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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 17β-estradiol, would modulate activity of VSNs. In this paper, we show that the estrogen receptors, GPR30 and ERα, were present in VSNs and that estradiol may be synthesized locally in the VNO. Our results also showed that 17β-estradiol decreased responses of isolated VSNs to dilute urine, a potent natural stimulus, with respect to current amplitudes and depolarization. Further, 17β-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 17β-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.

Key words: estrogen, vomeronasal organ, GRP30, ERα, 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 Gruenenberg 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

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Abbreviations: A7864, 5-androsten-3β,17β-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-3β-ol-20-one sulfate sodium salt; PBS, phosphate-buffered saline; Q1570, 4-pregnen-11β,21-diol-3,20-dione 21-sulfate sodium salt; TBST, Tris-buffered Saline and Tween20; TRPC2, Transient Receptor Potential Channel type 2; VNO, vomeronasal organ; VSN, vomeronasal sensory neuron.

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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 ERα (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 μg/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 $MgCl_2$, 2 $CaCl_2$, pH 7.4) with 11 μg/mL leupeptin was added to stop the activity of papain. After filtration through a 250-μm 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 μM was made using stocks of E1050 (1,3,5(10)-estratrien-3,17 diol disulfate, disodium salt), A7864 (5-androsten-3β,17β-diol disulfate, disodium salt), P8200 (4-Pregnean-3β-ol-20-one sulfate sodium salt), Q1570 (4-pregnen-11β,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). 17β-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.