test; Fig. 7B). As with our electrophysiology experiments, we tested 17β -estradiol by itself on VSNs but it was without effect ($n = 0/5$). Since the SS mix was dissolved in methanol, we tested to see if the vehicle had any effect on Ca^{2+} transients but no response was observed ($n = 0/3$). As a positive control, cells that did not respond to SS, ($n = 23$) were tested with ATP, since we have previously observed ATP to elicit robust Ca^{2+} transients in VSNs. Therefore, these non-SS responsive VSNs were capable of producing Ca^{2+} transients in response to ATP, but presumably did not have the correct receptors for any of the SS.

A two-tailed t -test was used to determine if sex of the mouse had any effect on SS-induced Ca^{2+} transients in the presence of 17β -estradiol. Our results showed that sex did not have any significant effect on SS-induced Ca^{2+} transients in the presence of 17β -estradiol (Fig. 7C). Interestingly, SS-mediated responses were larger for females than males, a finding similar to our observation of urine-induced inward currents ($p < 0.05$, two-tailed t -test; Fig. 7C inset).

Source and quantification of estradiol

Interestingly, both estrogen receptors were present in males and females and VSNs from both sexes showed 17β -estradiol modulation of chemosensory responses to urine and SS. This result was contrary to our hypothesis that 17β -estradiol would have its effects principally in females due to its higher levels in their circulation. In mice, 17β -estradiol reaches the VNO via three pathways (a) circulatory, (b) from the environment and (c) local production in the VNO. The VNO is heavily vascularized and thus is easily accessible to any hormone/peptide released into the circulatory system. 17β -estradiol is also present in urine and may reach VSNs via the lumen. However since estradiol can be locally produced in the CNS (Hojo et al., 2004), we hypothesized that estradiol could be locally produced within the VNO.

To determine if estradiol might be produced in the VNO, we first tested for the expression of aromatase, the enzyme necessary for the production of estradiol, using RT-PCR. Our data showed the presence of aromatase mRNA and the specificity of the band was confirmed by sequencing (Fig. 8A). However, having aromatase present does not demonstrate that estradiol is in fact being produced in the VNO. To further test our hypothesis, we quantified estradiol in the VNO by LC–MS/MS. Estrogen concentrations were determined by a well-established LC–MS approach with quantification specificity based on the retention time of

derivatized estradiol and its specific fragmentation route (unique MS/MS transition). The retention time and specific fragmentation pattern of the derivatized target analytes were identical to those of authentic 17β -estradiol, unambiguously confirming their identity as estradiol (Fig. 8B). The retention time of 17β -estradiol-IS and estradiol was within ± 0.1 min. Estradiol was quantified irrespective of its isomer, since 17α -estradiol and 17β -estradiol could not be separated in the chromatographic method used in this study. The concentrations of estradiol in VNOs from male, female, and OVX mice were determined based on constructed calibration curves of adequate linearity (r^2 equal or greater than 0.99) (Fig. 8C). Estradiol in five of the VNOs (1 male and 2 female) was undetected or detected below the limit of quantification (one female and one OVX). Limit of quantification is defined as the lowest amount of 17β -estradiol in samples that yielded an accuracy error within $\pm 20\%$ and with a CV below 20% (1.5 pg on column). The average accuracy of quantification (1.5, 3, 6, and 12 pg) was determined to be $104 \pm 16.1\%$, and coefficient of variations for replicate analyses of all calibration standards and samples were below 20%.

The retention time (top row) and specific fragmentation pattern (bottom row) of the derivatized target analytes were identical to those of authentic 17β -estradiol, unambiguously. Our results showed that estradiol was present in VNOs from male, female and OVX mice (Fig. 8D). Thus estrogen may be locally produced in the VNO regardless of the sex of the animal.

DISCUSSION

Estradiol has been shown to have rapid effects in the CNS, particularly in hippocampal and hypothalamic neurons where the physiological significance and mechanisms have been well characterized (Woolley, 2007). Estradiol is known to modulate pain sensation but its effects on other sensory systems such as vision, olfaction, or taste are less known (Chaban et al., 2003). To our knowledge, this is the first study that shows 17β -estradiol modulates the responses of mouse VSNs to odorants. In our study, 17β -estradiol (1) decreased urine-elicited depolarization, (2) increased the latency for the first urine-induced AP, (3) increased amplitude of APs, (4) decreased the inward currents elicited in response to urine, and, (5) decreased Ca^{2+} transients elicited in response to sulfated steroids. Furthermore, the presence of the estrogen receptors GPR30 and $\text{ER}\alpha$ in VSNs and the capacity for local synthesis of estradiol in the VNO support a role for estradiol in the VNO of both sexes.

Estrogen receptors $\text{ER}\alpha$ and GPR30 were differentially expressed in VSNs

We found that two estrogen receptors were present in VSNs, $\text{ER}\alpha$ and GPR30. We expected to see a difference in the labeling of the apical VSNs from the basal VSNs since they respond to different chemical stimuli (Leinders-Zufall et al., 2000, 2004). However, our results showed that most of the VSNs were labeled with the antibodies for $\text{ER}\alpha$ and GPR30.

$\text{ER}\alpha$ —Immunolabeling of $\text{ER}\alpha$ was seen in the dendrites, knobs, and axons of VSNs but not in the soma. This labeling pattern was consistently observed in sections of VNO and in isolated VSNs from mice of both sexes (Fig. 2A, B). In the CNS, there is a great degree of heterogeneity in the expression pattern of $\text{ER}\alpha$ across cell types. A study by Milner et al. (2001) shows that expression of $\text{ER}\alpha$ varies across cell types, but is present mostly in the

axons and dendrites, with limited expression in the soma of hippocampal CA1 pyramidal neurons. ER α receptors are present in the membranes at synaptic terminals in hippocampal neurons as well as along dendrites, supporting their role in rapid estradiol signaling (for a review, see Woolley, 2007). Our results showed a similar labeling pattern for ER α as observed in the hippocampus, suggesting that these receptors are localized to mediate rapid estrogen effects.

Our results suggest that the full length ER α receptor was present in the VNO. The 50-kDa and 80-kDa isoforms were present in the VNO and positive control tissues. Multiple ER α isoforms have been reported in both brain and mammary tissues (Bollig and Miksicek, 2000; Gorosito et al., 2008; Ishunina and Swaab, 2008; Dominguez and Micevych, 2010). Potentially, exons of the full length product may be deleted or duplicated leading to these multiple isoforms in the VNO. Additionally, the relative abundance of these isoforms varies across tissues as is evident from our results since the 67-kDa and 50-kDa isoforms appeared to be most abundant in the uterus while the 50 kDa was most abundant in the brain. Similar results were reported by Dominguez and Micevych (2010). In addition to our findings, Zhang et al., (2010) reported the presence of ER α mRNA in the VNO using microarray which correlates to a similar finding by Hagendorf et al. (2009) (personal communication).

GPR 30—GPR30 labeling was observed in the somas, dendrites, and knobs (Fig. 3). As with the ER α labeling, approximately 90 % of the isolated VSNs were labeled. This expression pattern is similar to that of human breast cancer cells and rat hippocampal CA2 neurons such that both cytosol and plasma membrane show GPR30 labeling (Thomas et al., 2005; Funakoshi et al., 2006; Filardo et al., 2007). The GPR30 receptor has also been found in the Golgi and endoplasmic reticulum (Revankar et al., 2005). We observed a similar restricted expression pattern in VSNs using a GPR30 antibody from Genetex (data not shown). We suspect this difference in the recognition of GPR30 receptor might be due to modification of GPR30 receptor as it is processed from the endoplasmic reticulum to the plasma membrane. This idea is supported by reports of homodimerization of GPR30 or heterodimerization with other GPCRs via C terminus (Akama et al., 2013). Both ER α and GPR30 mediate rapid estradiol effects in the CNS (Chu et al., 2009; Hammond and Gibbs, 2011). Our results suggest both ER α and GPR30 receptors are present in most VSNs and are positioned to mediate rapid estrogen effects.

Neuro-modulatory effects of estradiol on urine-induced responses in VSNs

Neurons encode information in the CNS via APs: (a) spike rate, (b) latency to the first spike, (c) spike amplitude, (d) spike width, and (e) afterhyperpolarization (AHP). We observed that 17 β -estradiol decreased urine-induced depolarization and altered AP firing patterns (Fig. 5A, B). Estradiol significantly increased the latency to the first AP and in some cells increased AP amplitude (Figs. 4A and 5C). The decrease in urine-induced depolarization by estradiol may contribute to this increase in amplitude of AP since the membrane potential was closer to resting levels observed in Ringers compared to the depolarization that occurred in the presence of estradiol.

We also observed that estradiol rapidly decreased the SS-induced Ca^{2+} transients (Fig. 7A) similar to the rapid effects of estradiol on urine-induced responses in Figs. 4A and 6A. In the CNS, such rapid estradiol effects that occur within few minutes have been reported. For example, estradiol decreases latency for firing in GnRH neurons through A-type potassium channels, thereby increasing neural excitability (DeFazio and Moenter, 2002). However, in the ventral medial vestibular neurons of the rat brain stem, estradiol reduces the spontaneous firing rate, thereby decreasing neural excitability (Grassi et al., 2010). This suggests the hypothesis that estradiol might be part of a negative feedback mechanism that regulates the excitability of VSNs. One previous report by Spehr et al., (2009) showed that VSNs undergo sensory adaptation via a Ca^{2+} –calmodulin feedback that inhibits TRPC2 channels. Interestingly, estrogen receptors in epithelial breast cancer cells associate with Ca^{2+} –calmodulin (Li et al., 2001). In VSNs if the Ca^{2+} –calmodulin is bound to estrogen receptor in an inactivated state and when 17β -estradiol binds with the estrogen receptor, Ca^{2+} –calmodulin is released, then more Ca^{2+} –calmodulin would be available to inhibit TRPC2 channels as Ca^{2+} enters the cell in response to a stimulus. However this remains to be determined.

The next question would be to ask what terminates the estrogen signal and thereby reverse its effects on VSNs. The actions of estradiol effect may possibly be limited via activation of steroid hydroxylase, which breaks down steroids into metabolites. Steroid hydroxylase activity has been demonstrated in mouse VNO microsomes using HPLC–GC–MS by determining the rate of total metabolite production from radiolabeled estradiol substrate (Gu et al., 1999). Hence, steroid hydroxylases may be essential in regulating the physiological effects of estradiol shown here.

Estradiol lacked sex-specific effects in VSNs

We initially hypothesized that the effects of estradiol would be observed in female VSNs alone due to the varying levels of circulating estradiol during their 4-day estrous cycle. This hypothesis was based on the findings by Dalton et al. (2002) that odor responses are enhanced (lowering the threshold detection of odors) with increases in blood serum estradiol in humans and rodents (Pietras and Moulton, 1974; Doty et al., 1981). However our results argue against any sex bias with respect to effects of estradiol in VSNs. Firstly, there was no difference in the expression pattern of $\text{ER}\alpha$ or GPR30 between males and females. This finding was similar to the expression of these receptors in the CNS. Although both urine responses and sulfated responses were larger in amplitude in females, the degree of the effect of estradiol on the responses was similar between sexes. Lastly, though traditionally thought of as a female hormone, estradiol is now widely accepted as a neurosteroid synthesized in the brain (Hojo et al., 2008). Our results suggest estradiol is locally produced in the VNO as evidenced from its presence in male and OVX female VNOs (Fig. 8D). Considering these results, as well as the expression of estrogen receptors and the physiological effects elicited by estradiol in males, our results support our new hypothesis that estradiol functions as a neurosteroid in the VNO of both sexes. We believe that the sex difference shown by Dalton et al., could be due to the fact that the behavioral responses they were studying were probably mediated through the main olfactory epithelium, septal organ and associated CNS structures rather than by the VNO. However, the lack of sex differences

with respect to estradiol in the VNO may not necessarily be reflected in the CNS as different neural circuits may be modulated.

Speculated mechanisms underlying estradiol production

We found that estradiol may be locally produced in the VNO as evidenced from the presence of aromatase mRNA and estradiol in male, female and OVX mice. It is interesting to speculate on the possible mechanisms that activate estradiol synthesis. Two possible mechanisms are (1) aromatase is constitutively active but is inactivated by increased estradiol levels (negative feedback), and (2) activation of aromatase is increased via activity-dependent events such as odorant responses (positive feedback).

Estradiol synthesis via aromatase may be affected by the local concentration of estradiol. Estradiol can reach the VSNs through the circulatory system or from the environment (such as urine or other body fluids). Additionally, sulfated estradiol in urine could be converted to estradiol via sulfatase enzymes in the lumen of the VNO. Estradiol from all of these sources would add to locally produced estradiol and thereby inhibit aromatase. An alternate mechanism is that activity-dependent events such as odorant responses can trigger aromatase activity. If aromatase is present in VSNs, it could be activated via increased intracellular Ca^{2+} that occurs in response to odors/pheromones, or ATP release that may occur during VNO pumping (Vick and Delay, 2012). These increases in intracellular Ca^{2+} within VSNs could lead to activation of calmodulin and thereby activate aromatase via phosphatases (Balthazart et al., 2005; Catalano et al., 2009).

CONCLUSION

We show that estradiol had acute effects on the odor-induced responses of VSNs, decreasing odor-induced responses and may be locally produced in the VNO. Our findings are novel in that (a) estradiol synthesis may occur in the VNO, (b) estradiol has a modulatory role in the physiology of VSNs and (c) these effects are sex independent contrary to previous speculations.

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Abbreviations

A7864 5-androsten-3 β ,17 β diol disulfate, disodium salt

ANOVA	analysis of variance
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
E1050	1,3,5(10)-estratrien-3,17 diol disulfate, disodium salt
EGTA	ethylene glycol tetra-acetic acid
ERα	estrogen receptor α
ERβ	estrogen receptor β
ESI	electrospray ionization
GFP	green fluorescent protein
GPR30	G-protein-coupled receptor 30
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ISI	interspike interval
OMP	olfactory marker protein
OVX	ovariectomized
P8200	4-pregnean-3 β -ol-20-one sulfate sodium salt
PBS	phosphate-buffered saline
Q1570	4-pregnen-11 β ,21-diol-3,20-dione 21-sulfate sodium salt
TBST	Tris-buffered Saline and Tween20
TRPC2	Transient Receptor Potential Channel type 2
VNO	vomer nasal organ
VSN	vomer nasal sensory neuron

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Fig. 1.

mRNA for ER α and GPR30 were present in mouse VNO. (A) RT-PCR results showed mRNA for ER α and GPR30 were present in both male and female mouse VNO. Mid brain regions of both male and female mice were used as positive controls. The length of ER α and GPR30 PCR products was 307 and 234 bp respectively. ER β (440 bp) was not present in VNO but was detected in brain. (+) indicates positive control while (-) indicates negative control (no reverse transcriptase) ($n = 4$ mice). VNO was used as tissue for negative control. (B) Western blot results showed ER α was present in both male and female VNOs. Tissue was taken from both male and female VNOs (lanes 1–4), MCF7 cells (lane 6), brain (lane 7) and uterus (lane 8). Lanes 1–4 show a dose dependence where 80- μ g and 40- μ g protein were loaded in lanes 1 and 2 for females and lanes 3 and 4 in males respectively. Eighty-micrograms of protein was loaded for the positive controls. No protein was loaded in lane 5. Pan α actin (45 kDa) was used as loading control (bottom panel). Three ER α isoforms of MW 67 kDa, 50 kDa and 80 kDa were seen.

Fig. 2.

ERα was expressed in sensory epithelium of VNO. (A) A coronal section from the VNO of an OMP-GFP female mouse showed strong labeling with the anti-ERα antibody. Labeling was seen mostly in the dendritic and knob regions of the sensory epithelium but was also observed below the basal lamina in the axon layer. Top panels show GFP (left panel) and ERα (right panel). Bottom panel shows merged images of ERα and OMP-GFP. Maximum intensity projection of z stack was made for these images. Scale bar = 10 μm. (B) ERα was labeled in dendrites of isolated VSNs. The panels showed antibody labeling for ERα in isolated VSNs (shown is from a female but similar results were observed in male) was seen mostly in the dendritic and knob regions of the cell. Top and bottom panels compare labeling for ERα between experimental and control (no primary) respectively. Maximum intensity projection of z stack was made for these images. Scale bar = 10 μm.

Fig. 3.

Label for the GPR30 antibody was detected in most isolated VSNs. (A) Coronal section of VNO from BALB/c mouse (male) showing labeling for GPR30. Labeling was seen throughout the epithelium. (B) GPR30 receptors were expressed in somas, dendrites and microvilli region of isolated VSNs from OMP-GFP mice (male) Top panels show GFP (left) and GPR30 (right). Bottom panels show merged and DIC image of the same cell. Scale bar = 10 μm. (C) GPR30 labeling seen in microvilli from an isolated cell from OMP-GFP mice (male). Labeling was observed in experimental (top left) but not in control group (top right).

Fig. 4.

17 β -estradiol affected urine-induced Responses in VSNs. (A) The response pattern varied from cell to cell; hence three representative VNS responses are shown. Cell 1 showed a moderate depolarization and had a uniform firing pattern. 17 β -estradiol (100 pM) greatly reduced the depolarization, delayed and decreased the frequency of action potentials (APs) to one spike. The urine response recovered to the initial pattern upon removal of 17 β -estradiol. In cell2 urine induced, a large depolarization with a few APs. 17 β -estradiol (100 pM) greatly decreased the depolarization to urine, delayed the first AP and increased the amplitude. The urine response recovered after removal of 17 β -estradiol. Cell3 showed a depolarization and non-uniform firing pattern to urine. Application of urine and 17 β -estradiol (100 pM) elicited a decrease in the depolarization compared to urine alone and the firing pattern appeared to be more uniform. With wash, the urine response somewhat recovered to that observed before 17 β -estradiol application. In all cells current was injected into VSNs so that their initial membrane potential was near -60 mV. Urine was applied with and without 17 β -estradiol for 2 s to evoke a response. A spike was defined by (a) crossing 0 mV as shown by dashed line and (b) if half width, measured as half of peak minus baseline for each peak was below 30 ms. Inset in cell 1 showed 17 β -estradiol by itself did not elicit any response. (B) To determine the latency for first AP we measured the difference between time from the start of stimulus application to the first peak from APs elicited by urine with and without estradiol. Top overlay shows the increase in latency of the first AP in the presence of 17 β -estradiol. It also shows the decrease in depolarization. The bottom trace shows how depolarization was measured (by taking the mean of distance from baseline (dark line) to base of each spike (dash line)).

Fig. 5.

17 β -estradiol decreased urine-induced depolarization, increased latency of first AP amplitude and increased amplitude of urine-induced spikes. (A) To compare the depolarization in response to urine we took the average depolarization and compared that to urine in .Urine-induced depolarizations (22.3 ± 3.5 mV) were reduced by 100 pM 17 β -estradiol (11.3 ± 1.8 mV) and recovered upon removal of (22.3 ± 3.7 mV). Data shown as Mean $\pm$ SEM ($n = 6/8$, $p < 0.05$ repeated measures ANOVA, Bonferroni post hoc). (B) 17 β -estradiol (100 pM) significantly increased the latency for first AP. Latency for first AP in urine (489.4 ± 36.1 ms) was increased by 17 β -estradiol (883.3 ± 67.3 ms). Data shown as Mean $\pm$ SEM. ($n = 6/10$, p value < 0.05 repeated measures ANOVA, Bonferroni post hoc). (C) 17 β -estradiol (100 pM) significantly increased the amplitude of APs in response to urine. Amplitude of AP induced by urine (66.9 ± 4.8 mV) was increased by 17 β -estradiol to 80.7 ± 4.4 mV. The response in wash (urine) recovered to 60.3 ± 5.7 mV. Data shown as Mean $\pm$ SEM. ($n = 5/7$, p value < 0.05 repeated measures ANOVA, Bonferroni post hoc).

Fig. 6.

17 β -estradiol decreased urine-induced currents. (A) Using perforated patch clamp technique, 17 β -estradiol decreased urine-induced currents in isolated VSNs. Shown is an example of urine response from an isolated VSN which produced a 45 pA inward current. One nanomolar 17 β -estradiol decreased this inward current to 15 pA and the response recovered with wash. The cell was voltage clamped at -80 mV and pooled mouse urine (1:500) with or without 1 nM 17 β -estradiol was applied for 500 ms to evoke inward currents. Treatments were followed by wash for 2 min in Ringers. (B) 17 β -estradiol significantly decreased urine induced currents. The mean urine induced (50.9 ± 10.1 pA) was reduced by 1 nM 17 β -estradiol (24.5 ± 5.4 pA), and recovered upon removal of 17 β -estradiol (49.2 ± 9.3 pA). Data shown as Mean $\pm$ SEM. ($n = 19/27$, $p < 0.0001$, repeated measures ANOVA, Bonferroni post hoc). (C) Two-tailed t -test showed no effect of sex on urine-induced currents in the presence of 17 β -estradiol. Surprisingly, urine-induced currents were larger in cells from females ($n = 15$) than in males ($n = 12$) (shown in inset; $p < 0.05$, two-tailed t -test).

Fig. 7.

17 β -estradiol decreased calcium response to sulfated steroids. (A) Sulfated steroids mix (SS) elicited calcium transients that were reduced in the presence of 100 pM 17 β -estradiol in isolated mouse VSNs ($n = 6/14$). Stimuli were applied for 15 s and washes were for 4 min. Shown is an isolated VSN that was stimulated three times with SS mix followed by two application of SS mix with 100 pM 17 β -estradiol. The bath was under constant perfusion. We also tested 17 β -estradiol by itself ($n = 0/5$) and the vehicle (0.1% methanol; $n = 0/3$) both of which did not elicit any Ca²⁺ transients in the cell. ATP (30 μ M) elicited a smaller Ca²⁺ transient. (B) We calculated the ratio of SS (predicted) or SS (with estradiol) responses to the SS response for each cell. There was a significant effect such that estradiol elicited a stronger decrease on SS induced Ca²⁺ transients than the predicted decrease due to desensitization ($n = 6/14$, $p < 0.05$, two-tailed t -test). Mean SS (predicted) value was calculated from linear regression of 1st and 2nd calcium transient peaks elicited by SS and SS (wash) respectively from $n = 14$ cells. (C) Two-tailed t -test showed no effect of 17 β -estradiol on SS induced Ca²⁺ transients between sexes. Consistent with the sex bias seen in the inset in Fig. 6C, SS-induced responses from cells of females ($n = 6$) were larger than those of males ($n = 8$) ($p < 0.05$, two-tailed t -test).

Fig. 8.

Estradiol may be locally produced in the VNO. (A) RT-PCR was used to determine the presence of aromatase (a key enzyme that converts testosterone to estradiol) mRNA in both male and female VNO. A 309-bp product was detected in both male and female VNO ($n = 4$) sequenced and identified as aromatase. (B) Representative extracted ion chromatograms (m/z 170.8–171.5) of dansyl chloride derivative-specific transition (17β -estradiol: m/z 506.3 $\rightarrow$ 171.1) in VNOs samples and 17β -estradiol authentic standard. Both peaks eluted symmetrically at the same retention time [± 0.1 min, peak width (FWHM): ~ 0.15 min] (*top row*). The retention time of 17β -estradiol-IS was 16.02 ± 0.11 min and that of estradiol in VNO was 16.05 ± 0.10 min in this particular experiment (*top row*). Full MS/MS spectra were acquired during the transition monitoring and compared against the standard. Fragmentation pattern of the target analyte (m/z 506.3) was identical to that of the estradiol standard (*bottom row*). (C) Calibration curve. Serial dilutions of 17β -estradiol ranging from 0.56 to 36 pg spiked with isotope standard 17β -estradiol-IS were freshly prepared and analyzed in three technical replicates. The plot shown was constructed based on three independent experiments performed on three separate days (9 replicates). The correlation coefficient (r^2) was equal or greater than 0.99, indicating an adequate linearity. The average of coefficient of variation (CV) over the entire range from 2.25 to 36 pg was 9.41% (6.6–12.4%), indicating sufficient measurement precision. (D) Estradiol concentrations from the VNOs dissected from a group of male ($n = 6/7$), female ($n = 5/7$) and ovariectomized (OVX) ($n = 4/4$) mice were determined based on the calibration curve [Area (Estradiol)/Area (IS)].