Estradiol Rapidly Attenuates ORL-1 Receptor-Mediated Inhibition of Proopiomelanocortin Neurons via G_q -Coupled, Membrane-Initiated Signaling

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Key Words

Estradiol · Proopiomelanocortin · Orphanin FQ ·
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Abstract

Estradiol rapidly regulates the activity of arcuate nucleus (ARH) proopiomelanocortin (POMC) neurons that project to the medial preoptic nucleus (MPN) to regulate lordosis. Orphanin FQ/nociceptin (OFQ/N) acts via opioid receptor-like (ORL)-1 receptors to inhibit these POMC neurons. Therefore, we tested the hypothesis that estradiol excites POMC neurons by rapidly attenuating inhibitory ORL-1 signaling in these cells. Hypothalamic slices through the ARH were prepared from ovariectomized rats injected with Fluorogold into the MPN. Electrophysiological recordings were generated in ARH neurons held at or near -60 mV, and neuronal phenotype was determined post hoc by immunohistofluorescence. OFQ/N application induced robust outward currents and hyperpolarizations via G protein-gated, inwardly rectifying K^+ (GIRK) channels that were attenuated by pretreatment with either 17- β estradiol (E_2) or E_2 conjugated to bovine serum albumin. This was blocked by the estrogen receptor (ER) antagonist ICI 182,780 and mimicked by the G_q -

coupled membrane ER (G_q -mER) ligand STX and the ER α agonist PPT. Inhibiting phosphatidylinositol-3-kinase (PI3K) blocked the estrogenic attenuation of ORL-1/GIRK currents. Antagonizing either phospholipase C (PLC), protein kinase C (PKC), protein kinase A (PKA) or neuronal nitric oxide synthase (nNOS) also abrogated E_2 inhibition of ORL-1/GIRK currents, whereas activation of PKC, PKA, protein kinase B (Akt) and nNOS substrate L-arginine all attenuated the OFQ/N response. This was observed in 92 MPN-projecting, POMC-positive ARH neurons. Thus, ORL-1 receptor-mediated inhibition of POMC neurons is rapidly and negatively modulated by E_2 , an effect which is stereoselective and membrane initiated via G_q -mER and ER α activation that signals through PLC, PKC, PKA, PI3K and nNOS.

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Introduction

17 β -Estradiol (E_2) exerts far-reaching effects on numerous biological systems. It is primarily synthesized in the ovaries, although small amounts are produced in other tissues (e.g. adrenal cortex, adipose tissue). Recent evidence suggests that it is also manufactured in the presynaptic bouton, where it could thus function as a neuro-

steroid [1]. In the female, levels of estrogen fluctuate naturally during the course of the estrous cycle. These fluctuations are responsible for numerous physiological and behavioral changes, including ovulation, uterine development and sexually receptive behavior (lordosis) [2]. This dynamic hormone also contributes to the maintenance of bone density, smooth muscle tone, memory, appetite and the coordination of female sexual behavior to optimize the likelihood of fertilization and implantation [3, 4]. Our laboratories have been investigating the temporal pattern of estrogen receptor (ER) signaling by E_2 using ovarian steroid-responsive hypothalamic opioid circuits in the rat that regulate sexual receptivity.

As mentioned above, E_2 is vital in the coordination of female sexual behavior, also known as lordosis or 'presenting', which is associated with copulation. The primary characteristics of lordosis are a lowering of the forelimbs with the rear limbs extended and hips raised, a lifting of the head, ventral arching of the spine and a movement of the tail to one side [5–7]. Initially, E_2 inhibits sexual receptivity during the metestrus/diestrus phase of the rodent ovarian cycle. This initial inhibition is required for subsequent sexual behavior. Shortly after E_2 peaks during early proestrus, progesterone will peak along with a LH and FSH surge; resulting in ovulation during late proestrus. These hormonal fluctuations set the stage for subsequent lordotic behavior during the estrus phase that optimizes the likelihood for fertilization and subsequent implantation [8, 9]. This natural cyclicity of female reproductive behavior is mimicked using a well-established ovariectomized female rat model [6, 10, 11]. Whereas low priming doses of E_2 (e.g., 2 μ g) alone inhibit lordotic behavior; they induce it when they are followed by progesterone (500 μ g) 26 h later. This regulation of behavior is occurring through multiple receptor signaling mechanisms, all of which appear to converge on hypothalamic arcuate nucleus (ARH) α -melanocortin (POMC) neurons that project to the medial preoptic nucleus (MPN) [12–14]. The initial inhibition of lordosis caused by low levels of E_2 is due to the excitation of these POMC neurons. This leads to an overriding tone of opioid activity that is inhibitory to lordotic behavior, confirmed in μ -opioid receptor-null mice or in rats pretreated intracerebroventricularly or site specifically into the MPN with the opioid receptor antagonist naloxone. In both instances, subsequent sexual receptivity was significantly inhibited in E_2 -primed, progesterone-treated females [7, 11]. This action is vital to the appropriate timing and subsequent expression of female sexual behavior.

The initial inhibition of sexual receptivity is ER mediated. However, there are many different subtypes of ER,

including ER α , ER β , G-protein coupled receptor-30 (GPR30) and a G_q -coupled membrane ER (G_q -mER), all of which have been implicated in playing some role in sexual behavior. For example, the ER α agonist PPT causes a clear change in sexual proceptive and receptive behavior (e.g., ear wiggling, hops and darts, lordosis quotient and decreased rejection), as well as increased [3 H]-inositol phosphate accumulation in the rat uterus, to a similar degree as seen with E_2 treatment [15, 16]. This sexual behavior is not seen upon administration of the ER β agonist diarylpropionitrile (DPN) [15]. When given simultaneously, however, DPN was able to modulate the activity of the PPT by eliminating the PPT-induced increase in expression of both receptive and proceptive female sexual behavior [15]. Furthermore, mice that lack ER α have stunted uterine development that results in infertility, whereas mice that lack ER β , experience normal uterine development [17]. These ER α -mediated effects on sexual receptivity are due, at least in part, to membrane-delimited interactions with specific metabotropic glutamate receptors [12, 14, 18]. Another route for estrogenic modulation is the GPR30 receptor [19]. GPR30 binds E_2 with high affinity, has been found to be involved in the rapid actions elicited by E_2 in peripheral reproductive tissue and activates the extracellular signal-regulated kinase (ERK) pathway independently of ER α or ER β [20]. Moreover, GPR30 is expressed in the ARH and in EB-primed rats. E_2 acting through GPR30 in the ARH can deactivate μ -opioid receptors in the MPN to facilitate lordosis within 30 min [21]. E_2 and the highly potent and selective agonist STX also activate another, more recently characterized G_q -mER. Similar to ER α , activation of the G_q -mER with STX increases intracellular Ca^{2+} levels and neuroprogesterone synthesis in hypothalamic astrocytes [22], stimulates MPN-projecting POMC neurons and elicits lordosis behavior in E_2 -primed females [13].

Rapid membrane-initiated signaling following activation of either ER α or G_q -mER triggers a signal transduction cascade involving phospholipase C (PLC), protein kinase C (PKC), protein kinase A (PKA), as well as phosphatidylinositol-3-kinase (PI3K), that significantly disrupts the ability of metabotropic, $G_{i/o}$ -coupled receptors such as the μ -opioid and GABA $_B$ receptors to activate inhibitory, G protein-gated, inwardly rectifying K $^+$ (GIRK) channels in ARH POMC neurons [3, 12, 23]. It is suggested that this uncoupling of metabotropic, $G_{i/o}$ -coupled receptors from their GIRK channels occurs via PKA-mediated phosphorylation in ARC POMC neurons [24]. This leads to the excitation of the ARH POMC neurons and the release of POMC-derived peptides like β -en-

dorphin [25, 26]. Moreover, rapid estrogenic signaling also occurs in GABAergic, gonadotropin-releasing hormone (GnRH) and A₁₂ dopamine neurons in the hypothalamus, which indicates that this rapid signaling is critical for normal estrogenic control of homeostatic functions [27–29].

Another likely substrate through which E₂ rapidly modulates the activity of POMC neurons is the orphanin FQ/nociceptin (OFQ/N)-opioid receptor-like (ORL)-1 system. OFQ/N is the endogenous ligand that binds to its cognate ORL-1 receptor, which is heavily expressed in neuronal populations throughout the mediobasal hypothalamus including MPN-projecting POMC neurons [30–32]. Like the GABA_B and μ -opioid receptors, the ORL-1 receptor is a metabotropic, G_{i/o} coupled receptor. The binding of OFQ/N to the ORL-1 receptor elicits a very robust, reversible outward current and hyperpolarization in ARH POMC neurons that can be antagonized by the GIRK channel blockers Ba²⁺ and tertiapin [33–35]. In addition, it can presynaptically inhibit the excitatory glutamatergic input impinging upon these cells [35]. We know that in vivo E₂ priming negatively modulates the postsynaptic inhibitory effects of ORL-1 activation on POMC neurons that project to the MPN, as well as the presynaptic inhibition of glutamate release onto them [35]. As mentioned above, there is compelling precedence for rapid estrogenic attenuation of metabotropic, G_{i/o}-coupled receptors like the GABA_B, μ -opioid and cannabinoid CB1 receptors from their effector systems. There is also a burgeoning yet incomplete understanding of the signaling molecules such as PLC, PKC, PKA and neuronal nitric oxide synthase (nNOS) through which this process takes place [36, 37]. Moreover, recent evidence indicates that E₂ rapidly attenuates ORL-1 receptor-mediated antinociception via an ERK2-dependent mechanism [38]. We therefore tested the hypothesis that E₂ rapidly attenuates the ORL-1 receptor-mediated activation of inhibitory GIRK channels in MPN-projecting POMC neurons that control female sexual receptivity via multiple ER receptor subtypes and signal transduction mechanisms.

Materials and Methods

Animals

Adult female Long-Evans rats (200–225g) were purchased from Charles River Laboratory Inc. (Wilmington, Mass., USA). Bilateral ovariectomies were performed by the supplier. The rats were 8–12 weeks of age at the time of ovariectomy, and were shipped to us 1 week later. After 1 week of quarantine, the rats re-

ceived their Fluorogold injection (see below), and 1 week later were used for experimentation. Thus, a total of 3 weeks had elapsed between the ovariectomies and the electrophysiology experiments (see below). Upon arrival, they received a 2- μ g priming dose of estradiol benzoate in order to help maintain steroid sensitivity. Rats were housed under a 12:12-hour light/dark cycle, with food and water available ad libitum. All procedures were approved by the Western University of Health Sciences IACUC in accordance with institutional guidelines based on NIH standards.

Drugs

For the electrophysiological experiments described below, all drugs used were purchased from Tocris Bioscience (Minneapolis, Minn., USA) unless stated otherwise. Tetrodotoxin (TTX; Na⁺ channel blocker; Alomone Labs, Jerusalem, Israel) was prepared as a 1 mM stock solution in UltraPure H₂O and diluted further with artificial cerebrospinal fluid (aCSF) to the working concentration of 500 nM. OFQ/N was prepared as a 1 mM stock solution in UltraPure H₂O and diluted further with aCSF to the working concentration of 1 μ M.

E₂ (Steraloids, Newport, R.I., USA) was prepared as a 1 mM stock solution in punctilious ethanol and diluted further with aCSF to the working concentration of 100 nM. 17 α -estradiol (17 α -E₂; Steraloids) was prepared as a 1 mM stock solution in punctilious ethanol and diluted further with aCSF to the working concentration of 100 nM. The membrane impermeant E₂ 17 hemisuccinate-bovine serum albumin (E₂-BSA; Steraloids) was prepared as a 1 mM stock solution in dimethyl sulfoxide (DMSO) and diluted further with aCSF to the working concentration of 100 nM. The ER antagonist 7 α ,17 β -[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) was prepared as a 1 mM stock solution in punctilious ethanol and diluted further with aCSF to the working concentration of 1 μ M.

STX (synthesized by AAPharma Syn, Ann Arbor according to the Tobias et al., ChemMedChem 2006 protocol) was prepared as a 1 mM stock solution in DMSO and further diluted with aCSF to the working concentration of 10 nM. The ER α agonist 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (propyl pyrazole triol/PPT) was prepared as a 10 mM stock solution in punctilious ethanol and diluted further with aCSF to the working concentration of 1 μ M. The GPR30 agonist ($\pm$)-1-[(3aR*, 4S*, 9bS*)-4-(6-bromo-1, 3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone (G1) was prepared as a 10 mM stock solution in DMSO and diluted further with aCSF to the working concentration of 3 μ M. The ER β agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) was prepared as a 10-mM stock solution in punctilious ethanol and diluted further with aCSF to the working concentration of 3 μ M.

The PI3K inhibitor 2-(4-morpholinyl)-8-(4-aminophenyl)-1-4H-1-benzopyran-4-one (PI828) was prepared as a 10 mM stock solution in DMSO and further diluted with aCSF to the working concentration of 10 μ M. The PLC inhibitor 1-[6-[[[(17 β)-3-methoxyestra-1, 3, 5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) was prepared as a 20 mM stock solution in DMSO and further diluted with aCSF to the working concentration of 20 μ M. The inactive U73122 analog 1-[6-[[[(17 β)-3-methoxyestra-1,3, 5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione (U73343) was prepared as a 20 mM stock solution in DMSO and further diluted with aCSF to the working concentration of 20 μ M. The PKC inhibitor (S)-2,6-diamino-N-[(1-(1-oxotridecyl)-

2-piperidinyl)methyl]hexanamide dihydrochloride hydrate (NPC 15437) was prepared as a 30 mM stock solution in DMSO and diluted further with aCSF to the working concentration of 30 μ M. The PKC activator phorbol 12,13-dibutyrate (PDBu) was prepared as a 10 mM stock solution in DMSO and further diluted with aCSF to the working concentration of 1 μ M. The PKA inhibitor (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3, 4-*i*] [1,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT5720) was prepared as a 300 μ M stock solution in DMSO and further diluted with aCSF to the working concentration of 300 nM. The PKA activator, (S)-adenosine, cyclic 3',5'-(hydrogen phosphorothioate) triethylammonium (Sp-cAMP) was prepared as a 10 mM stock solution in UltraPure H₂O and diluted further with aCSF to the working concentration of 100 μ M. The nNOS inhibitor N⁵-[imino(propylamino)methyl]-L-ornithine hydrochloride (N-propyl-L-arginine; NPLA) was prepared as a 10 mM stock solution in UltraPure H₂O and further diluted with aCSF to the working concentration of 10 μ M. The endogenous NOS substrate L-arginine was prepared as a 30 mM stock solution in UltraPure H₂O and diluted further with aCSF to the working concentration of 30 μ M. The Akt activator 2-amino-6-chloro- α -cyano-3-(ethoxycarbonyl)-4*H*-1-benzopyran-4-acetic acid ethyl ester (SC 79) was prepared as a 10 mM stock solution in DMSO and diluted further with aCSF to the working concentration of 10 μ M.

Stereotactic Surgery

All animals were focally injected with the retrograde tracer Fluorogold (Fluorochrome, LLC, Denver, Colo., USA) into the MPN 6–8 days prior to experimentation. They were fitted in a stereotactic apparatus (Digital Lab Standard; Stoelting Co., Wood Dale, Ill., USA) while under 3% isoflurane anesthesia. The scalp was opened with a 2- to 2.5-cm incision made down the midline of the skull, beginning at the front of the orbits towards the occipital lobe with a scalpel blade. The periosteum was rubbed from the scalp by sterile cotton-tipped applicators. A single hole was drilled, so that an injection needle could be slowly lowered into the MPN [coordinates from bregma, anterior, ($\pm$)0.1 mm; lateral, -0.75 mm; ventral, -6.0 mm from dura; tooth bar, -3.3 mm]. The injection needle was held at these coordinates for 1 min before the start of infusion. The retrograde tract tracer Fluorogold (5% dissolved in sterile saline; 0.7 μ l total volume) was injected slowly, over a period of 6 min, into the MPN using a Stoelting manual injector system. The injection needle remained in place for an additional 2 min after infusion to allow for diffusion from the tip and then slowly removed from the brain to reduce potential spread of Fluorogold. Sterile bone wax was placed in the hole to seal the cavity and help promote clotting. After surgery, the rats were given oral antibiotics in drinking water (0.5 mg/ml of sulphamethoxazole and 0.1 mg/ml of trimethoprim; Hi-Tech Pharmacal, Amityville, N.Y., USA), as well as Carprofen (5 mg/kg; s.c.; Sigma Aldrich Corp., St Louis, Mo., USA) to help control postoperative pain. Only those animals in which the Fluorogold was injected directly into the MPN were included in the present study.

Tissue Preparation

On the day of experimentation, the ovariectomized rat was anesthetized with 32% isoflurane and rapidly decapitated. The brain was removed from the skull and the hypothalamic area was dissected. We then mounted the resultant hypothalamic block on

a cutting platform that was secured in a vibratome well filled with ice-cold, oxygenated (95% O₂, 5% CO₂) aCSF in which the majority of sodium was replaced by sucrose (sucrose, 208; NaHCO₃, 26; KCl, 2; NaH₂PO₄, 1.25; dextrose, 10; HEPES, 10; MgSO₄, 2; MgCl₂, 1; CaCl₂, 1; in mM). Four to five coronal slices (300 μ m) through the rostrocaudal extent of the ARH were then cut at 1°C. The slices were transferred to an auxiliary chamber containing room-temperature oxygenated aCSF (NaCl, 124; NaHCO₃, 26; dextrose, 10; HEPES, 10; KCl, 5; NaH₂PO₄, 2.6; MgSO₄, 2; CaCl₂, 1; in mM) and kept there until electrophysiological recording. Slices were allowed at least 1 h of recovery before being transferred from the auxiliary chamber to the recording chamber.

Electrophysiology

During whole-cell patch recording from ARH neurons, slices were maintained in a chamber perfused with a warmed (35°C), oxygenated aCSF in which the CaCl₂ concentration was raised to 2 mM. aCSF and all drugs (diluted with aCSF) were perfused via a peristaltic pump at a rate of 1.5 ml/min. Patch electrodes are assembled from borosilicate glass (World Precision Instruments, Sarasota, Fla., USA; 1.5 mm OD) pulled on a P-97 Flaming Brown puller (Sutter Instrument Co., Novato, Calif., USA) and filled with the following (in mM): potassium gluconate, 128; NaCl, 10; MgCl₂, 1; EGTA, 11; HEPES, 10; ATP, 1; GTP, 0.25; 0.5% biocytin; adjusted to a pH of 7.3 with KOH. Electrode resistances varied from 3 to 8 M Ω . A Multiclamp 700A preamplifier (Axon Instruments, Foster City, Calif., USA) amplified potentials and passed current through the electrode. Membrane currents were recorded in voltage clamp (and membrane potential in current clamp) with access resistances that typically range from 8 to 22 M Ω , and underwent analog-digital conversion via a Digi-data 1322A interface coupled to pClamp 8.2 software (Axon Instruments). The access resistance (R_A), as well as the resting membrane potential and the input resistance (R_{in}), were monitored throughout the course of the recording. If the access resistance deviated >10% of its original value, the recording was ended. Low-pass filtering of the currents was conducted at a frequency of 2 kHz. The liquid junction potential was calculated to be -10 mV and corrected for during data analysis using pClamp software. Current-voltage (I/V) relationships were generated by administering pulses (10-mV increments; 150-ms duration) ranging from -50 to -130 mV. These were used to calculate OFQ/N-induced changes in slope conductance via linear regression in between -60 and -80 mV as well as -100 and -130 mV. All recordings were performed from a holding potential of -60 mV.

Baseline I/V relationships were generated after 3 min of perfusion with a solution containing TTX (500 nM) and either (1) vehicle, E₂, 17 α -E₂, or E₂-BSA; (2) vehicle, one of several ER agonists, or E₂ plus an ER antagonist, or (3) vehicle, one of several enzyme activators, or E₂ plus one of several enzyme inhibitors. After the baseline I/V, the solution was removed and replaced with a second solution containing the same mixture as the first, with the addition of OFQ/N (1 μ M). The membrane current (or potential) was continuously monitored until a new steady-state is reached, at which time a second I/V relationship is generated. The OFQ/N solution was then removed to allow the aCSF to clear the drugs from the slice. During the washout, the membrane current (or potential) was again monitored until it returned to its original baseline level, at which time a final I/V relationship was taken to ensure reversibility of the OFQ/N-induced effect. A schematic of the protocol for the drug administration is shown in figure 1. Control respons-

Fig. 1. Schematic representation of drug solution perfusion protocol used during electrophysiological recordings.

Fig. 2. A photomicrograph depicting a representative example of an MPN-projecting, POMC-positive, recorded ARH neuron. **a** Representative coronal section showing a typical injection site within the MPN and the spread of the Fluorogold after 7 days. **b** Biocytin labeling in the ARH neurons as visualized with streptavidin-cy2. **c** α -MSH immunoreactivity visualized with AF546. **d** Fluorogold labeling of the cell in **b**. **e** Composite overlay.

es are defined as an OFQ/N-induced hyperpolarization/outward current (and their corresponding I/V relationships) generated in the presence of the respective vehicle solutions for either E_2 , $17\alpha-E_2$, E_2 -BSA, ER agonists, the E_2 /ER antagonist cocktail, enzyme activators or the E_2 /enzyme inhibitor cocktails.

Immunohistochemistry

After conducting electrophysiological recording, slices were fixed with 4% paraformaldehyde in Sorensen's phosphate buffer (pH 7.4) for 90–180 min. They were then immersed overnight in 20% sucrose dissolved in Sorensen's buffer and frozen in Tissue-Tek embedding medium (Miles Inc., Elkhart, Ind., USA) the next day. Coronal sections (20 μ m) were cut on a cryostat and mounted on slides. These sections were washed with 0.1 M sodium phosphate buffer (pH 7.4) and then processed with either streptavidin-Cy2 (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) or streptavidin-Alexa Fluor 488 (Molecular Probes Inc., Eu-

gene, Oreg., USA) at a dilution of 1:300. After localizing the biocytin-filled neuron via fluorescence microscopy, the slides containing the appropriate sections were processed with polyclonal antibodies directed against either β -endorphin (Immunostar Inc., Hudson, Wis., USA; 1:400 dilution), α -melanocyte-stimulating hormone (α -MSH, Immunostar; 1:200 dilution) or cocaine- and amphetamine-regulated transcript (Phoenix Pharmaceuticals Inc., Burlingame, Calif., USA; 1:2,000 dilution) using fluorescence immunohistochemistry [39, 40].

Statistical Analyses

Comparisons between two groups were made with either the Student's *t* test or the Mann-Whitney U test. Comparisons between more than two groups were performed using the multifactorial analysis of variance (ANOVA) followed by the least significant difference test. Differences were considered statistically significant if the alpha probability was <0.05.

Fig. 3. Estradiol rapidly attenuates OFQ/N-induced postsynaptic currents in POMC neurons. Membrane current traces showing the OFQ/N-induced outward current observed during electrophysiological recordings from slices treated either with EtOH vehicle (**a**) or E₂ (100 nM; **b**). The concatenated, incremental current traces

reflect the I/V relationships generated prior to, and in the presence of, OFQ/N. Composite I/V plot (**c**) and bar graph (**d**) illustrating the marked reduction in slope conductance (ΔG) caused by E₂. * $p < 0.05$, multifactorial ANOVA/least significant difference.

Results

Experiment 1: The Effect of OFQ/N on MPN-Projecting, ARH POMC Neurons

We recorded from a total of 190 hypothalamic ARH neurons. These cells exhibited a resting membrane potential of -55.52 ± 0.67 mV and an R_{in} of 479.33 ± 17.99

MΩ. Ninety-eight of these cells were immunopositive for various markers of POMC neurons, and of these POMC-positive cells, 92 projected into the MPN. All recordings followed the same perfusion and recording protocol shown in figure 1. An example of an ARH POMC-positive neuron projecting to the MPN is represented in figure 2.

Fig. 4. Estradiol rapidly diminishes the OFQ/N-induced hyperpolarization of POMC neurons. Membrane current traces showing the hyperpolarization caused by OFQ/N during recordings in slices treated with EtOH vehicle (**a**) or E₂ (**b**). The composite bar graph (**c**) shows the estrogenic reduction of the OFQ/N-induced hyperpolarization. * $p < 0.05$, Student's t test.

OFQ/N has been known to regulate female sexual receptivity through the inhibition of POMC neurons [40]. In vehicle-treated slices, OFQ/N (1 μ M) produces a robust outward current that reverses upon clearance of the peptide much like the current shown in figure 3a. This response was associated with an increase in conductance and reversed polarity near the Nernst equilibrium potential for K⁺. The current elicited from ARH POMC neurons from ovariectomized female rats is significantly more sizeable compared to the responses seen in unidentified/POMC-negative ARH neurons (36.39 ± 7.21 vs. 13.39 ± 3.25 pA; Student's t test, $t = -2.06641$, $p < 0.05$; $n = 10$ –14). Therefore, all subsequent experimental analyses were performed on cellular cohorts enriched in pos-

itively identified POMC neurons (>57% total cells) that also contained unidentified cells bearing inherent characteristics of POMC neurons (i.e., robust OFQ/N responsiveness, expression of the hyperpolarization-activated cation current and/or the A-type K⁺ current [35, 41, 42]).

Experiment 2: The Receptor Subtypes through Which E₂ Rapidly Attenuates the OFQ/N Responsiveness of MPN-Projecting ARH POMC Neurons

In order to test our hypothesis that E₂ rapidly attenuates the inhibitory actions of ORL-1 signaling, OFQ/N was bath applied to a cell in both the presence and absence of E₂. Bath application of E₂ (100 nM) per se for a mere 8–10 min prior to OFQ/N had negligible effects on the

Fig. 5. The estrogenic modulation of the OFQ/N-induced outward current in POMC neurons is stereoselective, membrane delimited and ER mediated. Membrane current traces (**a–c**) and composite bar graph of ΔI and ΔG (**d**) illustrating the attenuation of OFQ/N-induced outward current caused by E_2 -BSA (100 nM) but not 17α - E_2 (100 nM) or E_2 + ICI 182,780 (1 μ M). * $p < 0.05$, Mann-Whitney U test.

holding current (2.94 ± 2.54 pA; $n = 21$) or the slope conductance between -60 and -80 mV (0.23 ± 0.03 nS; $n = 21$). In the presence of OFQ/N, however, E_2 markedly diminished the responsiveness of ARH POMC neurons to the neuropeptide (fig. 3b). Additionally, the composite I/V plot and conductance change (fig. 3c, d) further illustrate the attenuating effects of E_2 on the OFQ/N-induced outward current (fig. 3c: multifactorial ANOVA, $F_{steroid} = 15.24$, d.f. = 1, $p < 0.0001$, $F_{voltage} = 20.51$, d.f. = 8, $p < 0.0001$, $F_{interaction} = 7.42$, d.f. = 8, $p < 0.0001$; fig. 3d: multifactorial ANOVA, $F_{steroid} = 15.24$, d.f. = 1, $p < 0.0001$, $F_{voltage} = 1.69$, d.f. = 1, $p < 0.20$, $F_{interaction} = 1.52$, d.f. = 8, $p < 0.23$). The pronounced OFQ/N-induced outward cur-

rent seen in ARH POMC neurons from vehicle-treated slices is associated with a robust and reversible hyperpolarization that leads to the cessation of firing (fig. 4a). When OFQ/N was applied in the presence of E_2 , the magnitude the hyperpolarization was clearly decreased (fig. 4b). The composite graph of the hyperpolarization magnitude (fig. 4c) provides further evidence of the rapid attenuating effect of E_2 (Student's t test, $t = 5.68449$, $p < 0.0001$).

To determine the stereospecificity of the E_2 -ER binding responsible for the attenuation of the OFQ/N response, we pretreated slices with 17α - E_2 instead of E_2 along with OFQ/N. In this case, the OFQ/N-induced out-

Fig. 6. The estrogenic modulation of the OFQ/N-induced outward current in POMC neurons is due to activation of G_q -mER and ER α . Membrane current traces (a–d) and composite bar graph of ΔI and ΔG (e) showing inhibition of the OFQ/N-induced outward current caused by the G_q -mER ligand STX (10 nM) and ER α agonist PPT (1 μ M) but not the GPR30 agonist G1 (3 μ M) or the ER β agonist DPN (3 μ M). * $p < 0.05$, Mann-Whitney U test.

ward current was as robust as that seen in recordings from vehicle-treated control slices (fig. 5a); therefore, showing that the E₂ attenuation of the OFQ/N response is stereoselective. To test whether this estrogenic impairment is membrane initiated, we perfused the membrane impermeant E₂-BSA (100 nM) in lieu of E₂. Just as with E₂, E₂-BSA caused a significant reduction in the OFQ/N-outward current (fig. 5b), providing evidence that this E₂ effect is indeed membrane delimited. However, when the

ER antagonist ICI 182,780 (1 μ M) was co-perfused with E₂, the magnitude of the outward current was nearly identical to that observed in vehicle-treated slices (fig. 5c); providing a clear indication of the ER-mediated actions of E₂. Additionally, the composite current and conductance values (fig. 5d) corroborate these findings [ΔI (Mann-Whitney U test) – E₂: $W = 347.0$, $p < 0.002$, 17 α -E₂: $W = 91.0$, $p < 0.27$, E₂-BSA: $W = 126.0$, $p < 0.04$, E₂/ICI 182,780: $W = 160.0$, $p < 0.87$; ΔG (Mann-Whitney U

Fig. 7. The estrogenic modulation of the OFQ/N-induced outward current in POMC neurons is dependent on the activation of PI3K. Membrane current traces (**a**) and composite bar graph of ΔI and ΔG (**b**) showing how the PI3K inhibitor PI828 (10 μ M) rescues the OFQ/N-induced outward current in the presence of E_2 . * $p < 0.05$, Mann-Whitney U test.

Fig. 8. The estrogenic modulation of the OFQ/N-induced outward current in POMC neurons is dependent on the activation of PLC. Membrane current traces (**a**, **b**) and composite bar graph of ΔI and ΔG (**c**) showing how U73122 (20 μ M) but not U73343 (20 μ M) abrogates the E_2 -induced attenuation of the OFQ/N-induced outward current in ARH POMC neurons. * $p < 0.05$, Mann-Whitney U test.

Fig. 9. The estrogenic modulation of the OFQ/N-induced outward current in POMC neurons is blocked by inhibition and mimicked by activation of PKC. Membrane current traces (**a**, **b**) and composite bar graph of ΔI and ΔG (**c**) showing the restoration of the OFQ/N-induced outward current caused by the PKC inhibitor NPC 15437 (30 μ M) in E_2 -treated slices, and the attenuation caused by the PKC activator PDBu (1 μ M) per se. * $p < 0.05$, Mann-Whitney U test.

test) – E_2 : $W = 310.5$, $p < 0.0008$, 17α - E_2 : $W = 125.5$, $p < 0.66$, E_2 -BSA: $W = 121.5$, $p < 0.05$, E_2 /ICI 182,780: $W = 92.5$, $p < 0.56$].

Because there are multiple ER subtypes involved with E_2 signaling, it was crucial to determine which are involved specifically in estrogenic diminution of the OFQ/N response in ARH POMC neurons. We thus tested the ER agonists STX, PPT, G1 and DPN that are selective for G_q -mER, ER α , GPR30 and ER β , respectively. Both the G_q -mER ligand STX (10 nM) and the ER α agonist the PPT (1 μ M) reduced the OFQ/N outward current [fig. 6a, b, e; ΔI (Mann-Whitney U test) – STX: $W = 62.0$, $p < 0.02$, PPT: $W = 226.0$, $p < 0.04$; ΔG (Mann-Whitney U test) – STX: $W = 49.0$, $p < 0.02$, PPT: $W = 209.5$, $p < 0.006$], while the GPR30 agonist G1 (3 μ M) and the ER β (3 μ M) agonist DPN did not [fig. 6c–e; ΔI (Mann-Whitney U test) – G1: $W = 67.0$, $p < 0.30$, DPN: $W = 192.0$, $p < 0.32$; ΔG (Mann-Whitney U test) – G1: $W = 58.0$, $p < 0.27$, DPN: $W = 183.5$, $p < 0.07$]. Together, this shows that the G_q -mER

and ER α are responsible for the inhibition of the OFQ/N ORL-1 signaling, while GPR30 and ER β are not.

Experiment 3: The Signal Transduction Mechanisms Underlying E_2 -Mediated, Membrane-Initiated Disruption of OFQ/N-ORL1 Signaling in MPN-Projecting, ARH POMC Neurons

Now that we had an understanding of which ER subtypes were involved, we wanted to tease out the signaling mechanism responsible for the attenuating effects of E_2 . Based on previous findings [43–45], we speculated that PI3K would be a likely upstream signaling molecule. When we administered the PI3K inhibitor PI828 (10 μ M) along with E_2 , we observed an abrogation of the estrogenic impairment of the robust outward current [fig. 7; ΔI (Mann-Whitney U test): $W = 45.0$, $p < 0.35$; ΔG (Mann-Whitney U test): $W = 67.5$, $p < 0.66$], implicating the involvement of PI3K in the attenuation of the OFQ/N response.

Fig. 10. The estrogenic modulation of the OFQ/N-induced outward current in POMC neurons is blocked by inhibition and mimicked by activation of PKA. Membrane current traces (**a**, **b**) and composite bar graph of ΔI and ΔG (**c**) showing that the estrogenic attenuation of the OFQ/N-induced outward current is reversed by the PKA inhibitor KT5720 (300 nM) and mimicked by the PKA activator Sp-cAMP (100 μ M). * $p < 0.05$, Mann-Whitney U test.

Given that the attenuation of the OFQ/N response involved the activation of G_q-mERs, we then wanted to see if PLC plays a role in this estrogenic signaling cascade. When the PLC inhibitor U73122 (20 μ M) was perfused along with E₂, a robust outward current, comparable to the response in a vehicle-treated cell, was witnessed (fig. 8a). In order to assess the specificity of the compound, we also treated slices with an inactive analog of U73122 (U73343; 20 μ M) along with E₂. U73343 was without effect on the E₂-induced inhibition of the OFQ/N outward current (fig. 8b). The aggregate, OFQ-induced changes in current and slope conductance (fig. 8c) validate the conclusion that PLC is necessary for the estrogenic inhibition of the OFQ/N response [ΔI (Mann-Whitney U test) – U73122: $W = 122.0$, $p < 0.91$, U73343: $W = 188.0$, $p < 0.05$; ΔG (Mann-Whitney U test) – U73122: $W = 140.5$, $p < 0.50$, U73343: $W = 122.5$, $p < 0.002$].

Since PI3K and PLC were clearly essential for the attenuating effects of E₂ on the OFQ/N-induced outward

current, the next logical step was to see if PKC was involved. When we perfused the PKC inhibitor NPC 15437 (30 μ M) along with E₂, we observed a powerful OFQ/N outward current (fig. 9a). Conversely, the PKC activator PDBu (1 μ M) alone was able to induce an inhibition of the OFQ/N outward current to the same extent as E₂ (fig. 9b). Composite current and conductance changes provide further evidence for just how vital PKC is for the estrogenic inhibition of the OFQ/N-induced outward current [fig. 9c; ΔI (Mann-Whitney U test) – NPC 15437: $W = 122.0$, $p < 0.06$, PDBu: $W = 102.0$, $p < 0.002$; ΔG (Mann-Whitney U test) – NPC 15437: $W = 132.0$, $p < 0.10$, PDBu: $W = 86.0$, $p < 0.05$].

Because previous studies suggested that PKC upregulates PKA expression [36], we were interested in looking into the involvement of PKA. The PKA inhibitor KT5720 (300 nM) perfused along with E₂ reversed the estrogenic diminution of the OFQ/N-induced outward current (fig. 10a), while the cAMP activator Sp-cAMP (100 μ M)

Fig. 11. The estrogenic modulation of the OFQ/N-induced outward current in POMC neurons is blocked by inhibition and mimicked by activation of nNOS. Membrane current traces (**a**, **b**) and composite bar graph of ΔI and ΔG (**c**) showing that the estrogenic diminution of the OFQ/N-induced outward current is blocked by the nNOS inhibitor NPLA (10 μ M) and mirrored by the NOS substrate L-arginine (30 μ M). * $p < 0.05$, Mann-Whitney U test.

per se mimicked the effect of E₂ and suppressed the outward current (fig. 10b). The composite graph of the OFQ/N-induced changes in current and slope conductance values across the different treatment conditions corroborates these findings [fig. 10c; ΔI (Mann-Whitney U test) – KT5720: $W = 126.0$, $p < 0.90$, Sp-cAMP: $W = 4.0$, $p < 0.002$; ΔG (Mann-Whitney U test) – KT5720: $W = 170.0$, $p < 0.08$, Sp-cAMP: $W = 14.0$, $p < 0.04$].

We have established the importance of PI3K, PLC, PKA and PKC to the estrogenic impairment of OFQ/N-ORL-1 signaling in ARH POMC neurons. Given that NOS has been shown to be an important downstream signaling molecule for both peripheral [43] and central [44] effects of E₂, we took the opportunity to see if nNOS was critical for this inhibition of OFQ/N to take place. When the nNOS inhibitor NPLA (10 μ M) was perfused along with E₂ there was, again, a very robust and reversible outward current (fig. 11a). However, when the NOS substrate L-arginine (30 μ M) was applied in lieu of E₂, the

outward current was significantly inhibited (fig. 11b). The composite graph of the OFQ/N-induced changes in current and slope conductance across the different treatment conditions confirms these results [fig. 11c; ΔI (Mann-Whitney U test) – NPLA: $W = 108.0$, $p < 0.75$, L-arginine: $W = 66.0$, $p < 0.006$; ΔG (Mann-Whitney U test) – NPLA: $W = 65.0$, $p < 0.18$, L-arginine: $W = 85.5$, $p < 0.05$].

Additionally, evidence shows that PI3K signaling in the PVN and in the vascular endothelium can produce phosphatidylinositol (3,4,5) trisphosphate (PIP3) that subsequently stimulates Akt, which is then capable of activating nNOS [43, 44]. Therefore, it was important to establish if Akt plays a role in this signaling cascade. When the Akt activator SC 79 (10 μ M) was perfused in the absence of E₂, there was a reduction in the OFQ/N-induced outward current to a degree comparable with that produced by the steroid itself. Upon application of the nNOS inhibitor NPLA (10 μ M) in addition to SC 79, there was a

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(For legend see next page.)

clear rescuing effect that resulted in a robust OFQ/N-induced outward current (online suppl. fig. 1; see www.karger.com/doi/10.1159/000443765 for all online suppl. material).

Discussion

The present results demonstrate that E_2 rapidly attenuates OFQ/N-ORL-1 coupling in MPN-projecting ARH POMC neurons serving as a critical neuroanatomical substrate in a limbic-hypothalamic circuit that regulates sexual behavior in the female rat. These findings are based on the following observations: (1) Nearly half of the recorded ARH neurons were immunopositive for antigenic markers of POMC neurons and labeled with retrograde tracer injected into the MPH that contains the terminal fields of these cells; (2) OFQ/N evoked a robust outward current that hyperpolarized and inhibited firing in these cells via activation of GIRK channels, which was markedly diminished upon short-term exposure to E_2 . The findings also demonstrate that the attenuating effect of E_2 is stereoselective, membrane-delimited and ER-mediated via activation of ER α and G_q -mER. We base these conclusions on the observations that the E_2 -induced decrease in ORL-1/GIRK coupling is: (1) mimicked by the membrane impermeant E_2 -BSA conjugate but not by the 17 α enantiomer of E_2 ; (2) blocked by the ER antagonist ICI 162,780, and (3) mimicked by the ER α agonist PPT and the G_q -mER ligand STX but not the GPR30 or ER β agonists G1 and DPN, respectively. Lastly, our findings demonstrate this membrane-initiated estrogenic signaling utilizes a PLC/PKC/PI3K/nNOS pathway to decouple the ORL-1 receptors from their GIRK channels. We

base this on the fact that the E_2 -induced decrement in the outward current caused by OFQ/N is: (1) blocked by the PI3K inhibitor PI828, the PLC inhibitor U73122 (but not the inactive analog U73343), the PKC inhibitor NPC 15437, the PKA inhibitor KT5720 and the nNOS inhibitor NPLA, the Akt activator SC 79, and (2) mimicked by the PKC activator PDBu, the PKA activator Sp-cAMP, and the NOS substrate L-arginine.

This study provides expansive insight into preceding research on how E_2 regulates sexual behavior. We know that E_2 rapidly increases β -endorphin release from ARH POMC neurons, which activates μ -opioid receptors expressed in neurons in the MPN [25, 26]. The E_2 -induced stimulation of these POMC neurons initially suppresses lordosis, and this is essential for the subsequent expression of sexual behavior. We know this because pretreatment with the μ -opioid receptor-selective antagonist naltrexone prior to the initiation of estrogen priming significantly reduces lordosis in estrogen-primed, progesterone-treated female rats to a degree similar to that observed in μ -opioid receptor knockout mice [7, 11]. Thus, the uncoupling of the ORL-1 receptor from the GIRK channel in ARH POMC neurons that we see would serve to rapidly dampen this important inhibitory influence and thereby disinhibit these cells. This could explain, at least in part, the resultant increase in β -endorphin release in the MPN, which would then recalibrate the reproductive axis and set the stage for subsequent, appropriately timed sexual receptivity. These results are also consistent with the rapid attenuation of OFQ/N-induced spinal antinociception [38]. In addition, they are in line with the rapid modulation of other neuronal elements within the reproductive axis, as E_2 has long been known to directly hyperpolarize and thereby inhibit GnRH neurons within minutes of its application [27, 46]. Moreover, the ability of E_2 to rapidly disrupt the coupling of the ORL-1 receptor from the GIRK channel in ARH POMC neurons is congruent with that previously demonstrated for other metabotropic, $G_{i/o}$ -coupled receptors such as the μ -opioid and GABA $_B$ receptors in these cells [23, 46], as well as 5HT $_{1A}$ receptors in other systems [43, 44]. Since POMC neurons innervate GnRH neurons [47, 48], this collective body of evidence reinforces the pleiotropism through which E_2 exerts negative feedback on the reproductive axis and related behavior.

G_q -mER, ER α and GPR30 are the ERs implicated in the OFQ/N-induced spinal antinociception [38]. However, ER α and G_q -mER have been found to play a more significant role in controlling sexual receptivity by acting on $G_{i/o}$ -coupled receptors to modulate sexual receptivity

Fig. 12. a Schematic representation of how E_2 attenuates ORL-1/GIRK coupling in MPN-projecting, ARH POMC neurons. In the absence of ER activation, ORL-1/GIRK coupling is fully functional; resulting in the robust inhibition of MPN-projecting ARH POMC neurons. **b** E_2 activation of ER α or G_q -mER causes the dissociation of the α_q subunit to activate PLC. PLC activates PKC, which then phosphorylates adenylyl cyclase that converts ATP to cAMP. cAMP then activates PKA, which could phosphorylate either the ORL-1 receptor, RGS proteins (not shown) and/or GIRK channels. Additionally, ER α can activate PI3K, which stimulates the nNOS enzyme and subsequently guanylate cyclase that converts GTP to cGMP. cGMP activates PKG, which may also contribute to the phosphorylation of either the ORL-1 receptor, RGS proteins or GIRK channels. Collectively, this increases the excitability of MPN-projecting, ARH POMC neurons and thus the release of β -endorphin.

[13–15, 49]. These two membrane ERs differ from each other in that STX, the selective ligand for G_q -mER, does not bind to ER α [23, 50]. However, E_2 will bind and activate both ER α and G_q -mER. Additionally, these ERs have been found to facilitate the uncoupling of other receptors such as the μ -opioid receptor and the GABA $_B$ receptor [23, 36, 51]. Earlier immunohistochemical studies in the rat suggested that comparatively few POMC neurons (~4%) express ER α [48]. However, more recent studies in the mouse [52] and in particular the guinea pig [53] indicate that considerably more POMC express ER α (26–29 and 74%, respectively). Thus, it follows that the degree of the inhibitory effect on the OFQ/N-induced outward current in POMC neurons seen upon pretreatment with the ER α agonist PPT would be dependent on whether ER α is in fact expressed in these cells. It is now well established that E_2 can act within seconds to rapidly alter neuronal excitability via ‘membrane-initiated steroid signaling’, [24]. In order for this membrane-initiated steroid signaling to occur, ERs must first be trafficked to the plasma membrane. The localization of all sex steroid receptors to the plasma membrane caveolae rafts requires palmitoylation at a highly conserved sequence in the ligand-binding domain. Additionally, caveolin-1 is a necessary transporter of ER α to the rafts in the plasma membrane, and is essential for ER α -mediated regulation of sexual receptivity [49]. In cells lacking caveolin-1, endogenous ER α is only found intracellularly [54]. The localization of sex steroid receptors at the membrane requires the attachment of palmitic acid to an internal cysteine [54]. Palmitoylation occurs at a cysteine within a conserved 9-amino-acid motif in the ligand-binding domain [54]. Once localized to the plasma membrane, the receptors interact with scaffold and linker proteins, and can effectively activate G protein α - and $\beta\gamma$ -subunits. G protein activation results in the transduction of kinase cascades that occur in seconds to minutes after receptor binding by ligand [55, 56]. Moreover, both ER α and G_q -mER can associate with metabotropic glutamate receptors in the plasma membrane [13, 18, 57].

The present study elucidates the importance of the PI3K/PLC/PKC/PKA pathway with regard to E_2 inhibition of the OFQ/N-induced activation of GIRK channels. Our data have shown that PI3K, PLC, PKC and PKA all play a vital role in the signal transduction between E_2 and the ORL-1/GIRK coupling. These findings are consistent with those found in Small et al. [38] who provided strong evidence for rapid, nongenomic effects of estrogen mediated via ERK in the modulation of ORL1-mediated antinociceptive responses to OFQ/N. Additionally, our data

corroborate findings that ER α mediates rapid E_2 signal transduction via PKC that leads to MPN μ -opioid receptor internalization in the regulation of sexual receptivity [12, 18]. Finally, our findings also reflect similarities between the estrogenic effects on ORL-1/GIRK coupling and other metabotropic $G_{i/o}$ -coupled receptors such as μ -opioid receptors or GABA $_B$ receptors as both receptors couple to a GIRK channel and E_2 facilitates their uncoupling through PI3K/PLC/PKC/PKA signaling [36, 45].

It is our thought that this signaling cascade, initiated by E_2 binding to ER α or the G_q -mER, will begin with the disassociation of the α_q subunit to activate PLC. PLC then converts phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Recent evidence also suggests that α_q can promote Akt translocation to the membrane and PI3K-dependent phosphorylation [58, 59]. PI3K, which is an upstream mediator of E_2 signaling in the PVN as well as in the vascular endothelium [43, 44], is necessary for the facilitation of E_2 uncoupling of GABA $_B$ receptors to their GIRK channels leading to GABA $_B$ desensitization [45]. PI3K synthesizes phosphatidylinositol 3-phosphates, which regulate the activity of kinases like protein kinase B (Akt; fig. 12; online suppl. fig. 1). The serine/threonine kinase Akt is the principal downstream effector of PI3K, triggering several of its cellular effects, including activation of endothelial nitric oxide synthase. Results from the present study show that activation of Akt in the absence of E_2 can mimic the estradiol-induced attenuation of the OFQ/N induced outward current, while concomitant inhibition of the downstream nNOS will reverse the Akt-induced diminution; resulting in a robust OFQ/N induced outward current. This shows that nNOS is stimulated downstream of Akt activation. Furthermore, the E_2 -induced disassociation of the α_q subunit activates PLC. PLC then converts PIP2 to IP3 and DAG. Additionally, PI3K phosphorylates PIP2 to yield PIP3 and is involved with the downstream signaling. Because both PLC and PI3K utilize PIP2 as a substrate, it is important to note that the opening of GIRK channels via the activation of metabotropic $G_{i/o}$ protein-coupled receptors requires a permissive amount of PIP2, which is depleted by PLC and PI3K upon ER α and/or G_q -mER activation. Once the amount becomes insufficient, ORL-1/GIRK channel coupling becomes destabilized [60–62]. IP3 goes on to liberate intracellular calcium, and DAG activates PKC. PKC, which has been confirmed to be upstream of PKA [3], phosphorylates adenylyl cyclase, which converts ATP to cAMP. cAMP then activates PKA further downstream and phosphorylates either the GIRK channel or the ORL-

1 receptor [63, 64], diminishing receptor/effector coupling. It is also important to keep in mind that the regulator of G protein signaling (RGS) proteins also play an important role in the modulation of G protein-coupled receptors through GTPase activation [65, 66]. It has also been established that E_2 can increase the amount of RGS proteins within the PVN, and thus follows that RGSs may possibly play a role in the E_2 -induced uncoupling of the ORL-1 and GIRK channels just like they do in the desensitization of 5HT_{1A} receptors [67]. Thus, vital components of this mechanism have been shown in the case of the estrogenic regulation of GIRK channels linked to μ -opioid and GABA_B receptors, and we feel that this is reflective of what is occurring with OFQ/N-ORL-1 signaling. The convergence of ER α and G_q-mER on G_q-mediated signaling pathways that diminish the coupling between G_{i/o}-linked receptors and GIRK channels in POMC neurons sharply contrasts with what is observed in neuropeptide Y/agouti-related peptide neurons, in which ER α stimulation attenuates whereas G_q-mER stimulation potentiates GABA_B receptor-mediated activation of GIRK channels [51].

Additionally, we show that nNOS is involved in the ER α -mediated uncoupling of the ORL-1 and GIRK channel. This enzyme is also regulated by E_2 in the PVN, as is endothelial nitric oxide synthase in the vascular endothelium through an Src kinase (SRC)/PI3k/Akt pathway [43, 44], and we have shown previously that E_2 increases nNOS activity in the ARH [37]. The nNOS enzyme stimulates guanylate cyclase, which will convert GTP to cGMP. The newly produced cGMP can then activate protein kinase G (PKG) [68]. We suspect that the estrogenic activation of PKG can also contribute to the phosphorylation of either the ORL-1 receptor, RGS proteins or GIRK channel, thus leading to a disinhibition of MPN-project-

ing, ARH POMC neurons and a suppression of lordotic behavior (fig. 12). Alternatively, nNOS-induced formation of nitric oxide can lead to s-nitrosylation of thiol groups on cysteine residues in signaling molecules, ion channels, as well as ionotropic and metabotropic receptors, resulting in an alteration in the function of these proteins [69, 70]. Thus, it is possible that the E_2 -induced activation of nNOS causes s-nitrosylation that uncouples the ORL-1 receptor from its effector systems, as has been shown for other G_{i/o}-linked receptors like the M2 muscarinic receptor [70].

In conclusion, E_2 rapidly attenuates the ORL-1 receptor-mediated inhibition of POMC neurons in female rats by activating the PI3K/PLC/PKC/PKA/Akt/nNOS pathways. E_2 exerts far-reaching effects on vital, everyday physiological functions. When there is a defect in E_2 biosynthesis, bioavailability or ER-mediated signal transduction, there will be a clear decrease in quality of life. The unfortunate symptoms of such abnormalities can affect ovulation and uterine development that underlie fertility, not to mention bone density, cardiovascular function and memory [4]. By gaining a better understanding of the signal transduction mechanisms through which E_2 disrupts the coupling of ORL-1 receptors to their GIRK channels at a critical neuroanatomical substrate within the limbic-hypothalamic circuitry controlling female sexual receptivity, we can develop better treatments to fit individual needs for the treatment of infertility or the regulation of fertility.

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