Estradiol Upregulates Progesterone Receptor and Orphanin FQ Colocalization in Arcuate Nucleus Neurons and Opioid Receptor-Like Receptor-1 Expression in Proopiomelanocortin Neurons That Project to the Medial Preoptic Nucleus in the Female Rat

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Abstract

Background: Ovarian steroids regulate sexual receptivity in the female rat by acting on neurons that converge on proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus (ARH) that project to the medial preoptic nucleus (MPN). Estradiol rapidly activates these neurons to release β-endorphin that activates MPN μ-opioid receptors (MOP) to inhibit lordosis. Lordosis is facilitated by the subsequent action of progesterone that deactivates the estradiol-induced MPN MOP activation. Orphanin FQ (OFQ/N; also known as nociceptin) infusions into the ARH, like progesterone, deactivate MPN MOP and facilitate lordosis in estradiol-primed rats. OFQ/N reduces the activity of ARH β-endorphin neurons through post- and presynaptic mechanisms via its cognate receptor, ORL-1. Methods: We tested the hypotheses that progesterone receptors (PR) are expressed in ARH OFQ/N neurons by immunohistochemistry and ORL-1 is expressed in POMC neurons that project to the MPN by combining Fluoro-Gold injection into the MPN and double-label fluorescent in situ hybridization (FISH). We also hypothesized that estradiol increases coexpression of PR-OFQ/N and ORL-1-POMC in ARH neurons of ovariectomized rats. Results: The number of PR- and OFQ/N-immunopositive ARH neurons was increased as was their colocalization by estradiol treatment. FISH for ORL-1 and POMC mRNA revealed a subpopulation of ARH neurons that was triple labeled, indicating these neurons project to the MPN and coexpress ORL-1 and POMC mRNA. Estradiol was shown to upregulate ORL-1 and POMC expression in MPN-projecting ARH neurons. Conclusion: Estradiol upregulates the ARH OFQ/N-ORL-1 system projecting to the MPN that regulates lordosis.

Introduction

Proopiomelanocortin (POMC) neurons are located in the arcuate nucleus of the hypothalamus (ARH) and regulate reproduction and energy balance [1]. The activity of subsets of ARH POMC, putative β-endorphin (β-END), neurons is regulated by ovarian steroids that signal through multisynaptic neurocircuits that converge on...
POMC neurons to regulate the onset and termination of sexual receptivity in the female rat [2–5]. Estradiol initially inhibits sexual receptivity by activating a population of POMC neurons that project to the medial preoptic nucleus (MPN; fig. 1). These POMC neurons release β-END that activates and internalizes MPN μ-opioid receptors (MOP) to inhibit sexual receptivity [3, 4, 6]. Estradiol activates a membrane-associated estrogen receptor-α (ERα) that complexes with and signals through metabotropic glutamate receptor type 1a (mGluR1a; fig. 1) [7–11]. This ERα-mGluR1a signaling rapidly induces γ-aminobutyric acid (GABA) and neuropeptide Y (NPY) neurotransmission to induce β-END release that activates INTERNALIZES MPN MOP and inhibits sexual receptivity – lordosis (fig. 1) [4, 7–9].

Concurrent with activation of this lordosis inhibitory circuit, estradiol also primes neurocircuits for facilitation of lordosis [12–21]. For example, progesterone receptors (PR) necessary for facilitation of lordosis are upregulated, as are orphanin FQ (also known as nociceptin; OFQ/N) and opioid receptor-like receptor-1 (ORL-1, also known as NOP [22]) protein and mRNA expression, and the functional coupling of ORL-1 to its G protein in the mediobasal hypothalamus [12, 21, 23–26]. In the ARH-MPN

![ARH-MPN model lordosis circuit](image-url)
lوردозный нейротрансмиссионный цикл, OFQ/N и ORL-1 являются важными для ингибирования β-END нейронов, которые проявляются в МНП, чтобы содействовать лордозу (рис. 1). Следующие эффекты: OFQ/N, интрацеребровентрикулярного и специфического инъекционного введения OFQ/N, который активирует ORL-1 в вентромедial hypothalamus (VMH)-ARH областях активирует MPN MOP и содействует лордозу [5, 21, 23, 27].

Факторы, влияющие на лордозу, включая синергизм прогестерона или продлённого воздействия на эстрадиол, являются независимыми, что сопровождается уменьшением MPN MOP активации. Основной метод инициирования β-END фотосигнализации после эстрадиола может быть уменьшен через пред- и постсинаптические механизмы, которые активированы через ORL-1 в OFQ/N ORL-1 опиоидную систему [28–30]. OFQ/N прямо сокращает активность POMC нейронов через активацию ORL-1 на POMC нейронах, которые уменьшают содержание G protein-связанных нейтральных каналов (GIRK) (рис. 1) [28–30]. OFQ/N также было показано, что оно может прегназапнически уменьшить нейротрансмиссию через катехоламинергическую нейротрансмиссию к POMC нейронам, сокращая фронтальную активность после гипоталамического стресса, чтобы уменьшить потенциал калия через G protein-связанные интегрированные прямоугольные каналы (GIRK) (рис. 1) [28–30]. Основная часть нейротрансмиссии не приводит к синаптической активности на POMC нейронах, что является важным для ингибирования β-END нейронов [28–30]. Кроме того, эти пред- и постсинаптические эффекты OFQ/N-ORL-1 систем на POMC нейронах активации были показаны, что они могут быть регулированы эстрадиолом и прогестероном на уровне, который совпадает с экспрессией сексуальной receptivity (рис. 1) [29, 30].

В эксперименте I, мы тестировали гипотезу, что PR связаны со значительным двойным экспериментом OFQ/N нейронов, и что экспрессия PR в ARH нейронах увеличивается при двойной эксперименте OFQ/N нейронов [29, 30]. Дальнейшее исследование превентивных и постсинаптических действий OFQ/N-ORL-1 систем на POMC нейронах активации было показано, что они могут быть регулированы эстрадиолом и прогестероном в той же степени, что и ингибиторы GABAergic нейротрансмиссии к POMC нейронам [28–30]. Основная часть нейротрансмиссии не приводит к синаптической активности на POMC нейронах, что является важным для ингибирования β-END нейронов [28–30]. Кроме того, эти пред- и постсинаптические эффекты OFQ/N-ORL-1 систем на POMC нейронах активации были показаны, что они могут быть регулированы эстрадиолом и прогестероном на уровне, который совпадает с экспрессией сексуальной receptivity (рис. 1) [29, 30].

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PR and OFQ/N immunohistochemistry was conducted using a polyclonal PR antibody raised in rabbit (DAKO, Carpinteria, Calif., USA) that detects the PR isoforms A and B [34, 35] and a polyclonal OFQ/N antibody (Neuromics Inc., Edina, Minn., USA) raised in guinea pig. All labeling procedures were done at room temperature unless specified otherwise. Free-floating brain sections were washed in PBS followed by incubation in PBS containing 10% MeOH and 3% H2O2 for 10 min to quench endogenous peroxidase activity. Sections were washed in PBS containing 0.2% Triton X-100 (TX) and incubated in PBS containing 0.75% glycine for 30 min, and then incubated in a blocking solution of PBS containing 1% normal goat serum (NGS) and 1% bovine serum albumin (BSA) for 30 min. Sections were then incubated for 48 h at 4°C in PBS containing 1% NGS and the PR antibody (1:2,000) and then washed 3 times in PBS followed by a wash in Tris-buffered saline (TBS, pH 7.6). The PR primary antibody was labeled using a biotinylated goat anti-rabbit secondary antibody followed by labeling using fluorescein (FITC) Tyramide Signal Amplification kit (TSA kit, Perkin Elmer, Waltham, Mass., USA [5]). The sections were washed in PBS followed by an incubation in PBS blocking solution containing 1% NGS, 1% BSA and 0.5% TX for 60 min. Sections were then incubated for 48 h in the blocking solution containing OFQ/N antibody (1:500). OFQ/N primary antibody was visualized by a rhodamine-conjugated goat anti-guinea pig secondary antibody. Sections were rinsed in PBS followed by TBS and were incubated for 2 h in TBS containing 1% NGS and secondary antibody (1:200, Jackson ImmunoResearch Laboratories Inc., West Grove, Pa., USA). The sections were rinsed in TBS, transferred to Tris buffer and mounted onto Superfrost Plus slides (Fisher Scientific). Mounted sections were dried on a 37°C slide warmer and coveredslipped using Aqua-Poly/Mount (Polysciences Inc., Warrington, Pa., USA).

Immunohistochemistry Controls

The specificity of PR and OFQ/N antibody immunostaining was determined in a set of tissue sections that were incubated in either PR or OFQ/N primary antibody that had been preabsorbed overnight at 4°C with a 10-fold molar excess of the corresponding antibody peptide (PR amino acids 533–547; OFQ/N blocking peptide, Neuromics Inc.). In both sets of controls, no immunoreactive staining patterns were observed.

Analysis

To confirm colocalization of immunoreactivity for PR and OFQ/N, the slides were analyzed using an Olympus Fluoview 1000 confocal laser scanning system mounted on an inverted Olympus IX-81 microscope (Olympus America Inc., Center Valley, Pa., USA). Z-stacks were taken through the depth of the tissue within the areas of interest to allow for a three-dimensional reconstruction of neurons to demonstrate whether or not PR and OFQ/N immunoreactivity were colocalized [4]. The confocal was equipped with an argon ion laser with an output of 458, 488, and 514 nm, a green/yellow diode laser with an output of 559 nm, and a red diode laser with an output of 635 nm. FITC was visualized with a 465- to 495-nm emission filter and a 515- to 555-nm bandpass filter, and TRITC was visualized with a 515- to 550-nm emission filter and 600- to 640-nm bandpass filter.

The PR and OFQ/N double immunohistochemically-labeled sections were analyzed using a Zeiss Axioskop 2 epifluorescent microscope equipped with an AxioCam CCD camera, and a digital image analysis system (Carl Zeiss MicroImaging Inc., Thornwood, N.Y., USA). The images were adjusted using the Channel Mixer tool on Adobe Photoshop CS3 (Extended Version 10.0.1; Adobe, San Jose, Calif., USA). Quantitative measurements of PR and OFQ/N colocalization were performed by counting the number of cell bodies in the ARH which were immunoreactive for either PR only, OFQ/N only, or both PR and OFQ/N. The ARH was outlined, and the number of PR-immunopositive cells and OFQ/N-immunoreactive cells in ARH sections along the rostrocaudal axis at the level of the VMH and median eminence were counted using the National Institute of Health ImageJ software Cell Counter Plug-in (version 1.42; NIH, Bethesda, Md., USA). To avoid double counting of cells, new counts were adjusted using the Abercrombie correction [36] and the total numbers of single-labeled or double-labeled cell bodies were averaged across animals. In order to determine the proportion of PR– or OFQ/N– immunoreactive neurons that coexpress PR and OFQ/N, a percentage was obtained by dividing the total number of PR and OFQ/N colocalized neurons by either the total number of PR-only or OFQ/N-only immunoreactive neurons and then multiplying the calculated number by 100. The data were analyzed by SigmaStat 3.5 (Systat Software Inc., San Jose, Calif., USA) using 2-tailed t tests, where p < 0.05 was considered significant. Arcsin square root transformations were performed on percentage data prior to t test analysis.

Experiment II

To determine whether a population of ARH neurons projects to the MPN and expresses both POMC (putative β-END) and ORL-1, OVX rats were injected with 5% FG into the MPN. One week later, animals were perfused, and brains were collected 24 h after the third 2 μg EB or oil treatment (n = 4 per group). Brains were processed to visualize expression of POMC and ORL-1 mRNA and FG in the ARH.

MPN Fluoro-Gold Injection

Under isoflurane anesthesia and using standard stereotaxic procedures, a 29-gauge infusion needle was slowly lowered into the MPN [29]. The retrograde tract tracer FG (5% dissolved in 0.9% sterile saline) was pressure-injected into the MPN just dorsal to the MPN centralis (MPNc; coordinates from bregma, anterior, −0.1 mm; lateral, −0.8 mm, and ventral, −6.0 mm from dura; tooth bar, −3.3 mm [37]) using a Stoelting syringe pump (total volume 1 μl; 0.1 μl/min). The infusion needle was left in place for 10 min after infusion to allow for diffusion from the tip and then slowly removed from the brain to reduce potential spread of FG (see fig. 4g, h). The skull was sealed with bone wax and the wound closed with surgical wound clips. Rimadyl (5 mg/kg, s.c.) analgesic was given during the surgical procedure. Topical antibiotic was applied to the wound, and oral antibiotics were given in the drinking water (trimethoprim and sulfamethoxazole; 0.5 mg/ml).

Steroid Priming and Tissue Preparation

Animals received a subcutaneous injection of either EB or oil once every 4 days for 3 cycles. Steroid treatments were timed so that 1 week after FG injection and 24 h after the final oil or EB injection, the animals were deeply anesthetized with isoflurane and transcardially perfused with chilled 0.9% saline followed by 4% paraformaldehyde in Sorensen’s phosphate buffer, pH 7.4. Brains were postfixed overnight in 4% paraformaldehyde and then cryoprotected by transferring into 20% sucrose in a 0.1 M phosphate buffered saline solution for 1 week before being protected by transferring into 20% sucrose in a 0.1 M phosphate buffered saline solution for 1 week before being sectioned on a freezing microtome. Sections were collected at 30-μm thickness on 0.1% gelatin-coated slides and stored at −20°C. Thawed sections were permeabilized in 10% MeOH and 3% H2O2 for 10 min to quench endogenous peroxidase activity.
buffer solution (pH 7.5) and stored at 4°C until sectioning [5]. We chose the 24-hour time point for EB exposure because in previous studies POMC has been shown to be upregulated at this time point and also is associated with the upregulation of other systems that are important for the facilitation of lordosis such as PR, NPY and ORL-1 [13, 21, 38, 39].

For in situ hybridization, 20 μm thick coronal brain sections were collected on a cryostat with RNase-free paint brushes and placed directly into RNase-free 24-well plates containing RNase-free 0.1% diethylpyrocarbonate (DEPC)-treated PBS, pH 7.5. Within 48 h, these sections were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pa., USA). Mounted sections were dried on a 37°C slide warmer overnight and stored desiccated at –80°C until processing. The standard series of brain sections selected for labeling was 1:4.

Probe Preparation
ORL-1 in situ hybridization was performed using the plasmid vector pORL1.1 (courtesy of Dr. J. Boulter, Department of Psychiatry, UCLA) containing a 692-bp cDNA fragment of rat-ORL 1.1 (GenBank accession No. U05239; spanning bases 1–692). POMC in situ hybridization used the plasmid vector pPOMC (courtesy of Dr. Sylvie Jegou, University of Rouen, France) containing a 409-bp cDNA fragment corresponding to the third exon of the POMC gene, position 221–629 which encodes for the neuropeptide β-endorphin [40]. The cDNA from these plasmids was amplified using M13 forward and M13 reverse primers and gel purified using Epoch Biosystems silica spin columns according to the manufacturer’s instructions. The amplified cDNA served as the template for the in vitro transcription reaction in which T7 RNA polymerase (Ambion Inc.) was used to generate the antisense cRNA digoxigenin-11-UTP (DIG)-labeled ORL-1 probe and fluorescein-12-UTP (FITC)-labeled POMC probe. T3 RNA polymerase (Ambion Inc.) was used to generate the sense cRNA DIG-labeled ORL-1 control probe, and SP6 RNA polymerase (Ambion Inc.) was used to generate the sense cRNA FITC-labeled POMC control probe. Probes were precipitated using 3 M sodium acetate/100% ethanol (1:25 v/v) and reconstituted in 0.1% DEPC water.

Fluorescent in situ Hybridization
Mounted tissue was brought to room temperature in sealed boxes containing desiccant. The slides were rehydrated in 0.1% DEPC PBS for 30 min and treated with proteinase K (10 μg/ml, Sigma Aldrich) in 100 mM Tris-HCl and 1 mM EDTA, pH 8.0, for 10 min at 37°C while stirring to increase probe penetration. An incubation of 30 min in 3% H2O2 in PBS was used to inhibit endogenous peroxidase activity. The slides were then rinsed in 1× PBS, washed with 0.1 M triethanolamine (TEA) pH 8.0, for 5 min, and acetylated with 0.25% acetic anhydride in 0.1 M TEA, pH 8.0, for 10 min while stirring. Slides were then washed in 1× PBS 3 times for 5 min each and 2× SSC (0.3 M NaCl/0.08 M sodium citrate; pH 7.2) for 5 min. Tissue was dehydrated by a graded series of ethanol washes (50, 70, 95, and 100%) for 2 min each. The slides were air dried for 60 min and then stored at –80°C in sealed boxes containing desiccant until hybridization. For hybridization, slides were brought to room temperature. Hybridization buffer (100 μl per slide) containing 50% deionized formamide, 600 mM NaCl, 10 mM Tris-HCl pH 8.0, 1× Denhardt’s solution, 0.5 mg/ml yeast tRNA, 1 mM EDTA, 10% dextran sulfate, and 0.1 μg/ml of sheared salmon sperm DNA was heated on an 80°C heat block for 10 min. 500 ng of each probe was added to the hybridization buffer and incubated at 80°C for 5 min to denature the probe. Control slides received hybridization buffer with sense probe. The slides were hybridized in a humid hybridization chamber containing 2× SSC/50% formamide at 63°C for 16 h. Coverslips were individually removed by soaking in 5× SSC prewarmed to 65°C. The slides were then washed in 2× SSC/50% formamide at 65°C for 30 min, then transferred to a TNE (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA) wash for 10 min at 37°C with gentle agitation. To remove any unbound probe, the slides were treated with 20 μg/ml RNase A in TNE at 37°C for 30 min followed by a subsequent 10-min TNE wash at 37°C. Afterwards, sections were washed in 2× SSC, 1× SSC, and 0.5× SSC each for 20 min at 65°C while stirring.

Fluorescent detection of hapten-labeled probes with DIG and FITC was performed by immunohistochemistry. For double-label FISH, the ORL-1 DIG-labeled probe was detected with anti-DIG antibody (Roche Molecular Biochemicals, Mannheim, Germany) raised in sheep, followed by a rabbit anti-sheep antibody conjugated to DyLight 649 (Jackson Immunoresearch, West Grove, Pa., USA); POMC FITC-labeled probe was detected by anti-FITC monoclonal IgG subclass 1 antibody (Roche Molecular Biochemicals) raised in the mouse, followed by a goat anti-mouse IgG, Fcy subclass 1 specific antibody conjugated to DyLight 488 (Jackson Immunoresearch, West Grove, Pa., USA). The tissues were equilibrated in TBS for 10 min followed by a 60-min block in 1% Blocking Reagent (Roche Molecular Biochemicals) and a 60-min block in TBS containing 1% normal rabbit serum, 1% BSA, and 1% TX. Next, the slides were incubated with a 1:200 dilution of sheep anti-DIG in TBS block at 4°C for 42 h in a dark humid chamber. All following steps were carried out in the dark to avoid bleaching fluorescent signal. Three 20-min washes in block were followed by an incubation with secondary antibody (1:200 rabbit anti-sheep DyLight 649) for 2 h in a humid chamber for detection of ORL-1. Three washes in block for 10 min were followed by 3 TBS 10 min washes and a 60 min TBS block containing 1% NGS, 1% BSA, and 1% TX. Next, the slides were incubated with a 1:100 dilution of anti-FITC monoclonal IgG, Fcy subclass 1 primary antibody in TBS block at 4°C for 42 h in a dark humid chamber. Three 20-min washes in block followed to wash off unbound primary antibody from the slides. Incubation in the appropriate secondary antibody for 2 h was performed in a humid chamber for detection of POMC. Three washes in block for 10 min were followed by 3 10-min TBS washes, 2 TNT (0.05% Tween 20 in TN buffer) washes 10 min each, and rinsed in 0.1 M Tris-HCl pH 7.5. The slides were then dried on a 37°C slide warmer and coverslipped using Aqua-Poly/Mount (Polysciences, Warrington, Pa., USA).

POMC/ORL-1 FISH and FG Analysis
Colocalization was determined using the Leica DM6000 epiplane microscope, Leica DFC 360FX monochrome digital camera, and Leica AF-LAS microscope software to detect POMC, ORL-1, and FG through the use of FITC, Cy5, and FG filter cubes, respectively. POMC mRNA was visualized by FITC imaging at a 488-nm emission filter and a 515- to 540-nm bandpass filter. ORL-1 mRNA was visualized using a 649-nm emission filter and a 620- to 660-nm bandpass filter. FG was visualized using a wide-band ultraviolet excitation filter (excitation 331 nm, emission 418 nm at neutral pH). Images were adjusted for brightness and contrast using Adobe Photoshop (version 7.0). Files were imported to ImageJ for quantitative analysis of colocalization. The cell counter on Im-
overnight at 4 °C on an orbital shaker in one of two primary anti-orbital shaker. The following day, the membranes were incubated with goat anti-rabbit (1:10,000; Sigma-Aldrich) or rabbit anti-mouse (1:50,000; Sigma-Aldrich) secondary antibodies. This was followed by 3 washes with 1× TBST for 20 min each. The membranes were then incubated in West Pico chemiluminescent substrate (1:1 ratio of luminol:peroxide) for 5 min and then imaged using Biomax light film in the dark to visualize bands (Thermo Scientific).

The comparison of EB versus oil treatment on the number of ARH neurons that were positively single-, double- or triple-labeled for FG staining, POMC, or ORL-1 mRNA expression were analyzed by Student’s t test. The number of neurons in the ARH that colocalized POMC and ORL-1 mRNA was determined in EB- and oil-treated rats, and the percent of cells within each population that expressed the other mRNA was determined. The effects of EB compared to oil treatment on (1) the number of cells that expressed POMC and ORL-1 mRNA, (2) the percent of POMC cells that expressed ORL-1 mRNA, and (3) the percent of ORL-1 cells that expressed POMC mRNA were analyzed by Student’s t test (SigmaStat version 3.5). Similarly, Student’s t test was used to analyze the effects of EB on the number of triple-labeled cells and the percent of each triple-labeled cell population of (1) POMC, (2) ORL-1 and (3) FG.

**Experiment III**

To confirm the estradiol increase in ORL-1 expression in the ARH, ORL-1 protein levels were measured in whole tissue preparation of the ARH. As in experiment II, rats received either 2 μg EB (n = 6) or oil (n = 5) treatments for three cycles every 4 days, and 24 h after the third treatment the animals were deeply anesthetized with isoflurane and killed by decapitation.

Western Blot

Protein analysis and ARH block dissection were performed as described in Mahavongtrakul et al. [41]. Briefly, brains were quickly removed from the cranium, chilled in 4 °C PBS, and block dissections of the ARH 2 mm thick were collected into 1.5-ml Eppendorf tubes. The tissue was then flash frozen in a dry ice and ethanol bath and stored at –80 °C until processing. To extract total protein, the ARH tissues were individually homogenized in 2× gel-loading buffer [GLB; 0.5 M Tris, pH 6.8, 10% w/v sodium dodecyl sulfate (SDS), 20% glycerol] containing a 1:10 dilution of protease inhibitor cocktail (Sigma-Aldrich) and a 1:100 dilution of phenylmethanesulfonyl fluoride (Sigma-Aldrich) using a glass tissue grinder (Kimble Chase). A bicinchoninic acid assay (Thermo Pierce) was performed followed by UV-visible spectroscopy (Bio-Rad) at 562 nm to calculate total protein concentration. Total protein (20 μg) was denatured with 5% β-mercaptoethanol and loaded onto a SDS-PAGE gel for 1 h and 20 min at room temperature. The protein was then transferred onto nitrocellulose membranes by passing 100 V across the membranes at 4 °C for 1 h and 30 min (Bio-Rad). The membranes were then blocked in 10% nonfat milk and incubated in 1× TBST overnight at 4 °C on an orbital shaker. The following day, the membranes were incubated overnight at 4 °C on an orbital shaker in one of two primary antibodies: rabbit anti-nociceptin receptor (1:1,000; Abcam) or mouse anti-β-actin (1:100,000 in 3% nonfat milk; Sigma-Aldrich). Then, the membranes were washed 3 times for 10 min each with 1× TBST, followed by a 45-min incubation at room temperature on an orbital shaker in either affinity-purified peroxidase-conjugated goat anti-rabbit (1:10,000; Sigma-Aldrich) or rabbit anti-mouse (1:50,000; Sigma-Aldrich) secondary antibodies. This was followed by 3 washes with 1× TBST for 20 min each. The membranes were then incubated in West Pico chemiluminescent substrate (1:1 ratio of luminol:peroxide) for 5 min and then imaged using Biomax light film in the dark to visualize bands (Thermo Scientific).

Analysis

Densitometry of the Western blots was obtained using a Gel DocTM XR charged-coupled device camera and the Quantity One software (Bio-Rad). ORL-1 bands were normalized to β-actin. For each of the treatment groups, a mean normalized density was calculated. Then, the normalized densities were divided by the mean normalized density, resulting in a normalized mean of 1.0 for oil controls. The normalized densities for oil and 2 μg EB were compared to one another using Student’s t test with a significance threshold of p < 0.05 (SigmaStat 3.5).

**Results**

**Experiment I: Estradiol Regulation of PR and OFQ/N Expression in the ARH**

Within the ARH, populations of neurons were immunopositive for PR only, OFQ/N only, PR and OFQ/N, or not stained for either PR or OFQ/N (fig. 2). OFQ/N-immunopositive neurons were observed in the dorsomedial to the ventrolateral ARH (fig. 2a, d). The red fluorescent immunolabeling for the ligand OFQ/N was localized in the cytoplasm of the soma and some neural processes in colchicine-treated rats (fig. 2a, d). Neurons that were immunopositive for both PR and OFQ/N had either green fluorescent PR labeling in the nucleus surrounded by red fluorescent OFQ/N labeling in the soma and processes, or green and red labeling within the cytoplasm (fig. 2c, f). Colocalization of PR and OFQ/N immunoreactivity was confirmed by confocal microscopic three-dimensional reconstructions through the depth of the tissue (fig. 2g, h).

PR-immunopositive neurons were observed in the medial portion of the ARH and decreased in number towards the dorsomedial and ventrolateral regions of the ARH (fig. 2b, e). In estradiol-treated rats, immunopositive green fluorescent staining for PR was mainly localized in the nucleus of neurons (fig. 2e, g, h). In contrast, immunopositive staining for PR in the oil-treated control rats was observed both in the cytoplasm of some neurons and the nucleus of others (fig. 2b).

EB treatment significantly increased the number of OFQ/N-immunopositive neurons by 1.62-fold (fig. 3a; t test, d.f. = 8, t = 11.21, p < 0.001). The number of PR-immunopositive neurons was significantly increased by 2.48-fold in EB-treated rats compared to oil-treated control animals (fig. 3a; t test, d.f. = 8, t = 10.283, p < 0.001).

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Subpopulations of ARH neurons were immunopositive for both PR and OFQ/N, and EB treatment significantly increased the number of these neurons (fig. 3b; t test, d.f. = 8, t = 7.43, p < 0.001). The percentage of PR-immunopositive cells that colocalized with OFQ/N immunoreactivity in the ARH was not significantly increased with estradiol treatment (fig. 3c; t test, d.f. = 8, t = -1.219, p = 0.258). In contrast, the percentage of OFQ/N-immunopositive cells that expressed PR immunoreactivity in the ARH was significantly increased with estradiol treatment (fig. 3d; t test, d.f. = 8, t = 4.014, p = 0.004).
As in the ARH, subpopulations of neurons within the MPN, anteroventral periventricular nucleus (AVPV) and posterodorsal medial amygdala were observed to be immunopositive for both PR and OFQ/N (not shown).

Experiment II: FISH Visualization of ORL-1 mRNA Expression in POMC ARH Neurons That Project to the MPN

To determine whether ORL-1 are expressed in POMC neurons that project to the MPN, the retrograde tract tracer FG was injected into the MPN dorsal to the MPNc in OVX rats treated with either EB or oil, and tissue sections through the ARH were processed for ORL-1 and POMC mRNA expression by FISH. Positive POMC fluorescent mRNA staining was colorized green, and positive ORL-1 FISH mRNA fluorescent staining was computer-colorized red (fig. 4a, b). Both ORL-1 and POMC mRNA expression were observed in the perikarya. Retrogradely transported FG filled the soma and some processes with fluorescent staining (blue; fig. 4c). Colocalization analysis in the ARH revealed seven labeled populations of neurons: single-labeled for either POMC, ORL-1, or FG only; double-labeled for POMC-ORL-1, POMC-FG, or ORL-1-FG; triple-labeled for POMC, ORL-1 and FG (fig. 4), as well as nonlabeled neurons. A subpopulation of ARH neurons were triple-labeled (fig. 4d), supporting the hypothesis that ORL-1 are expressed in β-END neurons that project to the MPN.
ARH POMC Neurons Express ORL-1 and Project to MPN
Distribution of POMC mRNA-Expressing Neurons
FISH-labeled POMC mRNA expression was localized to neurons in the ARH and median eminence. In the ARH, POMC mRNA was expressed along the third ventricle in a dorsal-to-ventral pattern and a medial-to-lateral pattern along the ventral extent of the ARH. Although the median eminence contains few neuronal cell bodies, POMC mRNA expression was observed in neurons as reported previously [18]. Fluorescent-labeled POMC sense probe produced minimal nonspecific staining in the ARH (fig. 4 e).

Distribution of ORL-1 mRNA-Expressing Neurons
FISH-labeled ORL-1 mRNA expression was widespread within the rat brain and observed throughout the hypothalamus, as reported previously [21, 42]. ORL-1 mRNA expression was observed in ARH, VMH, dorsal medial hypothalamus, posterodorsal medial amygdala, cortex, and hippocampus. FISH ORL-1 mRNA labeled all regions of the VMH and exhibited a similar intense staining pattern in the ventrolateral portion of the VMH. Fluorescent-labeled ORL-1 sense probe produced minimal nonspecific staining in the ARH and VMH (fig. 4 f).

ARH Neurons Projecting to MPN
MPN FG injections produced FG-filled somas observed in various regions with known projections to the MPN (e.g. ARH, VMH, and posterodorsal medial amygdala [41]). FG retrograde labeling was observed through the rostrocaudal extent of the ARH as reported previously [4], primarily in the dorsal to ventral extent of the medial portion of the ARH as well as the mediolateral extent of the ventral ARH.

Estradiol Increased Number of Neurons That Express POMC and ORL-1 Expression, but Did Not Affect the Projections to MPN
Individually, EB treatment increased the number of ARH neurons that expressed either POMC or ORL-1 mRNA compared to oil-treated controls (fig. 5a, b; t test, p < 0.001, POMC, d.f. = 6, t = 8.422; ORL-1, d.f. = 6, t = 7.719). The number of ARH neurons that project to MPN was not affected by EB treatment (fig. 5c; t test, p = 0.561, d.f. = 6, t = 0.615).

Distribution of POMC and ORL-1 Double-Labeled Neurons
Neurons that stained positively for mRNA of both POMC and ORL-1 were present mainly in the dorsal ventral aspect of the ARH along the third ventricle. The distribution of double-labeled cells was similar to the POMC distribution since fewer cells expressed POMC.

Distribution of Triple-Labeled POMC and ORL-1 Neurons Projecting to the MPN
A subpopulation of POMC-ORL-1 mRNA-expressing neurons project to the MPN as indicated by the observation of ARH neurons triple-labeled with staining for FG, POMC mRNA and ORL-1 mRNA (fig. 4d). The distribution of triple-labeled cells (ORL-1/POMC neurons projecting to the MPN) within the ARH was mainly in the dorsal ventral aspect of the ARH along the third ventricle similar to that of double-labeled POMC- and ORL-1-expressing neurons. Because the FG probably did not perfuse the entire dorsal MPN region and not every POMC neuron terminal in the MPN took up FG, it is likely that

Fig. 5. Estradiol (2 μg EB, s.c. 24 h) treatment increases the number of neurons in the ARH that express POMC mRNA (a) or ORL-1 mRNA (b) FISH labeling compared to vehicle-treated (OIL) animals. c The number of ARH neurons that were labeled by FG MPN infusions was not affected. * p < 0.01, significantly greater than OIL treatment (t test).
Estradiol Increased ORL-1 and POMC mRNA Expression in ARH Neurons That Project to MPN

Estradiol increased the number of ARH neurons with coexpression of POMC and ORL-1 mRNA (fig. 6a; t test, d.f. = 6, t = 6.691, p < 0.001). The percentage of POMC mRNA cells expressing ORL-1 in the ARH was significantly increased by EB treatment (fig. 6b; t test, d.f. = 6, t = 2.530, p = 0.045), as was the percentage of ORL-1 that expressed POMC mRNA (fig. 6c; t test, d.f. = 6, t = 3.485, p = 0.013). In both oil- and EB-treated rats, over 50% of the POMC cells expressed ORL-1 mRNA, whereas less than 40% of ORL-1 cells in both treatment groups expressed POMC mRNA (fig. 6b, c). This is most likely due to more ORL-1 mRNA-expressing cells than POMC cells in the ARH (fig. 5a, b).

EB treatment increased the number of ORL-1 mRNA and POMC mRNA neurons that project to the MPN compared to oil-treated controls (fig. 7a; t test, d.f. = 6, t = 3.405, p = 0.014). Furthermore, the percentage of FG cells in the ARH that expressed POMC and ORL-1 mRNA was also increased by EB compared to oil (fig. 7b; t test, d.f. = 6, t = 2.733, p = 0.034). However, estradiol did not alter either the percentage of POMC cells that express ORL-1 and project to the MPN (fig. 7c; t test, d.f. = 6, t = 0.948, p = 0.380), or the percentage of ORL-1-labeled cells that contain POMC that project to the MPN (fig. 7d; t test, d.f. = 6, t = 1.367, p = 0.221). These data indicate that estradiol increases the total number of neurons that express POMC and ORL-1 mRNA in ARH neurons within the subpopulation of neurons that project to the MPN.

**Experiment III: Estradiol Regulation of ORL-1 Protein Levels in the ARH**

To confirm the estradiol-induced expression of ORL-1 mRNA in experiment II, Western blot analysis of whole tissue from ARH block dissections in estradiol- and oil-treated OVX rats was performed. Normalized to β-actin, ORL-1 levels were increased 24 h after 2 μg EB compared to the oil controls (fig. 8; t test, d.f. = 10, t = −2.322, p = 0.043).

**Discussion**

The present anatomical studies demonstrate that estradiol upregulates OFQ/N-ORL-1 and POMC systems in the ARH and in a subset of these neurons that project to the MPN. Estradiol upregulates the number of ARH neurons that (1) express PR and OFQ/N as well as their coexpression, and (2) express ORL-1 and POMC which project to the MPN and their coexpression. In general, our results show that estradiol upregulates the OFQ/N-ORL-1 system in the ARH that can directly regulate the activity of ARH POMC neurons. These systems within the ARH that project to the MPN may be associated with the regulation of sexual receptivity, whereas other POMC neurons express ORL-1 and project to MPN.
Neurons may be regulating energy balance and luteinizing hormone (LH) release [reviewed in 1]. Using double-label immunohistochemistry for PR and OFQ/N, we demonstrated that 48 h after estradiol treatment the number of PR- and OFQ/N-immunopositive neurons in the ARH is increased as well as their coexpression. This subpopulation of neurons with PR and OFQ/N suggests a potential mechanism for direct regulation of OFQ/N release by progesterone. In the ARH-MPN lordosis model circuit, a subset of these ARH-PR-OFQ/N neurons is hypothesized to synapse upon β-END-ORL-1 neurons (fig. 1). Given that numerous ARH neurons project to the MPN, it is possible that some of these PR-OFQ/N neurons may project to the MPN and are responsible for OFQ/N facilitation of lordosis in the MPN (fig. 1) [27]. Colocalization of PR and OFQ/N in ARH neurons has been shown in the ewe [43], and in other species estradiol has been shown to increase PR expression in the ARH [24–26]. However, this is the first study to demonstrate estradiol upregulation of their coexpression. These results support our hypothesis that estradiol priming increases the ability of progesterone to directly regulate OFQ/N neurotransmission.

Estradiol doubled the population of neurons that were immunopositive for PR and OFQ/N, indicating an increase in the responsiveness of this circuit to progesterone. This upregulation of OFQ/N by estradiol increases

**Fig. 7.** Effects of estradiol (2 μg EB) on a the number of POMC and ORL-1 mRNA-expressing ARH neurons that project to the MPN (FG labeled), b the percentage of FG-labeled neurons that are triple labeled, c the percentage of POMC mRNA-expressing neurons that are triple labeled, and d the number of ORL-1 mRNA-expressing neurons that are triple labeled. EB increased the number of POMC and ORL-1 mRNA-expressing neurons that project to the MPN compared to vehicle (OIL)-treated rats. However, estradiol did not alter the percent of either c POMC triple-labeled neurons or d ORL-1 triple-labeled neurons. * p < 0.01, significantly greater than OIL treatment (t test).

**Fig. 8.** Western blot analysis of estradiol regulation of ORL-1 protein levels in the ARH. a Representative Western blot of 20 μg total protein of ARH. Whole tissue preparations were loaded onto a SDS-PAGE gel and probed for ORL-1. ORL-1 levels were normalized to β-actin. b Graphical representation of ORL-1 protein levels in the ARH. Twenty-four hours after 2 μg EB (EB), whole tissue ORL-1 protein levels were increased in the ARH compared to oiltreated (OIL) OVX rats. * p < 0.05, significantly greater than oil treatment (t test).
the number of ARH neurons that could inhibit the activity of POMC (β-END) neurons that project to the MNP and regulate lordosis or those that project to other brain regions to regulate energy balance and feeding [reviewed in 1]. Subsequent progesterone or longer exposure to high levels of estradiol may be acting through these OFQ/N neurons to decrease estradiol-induced POMC neuronal activity [reviewed in 1].

Although estradiol increases OFQ/N expression, initially it is not released [21] since ARH-VMH infusions of OFQ/N rapidly induces sexual receptivity and deactivates MNP MOP in EB-primed nonreceptive OVX rats [5, 23, 27]. However, a high dose of EB (5–50 μg) does eventually stimulate the release of OFQ/N that is required for facilitation of lordosis [5, 27]. Thus, the release of OFQ/N is dependent on the dose of estradiol.

Estradiol also increased POMC and ORL-1 mRNA expression and their coexpression in ARH neurons and in a subpopulation of ARH neurons that project to the MNP. Previously, estradiol was not observed to increase ARH ORL-1 mRNA expression using radioisotopic methods [21]. The increase in ORL-1 mRNA expression was confirmed by measuring ARH ORL-1 protein levels (experiment III). Our current findings are also supported by estradiol increasing ORL-1 binding sites in the mediobasal hypothalamus [6, 12].

The regulation of POMC expression in ARH neurons has been shown to be estradiol dose and time dependent. What appear to be conflicting results on the ovarian steroid regulation of POMC expression are more congruent when considered in context with the steroid priming paradigm, and its effects on inducing sexual receptivity and the LH surge. In general, β-END release inhibits both lordosis and LH release [44]. We observed 24 h after EB priming that POMC expression is increased in OVX rats. A single dose of estradiol in OVX adult mice has also been shown to increase POMC mRNA levels at 12- and 24-hour intervals [13]. However, in rats implanted subcutaneously with a 2-mm estradiol-filled silastic capsule, no effect of estradiol was observed at 36 h, but 48 h after treatment POMC mRNA expression increased [18]. The 2 μg EB priming dose initially inhibits lordosis, increases ARH ORL-1 expression and is accompanied by the uncoupling of ORL-1 from the GIRQ channel in POMC neurons [29]. This reduces the ability of OFQ/N to inhibit β-END release in the MNP [29, 30] and allows for the maintenance of estradiol-induced excitation of POMC (β-END) neurons which inhibit lordosis. Steroid treatments that facilitate lordosis reduce MNP MOP activation by recoupling ORL-1 to the GIRQ channel and increase K+ hyperpolarizing currents to inhibit POMC neuronal transmission [29, 30].

OFQ/N also acts presynaptically to decrease the activity of POMC neurons. Estradiol and progesterone either enhance or decrease the activity of POMC neurons depending on whether the steroid milieu induces a sexually receptive state or not [29, 30]. Estradiol reduces the ability of OFQ/N to decrease glutamatergic excitatory neurotransmission onto ARH POMC neurons that project to...
the MPN [29, 30]. Progesterone restores the capability of OFQ/N to inhibit excitatory glutamatergic input to POMC neurons [30]. Thus, progesterone acting via OFQ/N-ORL-1 signaling decreases excitatory input and enhances ORL-1 signaling through GIRK channels on POMC neurons. These actions are part of the mechanism to reduce β-END release and deactivate MPN MOP and facilitate lordosis [30].

These same actions were also observed in POMC neurons that do not project to the MPN, indicating that the priming dose of EB acts through the OFQ/N-ORL-1 system to synchronize the effects of ovarian hormones on other systems regulated by ARH POMC neurons. POMC is a prohormone for several neuropeptides and peptide hormones that are responsive to ovarian hormones: α-melanocyte stimulating hormone, adrenocorticotropic hormone, γ-lipotropin, and corticotrophin-like intermediate lobe peptide [53, 54]. Therefore, ORL-1 may mediate ovarian hormone regulation of energy balance, feeding and hypothalamic feedback loops in addition to sexual receptivity that are controlled by products of the POMC gene [1, 43, 55]. For example, rising estradiol levels reduce appetite in female rodents [56–60]. In contrast, activation of the OFQ/N-ORL-1 system induces hyperphagia [61–65]. Energy homeostasis may be regulated by estradiol and the OFQ/N-ORL-1 system acting through POMC neurons that project to the paraventricular nucleus of the hypothalamus [55, 61, 66]. Estradiol decreases the ability of OFQ/N to pre- and postsynaptically reduce POMC activity in neurons that do not project to the MPN [29, 30] like those that do project to the MPN. This increases POMC neuronal activity to maintain neurotransmission that is inhibitory to food intake [28–30, 55]. Furthermore, OFQ/N reduces c-Fos expression in α-melanocyte stimulating hormone (POMC) neurons at the end of feeding [67].

Gonadotropin-releasing hormone (GnRH) feedback loops are also modulated by classical endogenous opioid peptides and the OFQ/N-ORL-1 system [43, 44, 68–78]. In vitro, OFQ/N inhibits GnRH release from hypothalamic and mediobasal hypothalamic sections from OVX rats [74, 79]. In female rats treated with estradiol and progesterone, OFQ/N infusion into the third ventricle reduced the LH surge magnitude, but did not eliminate the surge [44]. Since circulating progesterone levels rise concurrently or shortly after the onset of the LH surge in the rodent [80–82], progesterone may act to stimulate the release of OFQ/N to terminate the surge release of GnRH and LH. These neurons could receive direct input from PR-OFQ/N neurons located in the AVPV (also known as RP3V), MPN or even a population of ARH PR-OFQ/N neurons. Further, it is possible that ORL-1 activation is compartmentalized within the ARH for ovarian steroids to act through OFQ/N to regulate β-END release that modulates GnRH release [83]. Like the rodent, infusion of OFQ/N into the lateral ventricle of the ewe reduced LH secretion, and appeared to regulate LH pulse frequency and amplitude [73].

In summary, we observed that estradiol increases the number of ARH neurons that coexpress PR-OFQ/N and POMC-ORL-1. Ovarian hormone modulation of pre- and postsynaptic ORL-1 signaling that regulates neuronal activity of POMC neurons indicates that the OFQ/N-ORL-1 system could play an important role in synchronizing multiple homeostatic, reproductive and behavioral systems in addition to sexual receptivity that is controlled by products of the POMC gene [1, 55, 83].

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