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Plasma membrane G protein-coupled estrogen receptor 1 (GPER) mediates rapid estradiol facilitation of sexual receptivity through the orphanin-FQ-ORL-1 system in estradiol primed female rats

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ABSTRACT

In estradiol-primed nonreceptive ovariectomized rats, activation of G protein-coupled estrogen receptor 1 (GPER) in the arcuate nucleus of the hypothalamus (ARH) rapidly facilitates sexual receptivity (lordosis). Estradiol priming activates ARH β-endorphin (β-END) neurons that then activate medial preoptic (MPN) μ-opioid receptors (MOP) to inhibit lordosis. ARH infusion of non-esterified 17β-estradiol (E2) 47.5 h after 17β-estradiol benzoate (2 μg EB) priming deactivates MPN MOP and rapidly facilitates lordosis within 30 min via activation of GPER. Since it was unclear where GPERs were located in the neuron, we tested the hypothesis that GPER signaling is initiated at the plasma membrane. Membrane impermeable estradiol (17β-estradiol conjugated to biotin; E-Biotin) infused into the ARH of EB primed rats facilitated lordosis within 30 min, and MPN MOP was deactivated. These actions were blocked by pretreating with GPER antagonist, G-15. Further, we used cell fractionation and western blot techniques to demonstrate that GPER is expressed both in plasma membrane and cytosolic ARH fractions. In previous studies, the orphanin FQ/nociceptin-opioid receptor-like receptor-1 (OFQ/N-ORL-1) system mediated estradiol-only facilitation of lordosis. Therefore, we tested whether the OFQ/N-ORL-1 system mediates E-Biotin-GPER facilitation of lordosis. Pretreatment of UFP-101, an ORL-1 selective antagonist, blocked the facilitation of lordosis and deactivation of MPN MOP by ARH infusion of E-Biotin. Double-label immunohistochemistry revealed that GPER is expressed within approximately 70% of OFQ/N neurons. These data indicate that membrane GPER mediates the E2/E-Biotin facilitation of lordosis by inducing OFQ/N neurotransmission, which inhibits β-END neurotransmission to reduce MPN MOP activation.

1. Introduction

Estrogenic therapies have increased in number and include those for reproductive issues, estrogen responsive cancers, neurodegenerative diseases and psychological disorders (reviewed in Baez-Jurado et al., 2019). However, estrogens signal through multiple types of estrogen receptors (ER), which include ERα, ERβ, G protein-coupled estrogen receptor 1 (GPER; aka GPR30), ERx and the G protein q-coupled membrane estrogen receptor (Gq-mER; aka STX receptor). Adding to the complexity, these ERs are dynamically regulated over time by steroid exposure and can initiate multiple signaling pathways, in multiple subcellular compartments through interactions with other receptors, kinases, and signaling proteins (reviewed in Micevych et al., 2017; Micevych and Sinchak, 2018). Thus, understanding where and when the different types of ER signaling occur may allow for production of estrogenic therapies that have reduced side effects.

We have studied the dynamics of ER signaling over time in a model circuit that regulates sexual receptivity (lordosis) in female rats (Conde et al., 2016; Long et al., 2017; Long et al., 2014; Mahavongtrakul et al., 2013; Sanathara et al., 2011; Sinchak et al., 2013; Sinchak and Micevych, 2001; reviewed in Micevych et al., 2017; Micevych and Sinchak, 2018). Both classical genomic and non-classical extranuclear signaling pathways are essential for facilitation of sexual receptivity. Estradiol-only facilitation of lordosis requires the activity of multiple estradiol signaling pathways that are orchestrated over days and are initiated through multiple types of ERs (reviewed in Micevych et al., 2017; Micevych and Sinchak, 2018). We demonstrated previously that sexual receptivity can be facilitated by sequential activation of ERα and GPER. Estradiol initially acts through ERα to induce classical genomic regulation through an estrogen response element and rapidly initiate

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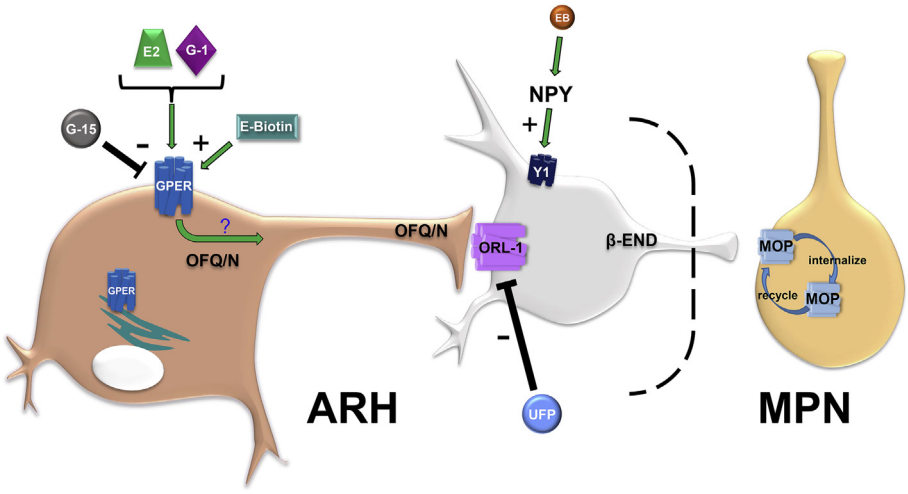

Fig. 1. Model of estradiol signaling through GPER in the arcuate nucleus (ARH) to rapidly facilitate sexual receptivity. Estradiol benzoate (2 μg EB) priming activates a plasma membrane associated estrogen receptor-α (ERα) that induces the release of neuropeptide Y (NPY) to activate the NPY-Y1 receptor (Y1) on ARH β-endorphin (β-END) neurons that project to the medial preoptic nucleus (MPN; Dewing et al., 2007; Dewing et al., 2008; Eckersell et al., 1998; Mills et al., 2004; Sinchak and Micevych, 2001). This rapidly activates and maintains activation of β-END neurotransmission that induces μ-opioid receptor (MOP) activation/internalization in the MPN to inhibit lordosis (Dewing et al., 2007; Dewing et al., 2008; Eckersell et al., 1998; Mills et al., 2004; Sinchak and Micevych, 2001). Deactivation (and recycling to the membrane) of MPN MOP 30 to 48 h after EB priming facilitates lordosis (Dewing et al., 2007; Long et al., 2017; Long et al., 2014; Sanathara et al., 2011; Sinchak et al., 2013; Sinchak and Micevych, 2001). Our laboratory recently demonstrated that activation of ARH GPER reduced MPN MOP activation and facilitated sexual receptivity within 30 min, 48 h after EB priming (Long et al., 2017; Long et al., 2014). Further, we showed that a single high dose of estradiol that facilitates lordosis is mediated by the orphanin FQ/nociceptin (OFQ/N) neuron that activates its receptor ORL-1 on β-END neurons to reduce MPN MOP activation/internalization (Sanathara et al., 2011; Sanathara et al., 2014). Thus, we tested the hypotheses that non-esterified 17β-estradiol (E2) rapid facilitation of lordosis is mediated by ARH GPER associated with the plasma membrane and requires ORL-1 activation in the ARH. Our results indicate that rapid facilitation of lordosis in EB-primed OVX rats is mediated by plasma membrane associated GPER since membrane impermeable 17β-estradiol conjugated to biotin (E-Biotin) facilitates lordosis like E2. Further, we demonstrated that GPER is localized to both plasma membrane and cytosolic fractions of ARH tissue. Inhibition of ORL-1 by UFP-101 (UFP) blocked facilitation of lordosis by E-Biotin, and the expression of GPER in ARH OFQ/N neurons indicates that E2 may directly regulate OFQ/N neurotransmission to inhibit β-END neurotransmission via ORL-1 activation (Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016). (Y1 = NPY-Y1 receptor).

signaling through membrane-associated pathways by complexing with and signaling through metabotropic glutamate receptor-1a (mGluR1a; Christensen et al., 2011, 2015; Dewing et al., 2007; Dewing et al., 2008; Micevych and Dewing, 2011; Micevych et al., 2003; Mills et al., 2004; Ogawa et al., 1998; Rissman et al., 1997; Sinchak et al., 2013). In the arcuate nucleus of the hypothalamus (ARH), estradiol initially activates an ERα-mGluR1a complex in the plasma membrane that induces the release of neuropeptide Y neurons to excite β-endorphin (β-END) neurons that project to the medial preoptic nucleus (MPN; Fig. 1; Christensen et al., 2011, 2015; Dewing et al., 2007; Dewing et al., 2008; Micevych and Dewing, 2011; Micevych et al., 2003; Mills et al., 2004; Sinchak et al., 2013). This β-END release rapidly activates MPN μ-opioid receptors (MOP) to inhibit lordosis (Fig. 1; Dewing et al., 2007; Eckersell et al., 1998; Sinchak and Micevych, 2001).

Facilitation of lordosis is dependent on the subsequent reduction in MPN MOP activation (Dewing et al., 2007; Long et al., 2017; Long et al., 2014; Sanathara et al., 2011; Sanathara et al., 2014; Sinchak et al., 2013; Sinchak and Micevych, 2001). In a 2 μg estradiol benzoate (EB) primed ovariectomized (OVX) rat, activation of GPER in the ARH 47.5 h after EB treatment reduces MPN MOP activation and facilitates lordosis in 30 min (Fig. 1; Long et al., 2017; Long et al., 2014). GPER is an extranuclear G protein-coupled receptor with a typical seven transmembrane motif that has been shown to be localized to multiple subcellular compartments that include the endoplasmic reticulum and the plasma membrane (Cheng et al., 2011a; Cheng et al., 2011b; Feri et al., 2016; Filardo et al., 2007; Filardo et al., 2006; Filardo et al., 2000; Filardo et al., 2002; Funakoshi et al., 2006; Noel et al., 2009; Otto et al., 2008b; Revankar et al., 2005; Terasawa et al., 2009; Thomas et al., 2005; but see Bondar et al., 2009; Gorosito et al., 2008; Kuo et al., 2010). When activated, plasma membrane associated GPER undergoes endocytosis to the perinuclear compartment and may be recycled back to the plasma membrane (Cheng et al., 2011a). GPER can initiate several signal transduction pathways that include PKC, MAPK, ERK1/2, PI3K, adenylyl cyclase, cAMP, and intracellular calcium (Filardo et al., 2000; Filardo et al., 2002; Gaudet et al., 2015; Kuo et al., 2010; Noel et al., 2009; Revankar et al., 2005; Sun et al., 2018; Terasawa et al., 2009; Thomas et al., 2005; reviewed in Prossnitz and Arterburn, 2015). It is unclear whether ARH GPER signaling that rapidly facilitates lordosis is initiated in the cytosol or the plasma membrane. Therefore, we tested the hypothesis that rapid facilitation of lordosis is mediated by plasma membrane associated GPER and observed that infusion of membrane impermeable estradiol conjugated to biotin (E-Biotin) into the ARH rapidly facilitated lordosis in EB primed rats. Further, using cell fractionation and western blot techniques, we showed that GPER is expressed on the plasma membrane and in the cytosolic fractions of tissue from the ARH of EB-primed OVX rat. In previous studies, we showed estradiol only facilitation of lordosis is mediated by the release of orphanin FQ (OFQ/N; aka nociceptin) that activates opioid receptor-like receptor-1 (ORL-1) to reduce β-END release in the MPN (Sanathara et al., 2011). Therefore, we also tested the hypothesis that the OFQ/N-ORL-1 system mediates the rapid facilitation of lordosis by estradiol activation of GPER. We also used double-label immunohistochemistry to demonstrate that a subpopulation of ARH OFQ/N neurons express GPER.

2. Materials and methods

2.1. Animals

Adult Long Evans OVX rats (200 to 225 g) and gonadally intact male rats (200 to 250 g) were obtained from Charles River Laboratory Inc., Wilmington, MA. The supplier performed the OVX surgeries, and rats were received within one week of OVX surgery. Rats were housed in light and climate-controlled room with food (PicoLab Rodent Diet 20; Newco Specialty Division, Rancho Cucamonga, CA) and water provided ad-libitum. Prior to stereotaxic surgery, sexually naïve females were double-housed in cages with pine bedding (P.J. Murphy Forest Products Sani-Chip; Newco Specialty Division, Rancho Cucamonga, CA). After cannulation, females were single housed to prevent potential head cap damage from the cage mate. Males were single or double housed. All procedures were approved by the California State University, Long Beach IACUC.

2.2. Subcutaneous steroid treatments

Steroids were dissolved in safflower oil (Oil), so that all doses were

injected in a volume of 0.1 ml. Steroid priming and Oil injections were delivered subcutaneously (s.c.) in the nape of the neck for all experiments. For all experiments, 2 μg EB was the priming dose administered. This dose of EB was given once every four days. This EB primes lordosis neurocircuits (e.g. upregulation of progesterone receptors; Sanathara et al., 2014) but does not induce sexual receptivity unless subsequent pharmacological or steroid treatments are given (Jones et al., 2013; Jones and Pfaus, 2014; Long et al., 2017; Long et al., 2014; Sanathara et al., 2011; Sinchak and Micevych, 2001). Further, it mimics proestrous circulating estradiol levels (Geary and Asarian, 1999; Micevych et al., 1996). In experiment 3, the dose of progesterone was 500 μg.

2.3. Experiment 1: Membrane initiated GPER signaling rapidly facilitates lordosis via the OFQ/N-ORL-1 system in EB primed rats

2.3.1. Experimental design

OVX rats were implanted with 21-gauge stainless-steel bilateral cannulae (PlasticsONE Inc., Roanoke, VA) aimed at the ARH using standard stereotaxic surgical procedures (coordinates from bregma; anterior −2.3 mm, lateral 0.7 mm, ventral −6.8 mm from dura; tooth bar set at −3.3; Sanathara et al., 2011; Sinchak et al., 2013). Animals were anesthetized with isoflurane and injected with an analgesic (Rimadyl, subcutaneous 5 mg/kg; Western Medical Supply, Arcadia, CA). Bilateral cannulae were secured to the skull with bone screws and dental cement. Dummy stylets were inserted into the cannulae and covered by screw-on head caps. EB priming started the day of surgery. Animals received antibiotics in their drinking water for four days after surgery (0.5 mg/ml of sulfamethoxazole and 0.1 mg/ml of trimethoprim; Hi-Tech Pharmacal, Amityville, NY; Sinchak et al., 2007) and were single housed for the rest of the experiment. Females received a single EB injection every four days for four “cycles” (Long et al., 2017; Long et al., 2014). On the third EB cycle, all female rats received two sequential infusions starting 47.25 h after EB priming. The first infusion was either 0.9% saline, G-15 (GPER antagonist; Dennis et al., 2009), or UFP (ORL-1 antagonist; Sanathara et al., 2011) and 15 min later, females were infused with either saline, nonesterified 17β-estradiol (E2), E-Biotin (estradiol conjugated to biotin at the sixth carbon), or biotin. Thirty minutes after the second ARH infusion, each female was placed in a plexiglass arena with a male rat to measure sexual receptivity. On the fourth EB cycle, females were infused with the same drug treatments. Thirty minutes after second infusion, rats were deeply anesthetized using 2–3% isoflurane and the brains were fixed for MOP immunohistochemistry via transcardial perfusion with chilled 0.9% saline followed by 4% paraformaldehyde in Sorensen's buffer (Dewing et al., 2007; Eckersell et al., 1998; Long et al., 2017; Long et al., 2014; Sanathara et al., 2011; Sinchak et al., 2013).

2.3.2. Drug infusions

Infusion drugs were dissolved in either 1 μl of DMSO or saline at the following concentrations: E2 (Steraloids, Newport, RI; 25 nmol), G-15 (Sigma-Aldrich; 70 nmol), UFP-101 (Sigma-Aldrich; 50 nmol; Sanathara et al., 2011), E-Biotin (conjugated at the sixth carbon; Sandia Biotech; 25 nmol), or biotin (Steraloids, Newport, RI; 25 nmol). Drugs were infused bilaterally into the ARH in 0.5 μl volume per side. These drugs were administered via a 25 μl Hamilton syringe attached to an infusion pump infusing at the rate of 0.5 μl/min (Stoelting Co., Wood Dale, IL; Long et al., 2017; Long et al., 2014; Sanathara et al., 2011). The 28-gauge infusion needles extended 2 mm past the opening of the cannulae and were left in the cannulae guide for 1 min after completion of infusion to allow for diffusion of drug treatment. Clean dummy stylets were inserted, cap replaced, and the female was placed back in her home cage until the start of behavioral testing or placement into isoflurane anesthesia chamber prior to perfusion.

2.3.3. Behavioral testing

At 48 h post EB priming, 30 min after the second infusion, females were placed in plexiglass arena with pine bedding (same as homecage) on the floor and with a sexually trained male rat. Male rats were allowed to perform ten vigorous mounts and sexual receptivity was measured by calculating the lordosis quotient (LQ). Sexual receptivity was indicated with a dorsiflexion of the back, extension of legs, and deflection of the tail. The LQ was calculated by dividing the number of times she displayed lordosis by ten and multiplied by 100 (Long et al., 2017; Long et al., 2014; Sinchak et al., 1997). Statistical analysis for LQ data underwent square root transformation and analyzed by a 1-way ANOVA. Post hoc analysis completed by Holm-Sidak (SigmaStat V3.5, Systat Software Inc) with effect size calculated by eta squared and pairwise effect size calculated by Cohen's *d*.

2.3.4. Tissue collection, brain sectioning, and cannula guide placement confirmation

Perfused brains were immediately extracted from the skull and postfixed overnight in 4% paraformaldehyde. The next day, brains were transferred to 20% sucrose in a 0.1 M phosphate buffer solution (pH 7.5) for cryoprotection. Brains were stored at 4 °C until cryostat sectioning was performed (Sanathara et al., 2011). Brains were blocked, mounted on a cryostat chuck, frozen rapidly using crushed dry ice, and cyrosectioned coronally at 20 μm thickness. Sections were collected with a wet paint brush into wells containing phosphate buffered saline (PBS; pH 7.5). The wells were stored at 4 °C until used for immunohistochemistry or cannula placement confirmation. For ARH cannula guide placement confirmation, brains were mounted onto a Superfrost Plus slide and thionin stained. Cannulae placement was confirmed using bright-field microscope (Sanathara et al., 2011).

2.3.5. MPN MOP immunohistochemistry and analysis

MOP immunohistochemistry was performed to measure MOP staining intensity which is positively correlated with MPN MOP activation/internalization following ARH drug infusions as previously describe (Dewing et al., 2007; Eckersell et al., 1998; Long et al., 2017; Long et al., 2014; Mills et al., 2004; Sanathara et al., 2011). Briefly, immunohistochemistry was performed on free-floating sections. Every fourth section through the MPN was washed in PBS, and endogenous peroxidases were removed (incubated in 10% methanol and 3% hydrogen peroxide in PBS for 10 min). Sections were washed in PBS and then incubated in MOP primary antibody raised in rabbit (Neuromics; 1:5000 dilution) at 4 °C for approximately 48 h. Sections were washed in Tris-buffered saline (TBS; pH 7.5) and Tyramide Signal Amplification kit (TSA kit; Perkin Elmer/Life Science Products, Boston MA) was used to visualize MOP antibody by Fluorescein (FITC). Tissue was imaged using Leica DM6000B epiluminescent microscope, Leica DFC 360FX monochrome digital camera, and Leica AF-LAS microscope software through FITC (480/40 nm excitation filter and a 527/30 nm bandpass filter). MPN MOP-immunoreactivity (MOPi) intensity was determined using epifluorescent photomicrographs taken from the dorsal region of MPN at the level of the MPN centralis (Long et al., 2017). Previous studies have demonstrated that levels of MOP internalization into early endosomes is positively correlated with MPN MOP intensity/fibers density levels. Quantification of the MPN MOP fiber density was determined using a gray-scale adjusted brightness and contrast image using Adobe Photoshop (version 7.0; Adobe Systems Inc., San Jose, CA) in one sitting (Long et al., 2017; Sanathara et al., 2011). MPN MOPi staining was calculated using Arbitrary units (AU) in ImageJ software (version 0.32j; National Institutes of Health, Bethesda, MD; Long et al., 2017; Sanathara et al., 2011) by decreasing the background to illuminate the MOP fibers. MPN MOPi intensity was analyzed via a 1-way ANOVA followed by post hoc Holm-Sidak test with a significance threshold of $P < 0.05$ (Sigmastat 3.5) and Cohen's *d* for pairwise effect size. Effect size estimates were calculated by eta squared for all ANOVAs.

2.4. Experiment 2: GPER localization in the plasma membrane in the ARH through western blot analysis

2.4.1. Animals

We tested the hypothesis that GPER is located on the plasma membrane in the ARH by western blot analysis. OVX rats were treated s.c. with either 2 μg EB or Oil. Twenty-six hours after their first injection, rats were treated s.c. with Oil or 500 μg of progesterone (P). The three treatment groups were Oil + Oil, EB + Oil, and EB + P. Four hours after the third steroid treatment cycle, the animals deeply anesthetized with isoflurane and killed by decapitation. Brains were quickly removed from the cranium and chilled in PBS at 4 °C. Block dissections of the ARH, hippocampus (HIPP), and amygdala (AMYG) were collected. The block dissections were placed in Eppendorf tubes and flash frozen by immersing tubes into a dry ice and ethanol bath. Tissue samples were stored at −80 °C until processing for total protein extraction, plasma membrane extraction and western blot analysis.

2.4.2. Plasma membrane and cytosolic protein extraction

In preparation for plasma membrane analysis, plasma membrane fractions were extracted from the ARH using the Plasma Membrane Protein Extraction Kit (Abcam, Cambridge, MA). To obtain enough ARH protein for analysis, ARH tissue samples were pooled from four different animals for each individual data point. HIPP and AMYG tissues from a single animal sufficed for sample analysis. Briefly, tissue samples were suspended in homogenization buffer containing Protease Inhibitor Cocktail (PIC) (1:500, supplied with the kit), homogenized with a glass tissue grinder, and centrifuged at 700 x g for 10 min at 4 °C (Kimble Chase). The pellet was discarded, and the supernatant was centrifuged at 10,000 ×*g* for 30 min at 4 °C to separate the cytosolic fraction (supernatant) from the membrane fraction (the pellet). Purified plasma membrane was extracted from the pellet according to manufacturer's instructions. After the final centrifugation, the purified plasma membrane fraction was resuspended in 100 μl of 2× Gel Loading Buffer (GLB) containing PIC (1:10) and phenylmethane sulfonyl fluoride (PMSF; 1:100). Protein concentrations of cytosolic and plasma membrane fractions were determined by BCA Assay. Absence of cytosolic contamination and presence of membrane were confirmed by western blot against the cytosolic marker LIM kinase-1 and the membrane marker NaK ATPase, respectively. Absence of NaK ATPase in cytoplasmic fractions were confirmed by using western blot.

2.4.3. Western blot

Plasma membrane and cytosolic fractions were thawed on a 95 °C heat block for 5 min. Twenty micrograms of protein from plasma membrane and cytosolic fractions from each sample was added to 5% β-mercapthoethanol and 45% 2× Laemmli buffer. Samples were run on a SDS-PAGE gel (4% stacking gel: 30% degassed acrylamide; 0.5 Tris-HCl, pH 6.8; 10% (wt/vol) SDS, containing ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine) at 120 V for 1 h and 20 min at room temperature in 1× running buffer (tris, glycine, SDS). The stacking gel was removed and then fixed into a transfer module (Bio-Rad Laboratories). To transfer the protein from the SDS-PAGE gel onto a nitrocellulose membrane, the transfer module was placed in transfer buffer (methanol, tris, and glycine), and the module ran at 100 V for 1 h at 4 °C. A removable Ponceau S stain was used to confirm the successful transfer of protein onto the nitrocellulose membrane (Fisher Scientific). The membrane was incubated in blocking buffer (5% nonfat milk) for an hour on an orbital shaker (Lab Scientific). The membrane was then incubated overnight at 4 °C on an orbital shaker with affinity-purified rabbit anti-GPR30 (1:1000, Alomone Labs, Jerusalem) and anti-alpha 1 Sodium Potassium ATPase (1:1000, Abcam, Cambridge, MA) primary antibodies. The following day, the membrane was washed for 5 min three times with 1× Tris-Buffered Saline with Tween 20 (TBST) and then incubated with an affinity-purified peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; Sigma-Aldrich) for 1 h on an orbital shaker at 4 °C. This was followed by three washes of 1× TBST for 15 min each. The membrane was incubated in West Pico chemiluminescent substrate (1:1 ratio of luminol and peroxide) for 5 min and sealed with saran wrap for imaging. Incubated in West Pico chemiluminescent substrate (1:1 ratio of luminol and peroxide) for 5 min and then placed in a cassette and imaged with FluorChem R (ProteinSimple).

2.4.4. Analysis

The membrane was imaged using FluorChem R machine (ProteinSimple) using the chemiluminescent protocol setting. Densitometry of the western blots was obtained with the AlphaView software for FluorChem Systems (ProteinSimple). For cytosolic and plasma membrane western blot analysis, bands were normalized to alpha 1 Sodium Potassium ATPase and LimK respectively. For each experimental group, a mean normalized intensity was calculated. Then, normalized intensities were divided by the mean normalized intensity. For the control animals, this resulted in a normalized average of 1.0, allowing comparison of all the experimental group means relative to 1.0. western blot intensities were analyzed by one-way ANOVA with a significance threshold of $P < 0.05$. All statistical analyses were done using SigmaStat 3.5.

2.5. Experiment 3: GPER is expressed in ARH OFQ/N neurons

2.5.1. Double-label immunohistochemistry

To determine whether OFQ/N neurons express GPER, double-label immunohistochemistry was performed. Brains were frozen in dry ice for 5 min then cryosectioned at 20 μm thickness through the ARH and stored at 4 °C in PBS for free-floating immunohistochemical analysis. Sections were rinsed with PBS and processed in 3% hydrogen peroxide and 10% methanol in PBS. Sections were blocked with 1% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in 0.5% triton-X (TX) in PBS for 1 h at room temperature. Sections were incubated in primary antibody (1:3000 anti-GPER, Novus) diluted in blocking buffer in 4 °C for 48 h and then washed with PBS and then tris-buffered saline (TBS). Sections were then washed with TNB (0.5% blocking reagent in TBS) and incubated in secondary antibody (biotinylated goat anti-rabbit, 1:200; Vector Laboratories) at room temperature for 1 h. Sections were then washed with 1× TNT (100 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) and incubated in streptavidin horseradish peroxidase (1:100 in TNB; SA-HRP) for 30 min followed by 3 washes with TNT and incubation in fluorescein (1:50 in AMP diluent; FITC) for 3 min. Sections were washed for 10 min, three times each with TNT, TBS and finally PBS, then incubated in blocking buffer for 1 h at room temperature and then transferred into primary antibody (1:10000 anti-OFQ/N; Neuromics) diluted in blocking buffer for 48 h at 4 °C. Sections were washed subsequently with PBS and TBS then incubated in secondary antibody in TBS (1:100 TRITC anti-goat). Sections were washed with TBS and TRIS buffers then mounted on superfrost slides (Fisher Scientific). Mounted sections were dried on a slide warmer and coverslipped using Aqua-Poly/Mount (Polysciences Inc., Warrington, PA). Staining was visualized via fluorescent microscopy (Leica DM6000, Leica Microsystems, Wetzlar, Germany) and Olympus Fluoview 1000 confocal laser scanning system (Olympus America Inc., Center Valley, PA). Cell counts were performed using ImageJ software (version 0.32j; National Institutes of Health, Bethesda, MD).

3. Results

3.1. Experiment 1: Activation of membrane GPER facilitates lordosis through the OFQ-ORL-1 system

3.1.1. Sexual receptivity

In EB-primed OVX rats, 30 min after bilateral ARH infusion of either E2 or E-Biotin, sexual receptivity was significantly increased as

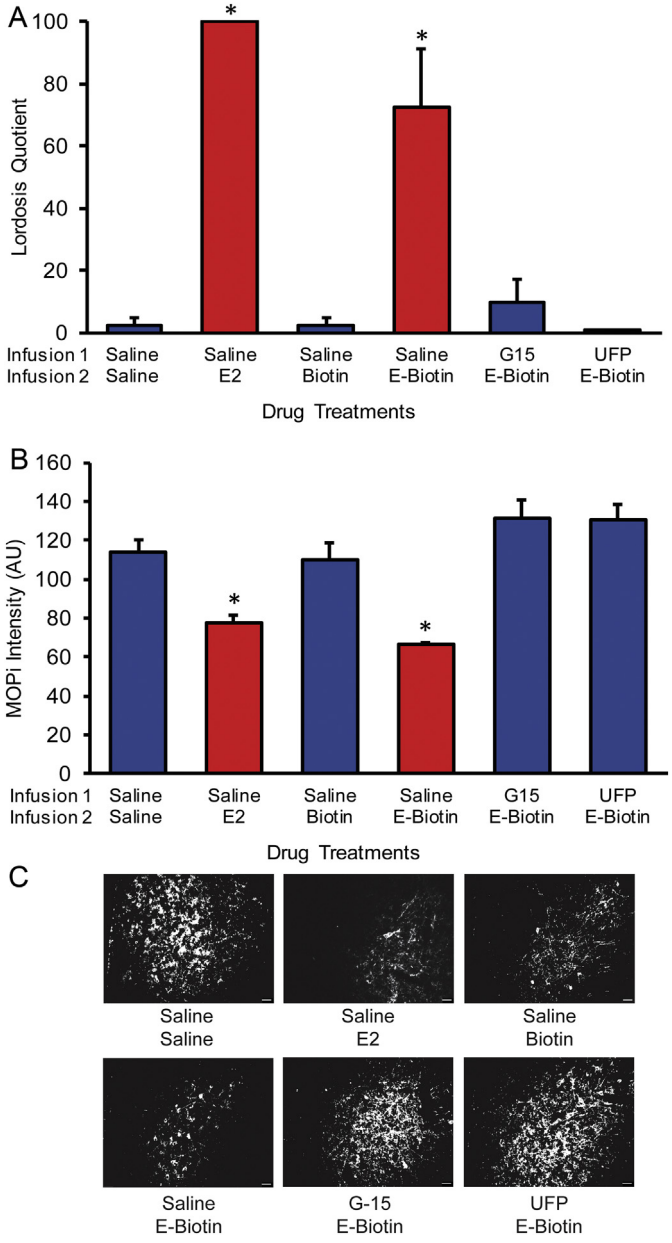

Fig. 2. In the arcuate nucleus (ARH), membrane GPER and ORL-1 mediate the rapid facilitation of lordosis and reduction in MPN MOP intensity by either non-esterified 17β-estradiol (E2) or membrane impermeable 17β-estradiol conjugated to biotin (E-Biotin). Ovariectomized rats received two sequential bilateral infusions into the ARH 47.25 h (Infusion 1) and 47.5 h (Infusion 2) after EB-priming (2 μg EB). Animals were tested for sexual receptivity (Lordosis Quotient, LQ) 30 min after Infusion 2. On the following EB cycle, animals received identical sequential infusions and 30 min after Infusion 2, brains were prepared for MOP immunohistochemistry. Quantification of MPN MOP immunopositive staining (MOPi) intensity was measured in arbitrary units (AU), which has been positively correlated with MPN MOP activation/internalization levels (Eckersell et al., 1998; Mills et al., 2004; Sanathara et al., 2011). A) Rats infused with either E2 or E-Biotin into the ARH had a higher LQ compared to Saline controls. Pretreatment with G-15 (GPER antagonist), or UFP-101 (ORL-1 antagonist) significantly reduced LQ's of rats infused with E-Biotin. B) ARH infusions of either E2 or E-biotin significantly reduced the levels of MPN MOPi staining intensity compared to Saline-infused controls. Pretreatment with G-15 or UFP-101 blocked E-Biotin induced reduction in MPN MOPi intensity levels. C) Examples of photomicrographs used to measure MPN MOPi intensity levels that had brightness and contrast adjusted prior to Image J analysis. * = significantly different from other groups. ($P < 0.05$; Holm-Sidak).

measured by LQ compared to saline controls (Fig. 2; 1-way ANOVA, df = 5,23, F = 27.25, $P < 0.001$, eta squared = 0.88; Holm-Sidak = 0.05; $P < 0.001$, $t = 8.25$, Saline-Saline v Saline-E2: Cohen's d = 1.37, $r = 0.57$; $P < 0.001$, $t = 5.93$, Saline-Saline v Saline-E-Biotin: Cohen's d = 2.60, $r = 0.80$). Pretreatment with either GPER antagonist, G-15, or ORL-1 antagonist, UFP-101, blocked E-Biotin induced sexual receptivity ($P < 0.001$; $t = 7.62$, G15-E-Biotin v Saline-E2, Cohen's d = 9.01, $r = 0.98$; $P < 0.001$, $t = 5.29$, G15-E-Biotin v Saline-E-Biotin, Cohen's d = 2.19, $r = 0.74$; $P < 0.001$, $t = 8.46$, UFP-E-Biotin v Saline-E2, Cohen's d = 1000, $r = 1$; $P < 0.001$, $t = 6.137$, UFP-E-Biotin v Saline-E-Biotin, Cohen's d = 2.716, $r = 0.805$). These data suggest that activation of membrane GPER rapidly facilitates sexual receptivity that is mediated by the OFQ-ORL-1 system.

3.1.2. MOP immunohistochemistry

Decreased MPN MOPi intensity levels were associated with increased LQ. Treatment of either E2 or E-Biotin that increased LQ levels significantly reduced MPN MOPi intensity levels compared to saline controls (Fig. 2, 1-way ANOVA df = 5,23, F = 15.20, $P < 0.001$, eta squared = 0.81; Holm-Sidak = 0.05; $P < 0.001$, $t = 3.63$, Saline-Saline v Saline-E2, Cohen's d = 3.48, $r = 0.87$; $P < 0.001$, $t = 4.80$, Saline-Saline v Saline-E-Biotin, Cohen's d = 5.16, $r = 0.93$). In contrast, infusion of G-15 or UFP prior to either agonist blocked the E-Biotin reduction in MPN MOPi intensity levels ($P < 0.05$; Holm-Sidak = 0.05; $P < 0.05$, $t = 5.43$, G15-E-Biotin v Saline-E2, Cohen's d = 3.71, $r = 0.88$; $P < 0.05$, $t = 6.61$, G15-E-Biotin v Saline-E-Biotin, Cohen's d = 4.76, $r = 0.92$; $P < 0.05$, $t = 5.36$, UFP-E-Biotin v Saline-E2, Cohen's d = 4.38, $r = 0.91$; $P < 0.05$, $t = 6.54$, UFP-E-Biotin v Saline-E-Biotin, Cohen's d = 5.77, $r = 0.95$; $P < 0.05$). These data indicate that estradiol is acting rapidly via membrane GPER through the OFQ-ORL-1 system to reduce MPN MOP activation/internalization to facilitate lordosis.

3.2. Experiment 2: GPER is expressed on the plasma membrane and cytoplasm in the ARH, HIPP, AMYG

In plasma membrane fractions from the ARH, HIPP, and AMYG, GPER immunoreactivity was observed by western blot analysis (Fig. 3). In each of these brain regions, steroid treatments (Oil + Oil, EB + Oil, and EB + P) did not alter plasma membrane levels of GPER in the ARH (1-way ANOVA, df = 2,10, F = 0.62, P = 0.564, equal variance, P = 0.59; Fig. 3), HIPP (1-way ANOVA, df = 2,11, F = 0.02, P = 0.977, equal variance, P = 0.58; Fig. 3), or the AMYG (1-way ANOVA, df = 2,11, F = 0.31, P = 0.739, equal variance, P = 0.64; Fig. 3). GPER reactivity was also observed in the cytosolic fractions from each brain region. Similar to the plasma membrane, steroid treatments had no effect on cytosolic GPER levels in ARH (1-way ANOVA, df = 2,11, F = 2.03, P = 0.187, equal variance, P = 0.76; Fig. 3), HIPP (1-way ANOVA, df = 2,11, F = 0.34, P = 0.722, equal variance, P = 0.66; Fig. 3), or AMYG (1-way ANOVA, df = 2,11, F = 0.21, P = 0.816, equal variance, P = 0.54; Fig. 3).

3.3. Experiment 3: GPER expression in ARH OFQ/N neurons

3.3.1. Immunohistochemistry

OFQ/N immunopositive (OFQ/Ni) cells were stained with TRITC (Red fluorescence) that appeared to be localized to the cytoplasmic compartment and may have extended into the proximal regions of the neuronal processes (Fig. 4). GPER immunopositive (GPERi) staining was green fluorescent label that was also cytoplasmic but appeared more punctate. As reported previously, GPER immunostaining was not associated with the plasma membrane (Long et al., 2014). A subpopulation of ARH neurons expressed both GPER and OFQ/Ni staining that overlapped within the cytoplasm (yellow confocal colorization) and distinct regions of the cytoplasm that had individual green or red staining.

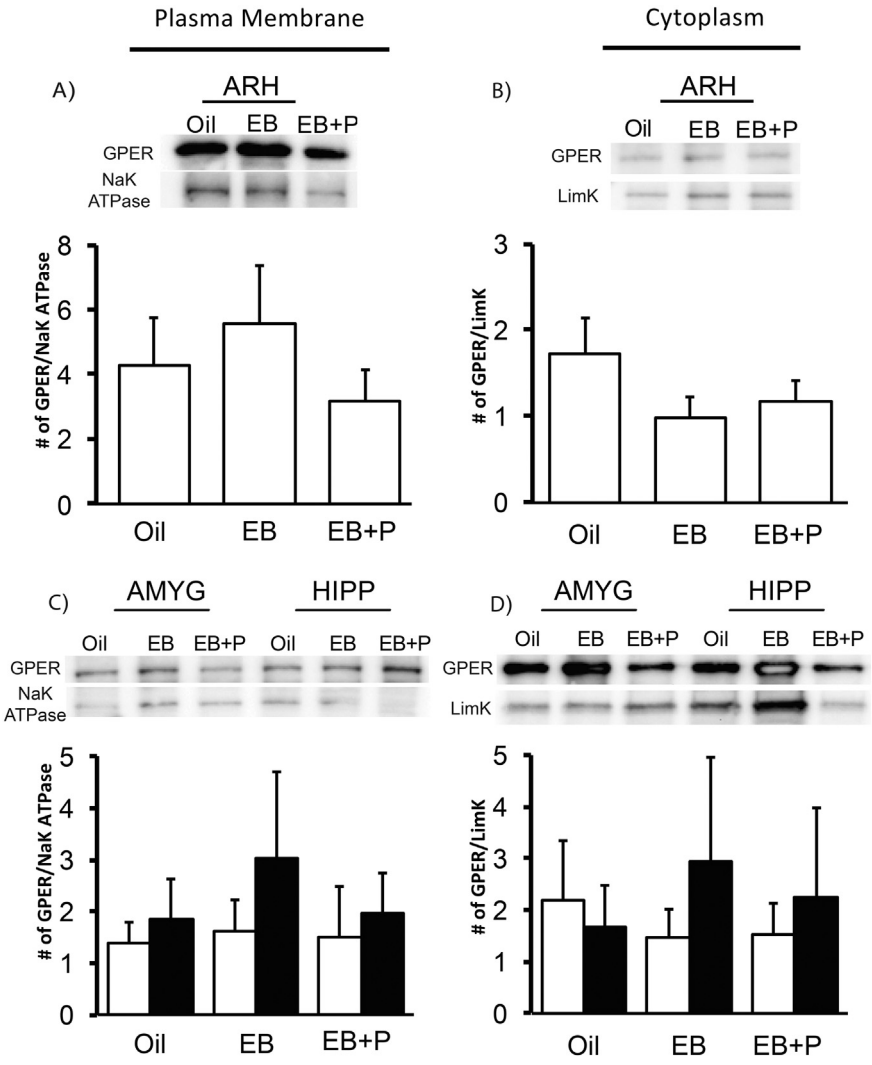

Fig. 3. GPER is localized to both the plasma membrane (PM) and cytosolic (CYTO) fractions of tissue from the arcuate nucleus (ARH), amygdala (AMYG) and hippocampus (HIPP) and levels of expression do not significantly fluctuate with steroid treatment. A–D) Western blot analyses and histograms of PM and CYTO of GPER in ARH, AMYG, and HIPP. Ovariectomized rats treated with either oil and 44 h later oil (Oil), 2 μg estradiol benzoate and 44 h later Oil (EB), or 2 μg estradiol benzoate and 44 h later 500 μg progesterone (EB + P) for three cycles. Four hours after the third set of treatments, brains were collected, areas dissected, and fractionated. Twenty micrograms of proteins extracted from either PM or CYTO were run on an SDS-PAGE gel and probed for GPER. PM fractions were normalized to sodium potassium ATPase (NaK ATPase) and CYTO fractions were normalized to Lim kinase (LimK). GPER was localized to both the PM and CYTO fractions of all these brain regions. However, GPER levels in the PM and CYTO were not regulated by EB treatment or EB + P treatment in the ARH, AMYG, or HIPP.

The number of ARH GPERi neurons was significantly increased by estradiol treatment (EB + Oil) compared to control treated rats (Oil + Oil) (1-way ANOVA df = 2,14, F = 6.70, P < 0.01; eta squared = 0.53; Holm-Sidak = 0.05; P < 0.01; Cohen's d = 2.13, r = 0.73, t = 3.35). Progesterone treatment (EB + P) reduced the number of GPER positive neurons compared to EB + Oil, which was equivalent to levels seen in control Oil + Oil (EB + Oil v EB + P; Holm-Sidak = 0.05; P ≤ 0.05; Cohen's d = 2.18, r = 0.74, t = 2.95; Oil + Oil v EB + P, P < 0.6, Cohen's d = 0.22, r = 0.11, t = 0.40). Like GPER, estradiol (EB + Oil) treatment increased the number of ARH OFQ/N neurons compared to Oil + Oil control treatment (1-way ANOVA df = 2,14, F = 9.41, P < 0.003, eta squared = 0.61; Holm-Sidak = 0.05; Oil + Oil v EB + Oil, P < 0.01, Cohen's d = 2.80, r = 0.81, t = 3.82). However, the number of OFQ/N immunopositive cells was not affected by subsequent progesterone treatment (EB + P) compared to EB + Oil (Oil + Oil v EB + P, P < 0.003, Cohen's d = 1.93, r = 0.69, t = 3.69; EB + Oil v EB + P, P < 0.9, Cohen's d = 0.09, r = 0.05, t = 0.13).

The number of neurons that expressed GPERi and OFQ/Ni staining neurons was significantly increased with estradiol (EB + Oil) treatment compared to Oil + Oil treated rats (1-way ANOVA df = 2,14, F = 10.42, P < 0.002, eta squared = 0.6346; Holm-Sidak = 0.05; P < 0.001, Cohen's d = 3.17, r = 0.85, t = 4.53). Subsequent progesterone treatment (EB + P) reduced the number of ARH neurons that expressed GPER and OFQ/N immunostaining compared to estradiol (EB + Oil) treated rats, which was equivalent to Oil + Oil control rats (EB + Oil v EB + P, P < 0.01, Cohen's d = 1.96, r = 0.70, t = 2.76; Oil + Oil v EB + P, P < 0.1, Cohen's d = 0.95, r = 0.43, t = 1.76). Approximately, 50% of the GPERi neurons expressed OFQ/Ni staining and steroid treatment had no effect on the percentage of GPER that expressed OFQ/N immuno staining (1-way ANOVA df = 2,14, F = 2.51, P < 0.1, eta squared = 0.30).

Estradiol treatment (EB + Oil) had no effect on the percentage of OFQ/Ni neurons that were GPERi. Interestingly, subsequent progesterone treatment (EB + P) significantly reduced the percentage of OFQ/Ni that were GPERi (1-way ANOVA df = 2,14, F = 13.0, P < 0.001, eta squared = 0.70; Holm-Sidak = 0.05; Oil + Oil v EB + P, P < 0.005, Cohen's d = 1.91, r = 0.69, t = 3.45; EB + Oil v EB + P, P < 0.003, Cohen's d = 4.59, r = 0.92, t = 5.19; Oil + Oil v EB + Oil, P < 0.1, Cohen's d = 1.02, r = 0.45, t = 1.75). Approximately, 70% of ARH OFQ/Ni neurons were GPERi.

4. Discussion

Previous studies in our laboratory demonstrated that GPER is expressed in ARH neurons and mediates rapid facilitation of lordosis and reduction of MPN MOP activation by estradiol in EB-primed rats (Long et al., 2017; Long et al., 2014). The current results extend these findings by demonstrating the rapid estradiol signaling that facilitates lordosis is mediated by a plasma membrane associated GPER in the ARH. Infusion of membrane impermeable estradiol, E-biotin, in EB-primed rats facilitated lordosis and reduced MPN MOP activity within 30 min as seen

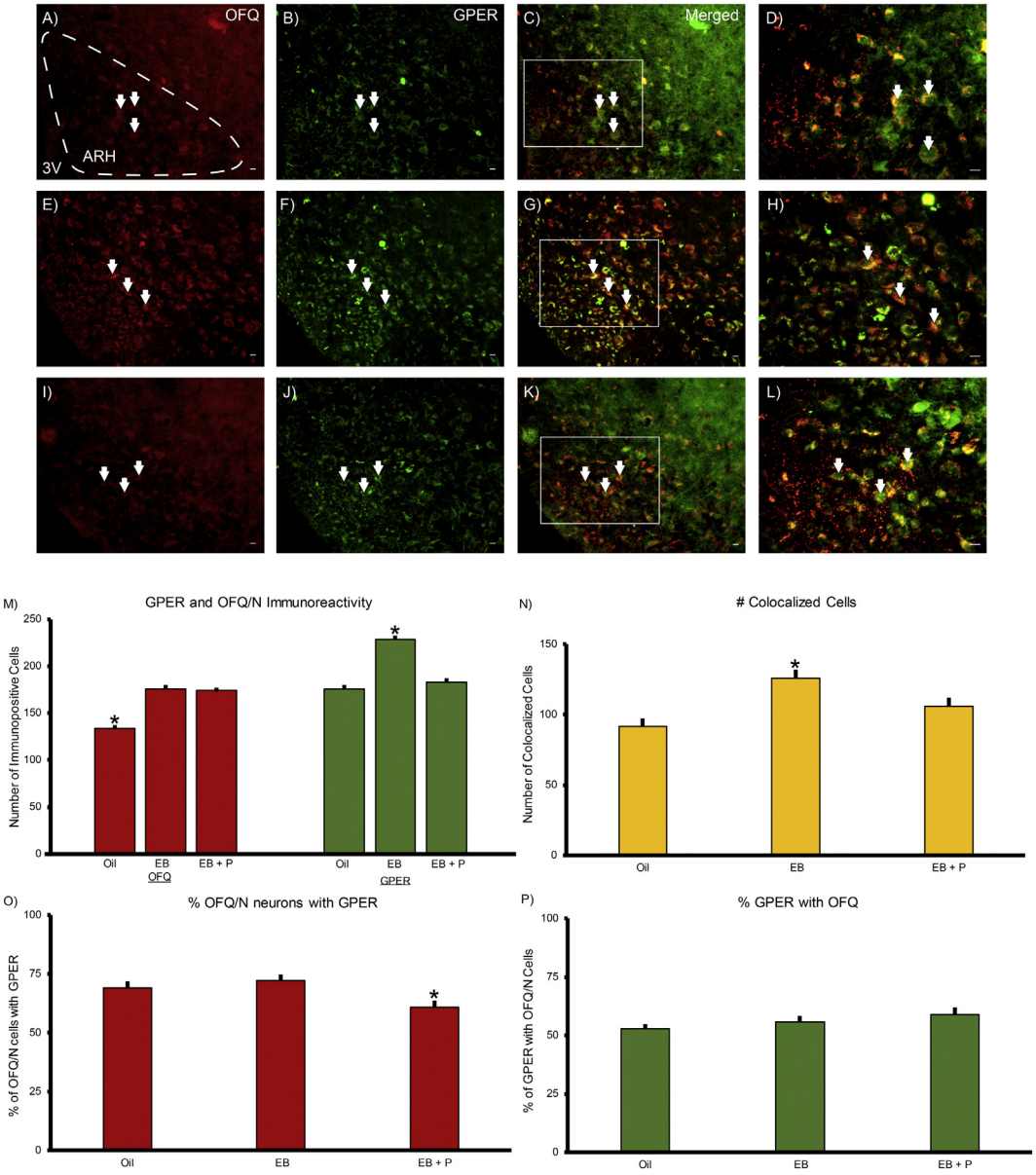

Fig. 4. Confocal photomicrographs of GPER and OFQ/N double-label immunohistochemistry in the arcuate nucleus of the hypothalamus (ARH). Ovariectomized rats were treated with either 17β-estradiol benzoate (2 μg EB) or safflower oil (Oil), and the 44 h later either Oil (Oil + Oil; EB + Oil) or 500 μg progesterone (EB + P) and tissues were collected 4 h later for immunohistochemistry. A, E, I) Labeling of OFQ/N immunoreactivity (TRITC, red) was also localized to the cytoplasm of ARH neurons. The area outlined is representative of the area counted for each section of the ARH. B, F, J) Immunopositive labeling of GPER (FITC, green) was mainly localized to the cytoplasm. Scale bar = 30 μm. C-D, G-H, K-L) A subpopulation of ARH neurons was labeled with both GPER and OFQ/N immunoreactivity (examples indicated by arrows). Where green and red labeling are contained within a given neuron, overlapping staining within the neuron is indicated by yellow computer colorization. Other ARH neurons were only labeled with either GPER or OFQ/N immunoreactivity or had no labeling. D, H, L) Higher power confocal photomicrographs are representative of the boxed regions in the merged ARH GPER and OFQ/N immunopositive staining (scale bar = 10 μm). M–P) Quantification of ARH neurons immunopositive for GPER and OFQ/N expression and colocalization. M) Number of neurons that were immunopositive for either GPER or OFQ/N. N) Number of neurons that were GPER and OFQ/N-immunopositive. O) Percentage of OFQ/N-immunopositive neurons that were GPER immunopositive. P) Percentage of GPER-immunopositive neurons that were OFQ/N immunopositive. 3 V = third ventricle. * = significantly different from other treatment groups within immunohistochemical staining group ($P < 0.05$; Holm-Sidak). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

previously with E2 (Fig. 2; Long et al., 2017; Long et al., 2014). Further, we demonstrated GPER is located in the plasma membrane in ARH tissue using cell fractionation and western blot techniques (Fig. 3). In addition, we demonstrated that GPER facilitation of lordosis and reduction of MPN MOP activation are mediated through the OFQ/N-ORL-1 system in the ARH. In a previous study, we showed that estradiol-only facilitation of lordosis required activation of ORL-1 in the ARH that inhibited the output of ARH β-END neurons that project to the MPN (Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016; Sanathara et al., 2011; Sanathara et al., 2014). Similarly, ARH infusion of the ORL-1 antagonist, UFP-101, blocked E-biotin facilitation of sexual receptivity and reduction of MPN MOP activation (Fig. 2). Moreover, using double-labeled immunohistochemistry in the ARH, we observed that approximately 55% of GPER positive neurons express OFQ/N and 70% of OFQ/N neurons express GPER (Fig. 4). Together, these data indicate that in EB-primed OVX rats, infusion of estradiol into the ARH rapidly activates a plasma membrane associated GPER that acts directly in ARH OFQ/N neurons to induce OFQ/N

neurotransmission that activates ORL-1 to inhibit the output of ARH β-END neurons that project to the MPN (Fig. 1; Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016; Sanathara et al., 2011; Sanathara et al., 2014).

Although the subcellular localization of GPER has been controversial, a consensus of studies supports that GPER is located in more than one subcellular compartment (plasma membrane, perinuclear compartment, golgi apparatus, and endoplasmic reticulum) in peripheral and nervous system tissues (Bondar et al., 2009; Canonaco et al., 2008; Cheng et al., 2011a; Cheng et al., 2011b; Filardo et al., 2007; Filardo et al., 2006; Funakoshi et al., 2006; Kuo et al., 2010; Levin, 2009; Otto et al., 2008a; Revankar et al., 2005; Thomas et al., 2005). Cheng et al., 2011a, transfected GPER into HEK-293 kidney cells that lack estrogen receptors to study the trafficking of GPER. Using immunohistochemical techniques, GPERi was initially observed in the membrane and then was trafficked to the perinuclear region of the cell by estradiol treatment, as well as in a ligand independent manner. GPERi then dispersed from the perinuclear area to other regions of the cytoplasm. Our previous ARH GPER immunohistochemistry observations indicated that GPER was located in the cytosol with much of the GPER immunopositive staining closely associated with the nucleus (Long et al., 2014). Similarly, we did not distinctly observe GPER immunopositive staining in the plasma membrane in this study either (Fig. 4). However, it is possible that the primary antibody does not recognize GPER in the plasma membrane, since our behavioral and western blot data clearly indicate that GPER is associated with the plasma membrane. In EB-primed rats, membrane impermeable E-Biotin facilitated lordosis, and these effects were blocked by pretreatment with G-15, a GPER antagonist, indicating that estradiol initiates rapid GPER signaling at the level of the plasma membrane (Fig. 2). This is supported by our observation of GPER in the plasma membrane fraction of ARH tissue (Fig. 3). Taken together, these data support our hypothesis that estradiol signals rapidly through GPER on the plasma membrane to facilitate sexual receptivity.

Since others observed GPER in the plasma membrane fraction of HIPP neurons (Akama et al., 2013; Funakoshi et al., 2006), we used the HIPP as a control for our cell fractionation and western blot techniques. Similarly, we observed GPER expression in plasma membrane fractions of the HIPP as well as the AMYG (Fig. 3). Additionally, GPER was also found in the cytosolic fraction of the tissue from the ARH, AMYG, and HIPP (Fig. 3). Thus, as observed by other investigators, our findings indicate that GPER is located in multiple subcellular compartments, and estradiol can initiate GPER signaling in the cytosolic compartment or at the level of the plasma membrane within neurons of the ARH, HIPP, and AMYG. Currently, it is unclear whether cytosolic GPER signaling is initiated in the ARH, and which cellular processes are regulated.

In previous studies, we demonstrated that a single dose of estradiol (EB 5–50 μg) that induces lordosis and deactivates MPN MOP 48 h after treatment requires activation of the ARH OFQ/N-ORL-1 system to directly reduce β-END neurotransmission to the MPN (Sanathara et al., 2011; Sanathara et al., 2014). This study tested whether GPER mediation of E2 rapid facilitation of lordosis required ARH ORL-1 activation, like a single bolus of EB (Sanathara et al., 2011). Similar to a single high dose subcutaneous injection of EB, infusion of E-biotin requires ARH ORL-1 activation to facilitate lordosis and reduce MPN MOP activation (Fig. 2). Inhibiting ARH ORL-1 with infusion of UFP, blocked the deactivation of MPN MOP and facilitation of lordosis by E-biotin. Previously, we demonstrated that ORL-1 is expressed in ARH β-END neurons that project to the MPN (Sanathara et al., 2014). Further, ORL-1 activation decreases excitation of β-END neurons by increasing K+ currents through the G protein-couple potassium channel type 1 (GIRK-1; Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016). Steroid treatments that facilitate lordosis increase the efficiency of the ORL-1-GIRK-1 signaling presumably to reduce β-END neurotransmission that we measure as a reduction in MPN MOP internalization (Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016; Sanathara et al., 2011). Although we know that the initial decoupling of ORL-1 and GIRK-1 is not mediated by GPER, GPER may mediate the recoupling of ORL-1 and GIRK-1 that is observed by other steroid treatments that facilitate lordosis (Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016).

Based on our current behavioral results, E2 activation of GPER induced ARH OFQ/N neurotransmission. Therefore, we tested in experiment 3 whether GPER may regulate the release of OFQ/N directly using double-labeled immunohistochemistry to demonstrate GPER expression in ARH OFQ/N neurons. In the ARH, we observed a subpopulation of neurons that were immunopositive for both GPER and OFQ/N (Fig. 4). Estradiol priming significantly increased the number of ARH neurons that individually expressed GPERi and OFQ/Ni staining as well as the number of number of neurons that co-expressed GPERi and OFQ/Ni staining (Fig. 4). Neither the percentage of ARH OFQ/Ni neurons that were GPERi positive nor the percentage of GPERi neurons that were OFQ/Ni positive was affected by estradiol priming (Fig. 4). Although estradiol priming increased the number of neurons that expressed and co-expressed GPERi and OFQ/Ni, the populations of single-labeled and double-labeled neurons maintained similar percentages of immunopositive neurons. Thus, it appears that estradiol priming increases the responsiveness of a subpopulation of ARH OFQ/N neurons to subsequent estradiol treatment by increasing the number of OFQ/N neurons that co-express GPER. Subsequent progesterone treatment that facilitates lordosis reversed the estradiol-induced increase in the number of GPERi neurons to levels observed in Oil treated rats (Fig. 4). This reduction in GPERi by progesterone appears responsible for reducing the number of neurons that co-expressed GPERi and OFQ/Ni and the percent of OFQ/Ni neurons that expressed GPERi. In contrast, progesterone had no effect on estradiol-induced levels of OFQ/Ni neurons or the percent of GPERi neurons that expressed OFQ/Ni (Fig. 4). These immunohistochemistry results support the notion that estradiol-GPER signaling is possible in a subset of OFQ/N neurons, and that estradiol increases the number of OFQ/N neurons that are responsive to estradiol via GPER. However, our western blot analysis of GPER expression in the ARH did not reflect the estradiol induced increase in GPER that we observed in the immunohistochemical analysis (Figs. 3 and 4). Estradiol priming did not alter the levels of GPER protein in either the membrane or cytosol as measured by western blot of block dissections (Fig. 3). These results also support the idea that the immunohistochemical staining does not reveal all of the subcellular populations of GPER. For example, as mentioned previously, we did not observe GPERi that was specifically associated with the plasma membrane in either the current or previously study (Long et al., 2014). Thus, steroid treatments may alter the subcellular location or conformation of GPER so that optimal staining is reduced because the antigen site is not as accessible to the primary antibody. Therefore, we may have undercounted the total population of neurons expressing GPER.

Our present findings indicate that in the ARH E2 rapid facilitation of lordosis is mediated by plasma membrane GPER in EB-primed rats. The SERMs, tamoxifen and ICI 182,780, act as antagonists of ERα and ERβ, but are agonists of GPER (Filardo et al., 2000; Ignatov et al., 2010; Long et al., 2017; Mo et al., 2013; Revankar et al., 2005). We demonstrated that site specific ARH infusions of tamoxifen and ICI 182,780 (Chokr et al., 2016) as well as subcutaneous tamoxifen treatment or ICI 182,780 infusion into the third ventricle facilitated lordosis in EB primed rats (Long et al., 2017; Long et al., 2014). These results indicate that the late actions of E2-GPER signaling that facilitate lordosis do not require ERα or ERβ signaling. If ERα or ERβ activation were necessary for rapid facilitation of lordosis, then tamoxifen or ICI 182,780 infusions should have not facilitated lordosis, unless ERα or ERβ activation activated lordosis circuits that are “upstream” of ARH GPER actions. Others have also demonstrated that E2 rapidly facilitates lordosis and acts through GPER when infused into the lateral ventricle (Dominguez-Ordonez et al., 2015; Dominguez-Ordonez et al., 2018). However, under these conditions, GPER facilitation of lordosis was blocked by

tamoxifen. Further, a progesterone receptor antagonist (RU486) and a GnRH-1 receptor antagonist (Antide) also blocked GPER facilitation of lordosis (Dominguez-Ordonez et al., 2018). This group also observed that activation of either ERα or ERβ by PPT (ERα agonist) or DPN (ERβ agonist) infusion into the lateral ventricle rapidly facilitated lordosis (Dominguez-Ordonez et al., 2016). These effects were blocked by specific ERα or ERβ antagonists (Dominguez-Ordonez et al., 2016). The discrepancies among the studies may be due to strain differences (Long Evans vs Sprague Dawley) or the routes of administration. However, both studies administered tamoxifen in a manner that would act globally in the brain (s.c., third ventricle and lateral ventricle) that produced opposite results. Our results indicate that ARH GPER mediating the rapid actions of E2 do not require ERα and ERβ signaling during the E2-GPER facilitation of lordosis (Chokr et al., 2016; Long et al., 2017).

GPER is a GPCR that signals through multiple G proteins that include the $G\alpha_s$ as well as a $G\alpha_{i/o}$ subunit that is pertussis toxin sensitive (Filardo et al., 2000; Thomas et al., 2005). In turn, GPER initiates the activity of numerous intracellular signaling pathways (reviewed in Baez-Jurado et al., 2019). These include adenylate cyclase-cAMP-PKA, Akt, ERK1/-2 and PI3K (Arevalo et al., 2010; Filardo et al., 2000; Karki et al., 2013; Karki et al., 2014; Prossnitz and Maggiolini, 2009; Tang et al., 2014; Thomas et al., 2005). When coupled to the $G\alpha_{i/o}$ subunit, GPER initiates signaling through phosphoinositide 3-kinase or Src family kinase, potentially regulating intracellular calcium release (Prossnitz and Barton, 2011; Revankar et al., 2005). Intracellular calcium regulation by GPER also occurs through multiple mechanisms that include signaling through phospholipase C, and the inositol and ryanodine receptors, as well as the voltage-gated calcium channels (Abdelhamid et al., 2011; Ariazi et al., 2010; Revankar et al., 2005; Sun et al., 2010). Activation of GPER has been shown to transactivate the epidermal growth factor receptor via Src kinase (Lee et al., 2012; Mo et al., 2013; Prossnitz and Barton, 2011). A promising pathway that GPER may activate for rapidly facilitating lordosis is the ERK1/-2 signaling pathway. An ERK/MAPK pathway has been shown to mediate lordosis in estradiol primed rats that received intracerebroventricular (icv) infusions (Gonzalez-Flores et al., 2009). Further, ICI 182, 780 has been shown also to activate ERK1/-2 through GPER (Filardo et al., 2000; Prossnitz and Maggiolini, 2009). Previous studies indicate GPER may be regulating GnRH neuronal activity and that lordosis may be regulated through the adenylate cyclase-cAMP-PKA pathway (Dominguez-Ordonez et al., 2018; Terasawa et al., 2009). However, because icv infusions flood the brain, the origin of the ERK and adenylate cyclase signaling pathways that facilitate lordosis has not been localized.

The initial rapid actions of estradiol in the ARH-MPN lordosis regulating pathway model are mediated through ER rather than GPER (Fig. 1; Dewing et al., 2007; Sinchak et al., 2005). The initial rapid and maintained estradiol priming effects in the ARH are mediated mainly through ERα that complexes with and transactivates mGluR1a to induce NPY-Y1 activation of β-End neurons that project to the MPN (Christensen et al., 2011; Dewing et al., 2007; Dewing et al., 2008; Mills et al., 2004; Sinchak et al., 2005). This increases the activation and internalization of MPN MOP, which inhibits lordosis and allows for estradiol priming effects for subsequent signaling to facilitate lordosis (Fig. 1; Long et al., 2017; Long et al., 2014; Sanathara et al., 2011; Sinchak and Micevych, 2001). Interestingly, activation of the putative Gq-coupled membrane ER (Gq-mER; Qiu et al., 2003) by infusion of STX into the ARH produced a similar activation of MPN MOP that is mediated via mGlurR1a as well (Christensen and Micevych, 2013; Micevych and Dewing, 2011). However, infusion of G1 (GPER agonist) did not increase the internalization/activation of MPN MOP, indicating GPER does not initiate the activation of the ARH-MPN lordosis circuit (Dewing et al., 2007). Behavioral data in mice indicate that GPER may have priming effects by upregulating progesterone receptors. Mice were treated with G1 instead of estradiol. Subsequent progesterone treatment facilitated moderate levels of lordosis indicating that GPER may mediate some of estradiol's priming effects on progesterone receptor expression and trafficking (Anchan et al., 2014). Further support of this is that in ERαKO mice, progesterone receptor expression is increased by estradiol priming (Moffatt et al., 1998). Moreover, in the rat, neither individual nor simultaneous activation of ERα and ERβ up regulated or trafficked progesterone receptors to the same levels as estradiol (Sa et al., 2015). Our current results demonstrate that EB priming increases ARH GPER expression in the OFQ/N neurons (Fig. 4). Although cell fractionation and western blot techniques indicated that GPER is located in the plasma membrane and cytosolic compartments, subsequent ARH infusion of membrane impermeable E-biotin demonstrated that GPER located in the plasma membrane mediate the facilitation of lordosis (Fig. 1). Our findings also support the idea that GPER activation appears to directly induce OFQ/N neurotransmission. A subpopulation of ARH OFQ/N neuron express GPER and blocking the activation of ORL-1 with UFP inhibited E-biotin facilitation of lordosis (Fig. 4). We previously demonstrated that ORL-1 is expressed in β-END neurons that project to the MPN and their activation by OFQ/N produces inhibitory potassium currents via GIRK-1 channels (Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016; Sanathara et al., 2014). In turn, ORL-1 activation reduces β-END release into the MPN and reduces MOP activation (Fig. 1; MOP recycle back to plasma membrane) that facilitates sexual receptivity (Sanathara et al., 2011). Currently, the signaling pathway that GPER activates to induce OFQ/N transmission is not known.

5. Conclusions

Our data demonstrated that in EB-primed female rats E2 facilitation of lordosis is mediated by ARH GPER located in the plasma membrane. Using western blot and cell fractionation techniques, GPER was located in plasma membrane ARH fractions as well as in the cytosolic compartment. ARH infusion of membrane impermeable E-Biotin facilitated lordosis, which was inhibited by pretreatment with a GPER antagonist. E2 activation of GPER appears to directly induce OFQ/N neurotransmission in the ARH. Estradiol priming increased the number of OFQ/N neurons that express GPER and inhibition of the OFQ/N receptor ORL-1, GPER induced lordosis was blocked. Taken together, these data indicated that estradiol priming initially upregulates GPER and OFQ/N expression and that subsequent E2 infused into the ARH acts on membrane associated GPER in OFQ/N neurons to induce OFQ/N neurotransmission that activates ORL-1 to reduce β-END neurotransmission/MPN MOP activity and facilitates sexual receptivity. Currently, it is unclear which signaling mechanism GPER is activating in the ARH to facilitate lordosis.

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