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Extranuclear signaling by ovarian steroids in the regulation of sexual receptivity

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INTRODUCTION

This year marks the 50th anniversary of the publications describing a uterine estrogen-specific binding protein, an estrogen receptor (ER) (Gorski et al., 1968; Jensen et al., 1968). The following year, ER was demonstrated to be expressed in the hypothalamus (Eisenfeld, 1969) and helped explain estradiol accumulation in neurons (Pfaff, 1968; Sar and Stumpf, 1973). In the mid-1980's, new techniques in molecular biology led to important steroid receptor discoveries: the production of antibodies (Clarke et al., 1987a; Clarke et al., 1987b; Greene et al., 1984; Sullivan et al., 1986), the cloning of ER α (Green et al., 1986; Koike et al., 1987a; Walter et al., 1985), and at the time, the discovery of what was the only progesterone receptor (PGR; Conneely et al., 1986; Jeltsch et al., 1986). Also in the mid-80's, immunohistochemistry techniques revealed two distinct forms of ERs, designated ER α and ER β (Giambiagi et al., 1984). ER β was subsequently cloned about a decade later (Kuiper et al., 1996; Tremblay et al., 1997). This produced a rapid expansion of our understanding of the anatomical locations of ERs and PGRs throughout the brain (Akesson et al., 1988; Hagihara et al., 1992; Lubischer and Arnold, 1990; Perrot-Applanat et al., 1985; Shughrue et al., 1997a; Simerly, 1990; Warembourg et al., 1986). Initial immunohistochemical studies suggested that unoccupied ERs were found in the cytoplasm and translocated to the nucleus, where they were thought to regulate transcription (Clarke et al., 1987b; Greene et al., 1984; Roy and McEwen, 1979). However, it was subsequently established that unoccupied ERs and PGRs were localized in the nucleus (Welshons et al., 1985; Welshons et al., 1984). These findings led to research emphasizing nuclear actions of ER and PGR mediating cellular (neural) functions (Koike et al., 1987b; Walter et al., 1985;

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White et al., 1987) through response elements in the promoter regions of specific genes (Couse and Korach, 1999; Klinge, 2001; Klinge et al., 2001).

In spite of a preponderance of evidence that ERs and PGRs are ligand-activated transcription factors, there were indications that steroid receptors could rapidly alter cell signaling pathways. For example, in endometrial cells, estradiol produced rapid physiological responses (Pietras and Szego, 1975; Rambo and Szego, 1983; Szego and Davis, 1967, 1969), and that ER was located on the plasma membrane of these tissues (Pietras and Szego, 1977, 1979). Further, Martin Kelly and Robert Moss demonstrated that estradiol rapidly alters neuronal electrophysiological properties in the medial preoptic-septal region (Kelly et al., 1976, 1977a, 1978; Kelly et al., 1977b). Despite this substantial evidence for alternate mechanisms of actions, the dogma was that steroid receptors acted in the nucleus (reviewed in Micevych et al., 2015). Research into membrane initiated, so-called “rapid actions,” was not popular due to the expectation that a new receptor was needed to initiate membrane signaling. This was shown to be untrue. In a series of elegant studies, Elis Levin demonstrated that the so-called “nuclear ERs,” ER α and ER β , can be trafficked to the nucleus and initiate intracellular signaling (Razandi et al., 1999). Shortly thereafter, this was also demonstrated in the brain (Abraham et al., 2004). Since then, membrane ER α and ER β have been shown to complex with and signal through metabotropic glutamate receptors (mGluRs) (Boulware et al., 2005; Dewing et al., 2007; Grove-Strawser et al., 2010; reviewed in Meitzen and Mermelstein, 2011; Micevych et al., 2017). Thus, many of the same intracellular signaling molecules known to function in glutamate pathways (kinases, etc.) are integral players in estrogen signaling.

Beyond ER α / β interactions with mGluRs, multiple other steroid receptor relationships have been revealed. For example, ER interacts with insulin growth factor-1 (IGF-1), which has been implicated in reproduction (Quesada and Etgen, 2002) and neuroprotection (Quesada et al., 2007). In addition to the full-length ER α , we and others have found a splice variate missing exon 4, ER α 4 on the membrane (Christensen and Micevych, 2012; Dominguez et al., 2013; Gorosito et al., 2008), whose mRNA was described in the 1990s (Skipper, 1993). Although PGR has many of the features that allow membrane ER α /ER β to transactivate mGluRs, the best evidence indicates PGR directly activating Src and downstream kinases (Boonyaratanakornkit et al., 2007; Boonyaratanakornkit et al., 2001; Mittelman-Smith et al., 2017; Phan et al., 2014a; Serey et al., 2014b).

As the field expected, several novel membrane-associated estrogen binding proteins have been discovered. The best characterized is the orphan G protein-coupled receptor, GPR30, which is also known as G protein ER (GPER). This extranuclear ER is associated with the endoplasmic reticulum as well as 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., 2008; Revankar et al., 2005; Terasawa et al., 2009; Thomas et al., 2005; but see Bondar et al., 2009). GPER activates a number of signaling pathways that include adenylyl cyclase, cAMP, and intracellular calcium (Filardo et al., 2000; Filardo et al., 2002; Kuo et al., 2010; Noel et al., 2009; Revankar et al., 2005; Terasawa et al., 2009; Thomas et al., 2005). A putative membrane ER, is the G protein q-coupled membrane estrogen receptor (Gq-mER, aka STX

receptor). Gq-mER does not have an established binding protein/receptor (Qiu et al., 2003; Qiu et al., 2006). Although not cloned, Gq-mER behaves like a Gαq protein-coupled membrane-associated binding protein/receptor that is activated by estradiol, and STX, a diphenylacrylamide derivative. This putative receptor initiates signaling through a number of pathways that include PLC, PKC-PKA, and calcium release (Kuo et al., 2010; Qiu et al., 2003; Zhang et al., 2010), and is inhibited by ICI 182,780 (Qiu et al., 2003; Qiu et al., 2006). Interestingly, STX activation of calcium flux is blocked with the mGluR1a antagonist, LY367,385 – just like ERα (Kuo et al., 2010).

Thus, like other transmitters in the brain, estradiol can act through multiple receptors (Rudolph et al., 2016), which makes understanding estradiol facilitation of sexual receptivity much more difficult. ERα is critical, but there are solid indications that not all ERα actions are directly nuclear, and that GPER and even Gq-mER are involved (Christensen and Micevych, 2013; Long et al., 2017; Long et al., 2014). Perhaps the best and most well-studied behavioral response in neuroscience is lordosis behavior of a receptive female. Starting with Frank Beach, generations of neuroendocrinologists have used this behavior to investigate the temporal pattern of estradiol and progesterone activation of neural circuits that regulate sexual receptivity in the brain (Beach, 1948). For much of that time, it was assumed that steroids acted directly in the nucleus and that these genomic actions alone were essential for facilitating lordosis. It has become apparent that estradiol also acts through extranuclear signaling pathways to rapidly alter neuronal activity, a necessary step in the estrogenic induction of lordosis. To illustrate this, we review the evidence for estradiol and progesterone regulation of an important subcircuit of sexual receptivity. We have explored the role of estradiol and progesterone signaling in the arcuate nucleus of the hypothalamus (ARH). Signaling begins here and then involves neurons in the medial preoptic nucleus (MPN) – a critical node for integrating steroid, and limbic system input – which in turn project to the ventromedial hypothalamic nucleus (VMH; reviewed in Micevych and Sinchak, 2013). The VMH is the common, final output of the “hypothalamic module” that activates downstream centers all the way to spinal motoneurons innervating trunk musculature that produces the lordotic posture (Pfaff et al., 2008).

Steroid activation of sexual receptivity

The gonadally intact adult rat spontaneously ovulates and has regular 4-5 day estrous cycles. Sexual receptivity is facilitated through the sequential exposure of neural lordosis circuits to ovarian estradiol and then progesterone (Powers, 1970). In fact, multiple steroid replacement paradigms induce lordosis in ovariectomized rats. In our model, estradiol levels of the estrous cycle are mimicked in ovariectomized Long Evans rats by 2 µg estradiol benzoate (EB; subcutaneously, s.c.) once every four days. This produces peak circulating levels of estradiol that are similar to levels seen during proestrus without inducing sexual receptivity, and “primes” steroid-sensitive circuits (Geary and Asarian, 1999; Jones et al., 2013; Micevych et al., 1996). Subsequent treatment with either molecular or pharmacological agents 26-48 hours later allow the study of estrogen-induced actions that induce proteins (e.g., neuropeptides, enzymes, and receptors) as well as modulating the functional coupling of G protein-coupled receptors (Kellert et al., 2009; Kelly et al., 1992; Lagrange et al., 1994; Qiu et al., 2003; Quesada et al., 2008; Tang et al., 2005). The canonical ERα dependent

induction of PGR in the mediobasal hypothalamus (ARH, MPN and VMH) requires estradiol exposure for 20-24 hours (Alves et al., 2000; Blaustein and Feder, 1979; Kraus et al., 1994; MacLusky and McEwen, 1978, 1980; Mittelman-Smith et al., 2017; Moffatt et al., 1998; Moguilewsky and Raynaud, 1979; Shughrue et al., 1997b). Progesterone (250-500 µg; s.c.) given 20 to 56 hours after estradiol produces maximal levels of sexual receptivity 4 hours after progesterone treatment (Sinchak and Micevych, 2001). However, when infused directly into the hypothalamus of an estradiol primed rat, progesterone facilitates lordosis within 30 minutes (Mani et al., 1994b; Serey et al., 2014a). Progesterone has biphasic actions: it produces a window of sexual receptivity that lasts about 12 hours and then terminates sexually receptivity (reviewed in Clemens and Weaver, 1985).

The estradiol + progesterone hormone replacement mimics the gonadally intact rat's activation of lordosis, but sexual receptivity can also be induced by treating an ovariectomized rat with estradiol only. There are two main EB priming paradigms: i) a single high dose of EB (5-50 µg; s.c.), which produces high circulating levels of estradiol for more than 48 hours with maximal sexual receptivity 48 hours later; ii) two sequential treatments of estradiol: a 2 µg EB priming dose followed 44-48 hours later by nonesterified estradiol subcutaneously, intracerebroventricularly (icv) or into a specific brain regions. Like progesterone, subcutaneous injections require about 4 hours to induce maximal sexual receptivity, whereas icv and or site specific brain infusions facilitate sexual receptivity within 30 minutes (Long et al., 2014; Micevych and Sinchak, 2013; Parsons et al., 1984).

An ARH-MPN circuit regulates lordosis

The MPN has long been recognized as an inhibitory node for female sexual receptivity. Stimulation of the MPN inhibits lordosis, whereas MPN lesions facilitate lordosis in rats treated with subthreshold doses of estradiol (Moss, 1974; Nance et al., 1977; Powers and Valenstein, 1972; Takeo, 1993; Sakuma, 1994; Spiteri et al., 2012). It slowly emerged that activation of opioid receptors, especially μ -opioid receptors (MORs), was part of this inhibition (Acosta-Martinez and Etgen, 2002; Pfaus and Pfaff, 1992; Sinchak and Micevych, 2001; Sinchak et al., 2004; Sinchak et al., 2005; Sirinathsinghji et al., 1986). Interestingly, the source of β -endorphin (β -End), the primary endogenous ligand of MOR, is the ARH (Bouret et al., 2004; Mills et al., 2004). The ARH is an important integrative center for energy balance, cardiovascular regulation, GnRH secretion, temperature regulation, and female reproduction (Coppari et al., 2005; Mittelman-Smith et al., 2016; Mittelman-Smith et al., 2012; Mittelman-Smith et al., 2017; Rance et al., 2013; Voogt et al., 2001).

Counterintuitively, without the opioid inhibition in the MPN, full sexual receptivity is not reached, even with sufficient doses of estradiol and progesterone that in control animals induce maximal lordosis behavior (Torii and Kubo, 1994; Torii et al., 1995, 1996, 1999). Sexual receptivity was also reduced in estradiol and progesterone primed MOR knock-out mice (Sinchak et al., 2005). To understand steroid signaling mechanisms regulating sexual receptivity, we have examined the activation of the ARH β -End circuit that regulates MOR in the MPN and sequentially influences the output of the VMH (reviewed in Fahrbach et al., 1989; Micevych and Sinchak, 2013; Sinchak and Wagner, 2012; Takeo, 1993). These studies indicate that estradiol and progesterone regulate neuropeptide Y (NPY), orphanin FQ (OFQ/N; aka nociceptin) and β -End neuronal activity modulating sexual receptivity.

Treatment of ovariectomized rats with estradiol rapidly induces MOR activation as visualized by MOR internalization (Eckersell et al., 1998). Estradiol does not bind to MOR, nor does it have this effect when administered directly into the MPN. However, estradiol does induce internalization when administered into the ARH, the origin of β -End fibers in the MPN. For these reasons, we concentrated on circuits located in the ARH (Dewing et al., 2007; Mills et al., 2004). We observed that β -End neurons that project to the MPN have NPY-Y1 receptors that are activated after estradiol treatment. We also found that blocking these Y1 receptors prevented the activation/internalization of MOR in the MPN (Mills et al., 2004). Thus, we surmise that the ARH-MPN MOR system is regulating the onset of sexual receptivity by preventing copulation from occurring until all the reproductive organs are exposed to the necessary levels and duration of steroid hormones. This ensures that sexual activity is coordinated with ovulation, maximizing the chances for pregnancy.

Initial actions of estradiol controlling lordosis

Lordosis behavior is dependent on the specific timing of ER α activation (Ogawa et al., 1998; Rissman et al., 1997). Both direct nuclear and membrane-initiated mechanisms are needed to induce sexual receptivity along with activation of GPER and Gq-mER (Christensen and Micevych, 2013; Micevych et al., 2017). Immediately after estradiol treatment of an ovariectomized rodent, sexual receptivity is repressed. As mentioned, this active inhibition is mediated by β -End released from ARH proopiomelanocortin (POMC) neurons that project to the MPN. Several lines of evidence point to membrane-initiated estradiol actions underlying this activation: MOR activation is observed within minutes after estradiol treatment, a membrane-constrained estradiol (E-6-biotin) mimics the actions of estradiol when injected into the ARH, and blocking ERs with ICI 164,384 abrogates the effect. These early actions of estradiol are dependent on ER α (Micevych et al., 2003). Although a member of the nuclear receptor family, ER α is trafficked to the membrane in association with a chaperone, caveolin, which determines signaling by allowing association with specific mGluRs (Boulware et al., 2007; Mermelstein and Micevych, 2008; Stern and Mermelstein, 2010). In the ARH, the estradiol membrane initiated signaling regulating MOR activation and lordosis inhibition is through the transactivation of mGluR1a (Dewing et al., 2007; Dewing et al., 2008). Activating mGluR1a mimics estradiol treatment, and blocking the mGluR1a prevents estradiol-induced MOR activation. Activating PKC, a downstream target of ER α -mGluR1a signaling, in these animals activates MOR and inhibits lordosis behavior. Moreover, in the ARH, knockdown of caveolin-1, necessary for ER α -mGluR1a trafficking to the membrane, abrogates estradiol-induced lordosis behavior (Christensen and Micevych, 2012). β -arrestin, usually considered a mediator of membrane receptor internalization, is involved with both trafficking of ER α to the membrane and its internalization, a hallmark of membrane receptors after they have bound their natural ligand (Wong et al., 2015; reviewed in Sinchak and Micevych, 2003). Receptor internalization is necessary to remove the ligand from the receptor. Recycling the empty ER α to the membrane allows reactivation by estradiol. Knockdown of β -arrestin prevents both ER α trafficking to the membrane and internalization, which compromises estradiol signaling - disrupting lordosis behavior. In vitro, treatment with estradiol eventually leads to down regulation of ER α on the membrane due to degradation of the receptor in lysosomes (Bondar et al., 2009; Dominguez and Micevych, 2010). As the levels of membrane ER α

decrease, estradiol-induced MOR activation is terminated. In ovariectomized rodents, large doses of estradiol benzoate (EB; 50 μ g) induce lordosis within 48 hours. Doses that are closer to physiological levels (e.g., 2 μ g EB) do not induce lordosis (e.g., Mahavongtrakul et al., 2013; Sanathara et al., 2011). This may be due to a differential action of high vs. low levels of estradiol on ER α -mGluR1a complexes on the membrane that are required for membrane-initiated estradiol signaling. Low dose EB (2 μ g) produces elevated mER α -mGluR1a levels in the ARH; in contrast, 48 hours after a high dose of EB (50 μ g), mER α -mGluR1a levels in the ARH are significantly reduced (Mahavongtrakul et al., 2013). Thus, a priming dose may not induce lordosis because mER α -mGluR1a signaling maintains opioid inhibition, but a high dose of EB reduces mER α -mGluR1a due to receptor internalization (Dominguez and Micevych, 2010). Behavioral inhibition can be alleviated approximately 20 hours after estradiol treatment by progesterone, which mimicks the induction of lordosis in gonadally intact rats. Progesterone, it turns out, terminates the estradiol-induced opioid inhibition of lordosis (Sinchak and Micevych, 2001).

Later extranuclear actions of steroids that facilitate lordosis

Following the initial actions of estradiol that activate the ARH-MPN pathway, the deactivation of MPN MOR is important for facilitation of lordosis (Borgquist et al., 2013; Borgquist et al., 2014; Cheung et al., 1995; Christensen et al., 2011; Christensen and Micevych, 2012, 2013; Dewing et al., 2007; Dewing et al., 2008; Long et al., 2017; Long et al., 2014; Long et al., 2012; Micevych et al., 2003; Mills et al., 2004; Sanathara et al., 2011; Sanathara et al., 2014; Sinchak et al., 2013; Sinchak and Micevych, 2001; Sinchak et al., 2005). We also observed this pattern of MPN MOR activity in intact cycling rats. MPN MOR activation is higher during diestrus days I & II and estrus (relatively lower levels of estradiol) compared to the evening of proestrus when the rats are sexually receptive and estradiol levels are high (Sinchak et al., 2003). Although each steroid paradigm reduces MPN MOR activity and facilitates lordosis, the deactivation of β -End neurotransmission is regulated by several pathways (Sanathara et al., 2011). Both the single high dose and sequential low doses of estradiol that facilitate lordosis require presynaptic OFQ/N neurotransmission that activates its cognate receptor, ORL-1 (opioid receptor-like receptor-1; aka nociceptin receptor), on ARH β -End neurons (Chokr et al., 2016; Long et al., 2015; Sanathara et al., 2011). In contrast, estradiol + progesterone treatment does not require the activation of the OFQ/N-ORL-1 system and appears to act directly on β -End neurons that project to the MPN (Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016; Eskander et al., 2017; Jones et al., 2015; Sanathara et al., 2011; Sanathara et al., 2014).

Estradiol deactivation of MPN MOR

Initially, whether given a high dose or priming dose of estradiol, ARH ER α -mGluR1a signaling activates MPN MOR to inhibit lordosis (Dewing et al., 2007; Eckersell et al., 1998; Mills et al., 2004; Sanathara et al., 2011; Sinchak et al., 2013; Sinchak and Micevych, 2001). Forty-eight hours later, ARH levels of membrane ER α -mGluR1a complexes are determined by estradiol dose (Mahavongtrakul et al., 2013). Additionally, our studies have identified other pathways through which estradiol may act to relieve the opioid inhibition of sexual receptivity. The effects of estradiol on PGR expression are not needed for estradiol-only facilitation of sexual receptivity. Estradiol-only facilitation of lordosis is not dependent

on upregulation or activation of classical PGR (Blaustein et al., 1987; Jones et al., 2015; Mani et al., 1997; but see Dominguez-Ordóñez et al., 2018). Although this review concentrates on rapid actions of GPER in the ARH to facilitate lordosis, others have shown in estradiol primed rats that activation of either ER α or ER β via intracerebral ventricular (icv) infusions rapidly facilitates lordosis, and that rapid estradiol facilitation of lordosis is blocked by specific ER α or ER β antagonists (Dominguez-Ordóñez et al., 2018). Because of the lack of specificity of the icv infusion, it is difficult to ascertain where these signaling pathways are being activated.

GPER rapid facilitation of lordosis

GPER is expressed in the ARH (Long et al., 2014). GPER mediates the rapid effects of nonesterified 17 β -estradiol (E2) ARH infusions in EB primed rats. This activation of GPER reduces MPN MOR activation and facilitates lordosis within thirty minutes (Long et al., 2017; Long et al., 2014). These rapid effects of E2 are mimicked by G1, a GPER agonist, and blocked by pretreating with the GPER antagonist G15 (Long et al., 2014). In an experiment that was designed to test whether removing mER α -mGluR1a signaling after estradiol priming facilitates lordosis, Long Evans ovx rats were treated with either tamoxifen or ICI 182,780 (ICI; aka fulvestrant) 44 hours after EB priming. Both of these selective ER mediators (SERMs) reduced MPN MOR activity and facilitated lordosis supporting our idea that removal mER α -mGluR1a signaling was important to facilitating lordosis (Garcia et al., 2010). However, tamoxifen and ICI 182,780 are also GPER agonists (Filardo et al., 2000; Filardo et al., 2002; Revankar et al., 2005; Vivacqua et al., 2006). Like ARH infusions of E2 and G1, these SERMs rapidly reduced MPN MOR activity and facilitated lordosis within 30 minutes, which was blocked by pretreatment of G15 (Long et al., 2017). These results indicated that lordosis was facilitated by the SERMs activating GPER and not through the inhibition of mER α -mGluR1a signaling. Our preliminary data indicate that estradiol acts through a plasma membrane GPER to affect OFQ/N neurotransmission, which in turn facilitates lordosis by inhibiting ARH β -End neurons (Borgquist et al., 2013; Borgquist et al., 2014; Conde et al., 2016). In EB-primed rats, ARH infusions of membrane impermeable E-6-biotin rapidly reduced MPN MOR activity and facilitated lordosis (Chokr et al., 2017), indicating GPER initiate signaling at the level of the plasma membrane. Indeed, we observed that ARH plasma membrane fractions contain GPER (Feri et al., 2016). ARH infusion of an ORL-1 antagonist, UFP101, blocks GPER facilitation of lordosis (Chokr et al., 2017; Chokr et al., 2016). Further, GPER is expressed in ARH OFQ/N neurons indicating that GPER may directly induce OFQ/N neurotransmission that acts on ORL-1 expressed in MPN projecting β -End neurons to reduce MPN MOP activation (Figure 1; Chokr et al., 2016; Tran et al., 2015). Although OFQ/N mediates facilitation of lordosis by both the high single-dose and sequential treatments of estradiol, it is unclear whether the high dose signals through GPER to induce OFQ/N release (Chokr et al., 2016; Long et al., 2015; Sanathara et al., 2011; Sanathara et al., 2014).

Steroid regulation of the ORL-1

During the 1990's another opioid receptor was cloned, the ORL-1 (Bunzow et al., 1994; Chen et al., 1994; Fukuda et al., 1994; Lachowicz et al., 1995; Marchese et al., 1994; Mollereau et al., 1994; Wang et al., 1994; Wick et al., 1994). Its endogenous ligand, OFQ/N,

is a heptadecapeptide (Meunier et al., 1995; Reinscheid et al., 1995; Saito et al., 1996; Saito et al., 1995). Although ORL-1 is in the family of opioid receptors, ORL-1 is not grouped with the classical opioid receptors (MOR, delta and kappa) and is not antagonized by naloxone (Minami et al., 1993; Mollereau et al., 1994). ORL-1 is expressed in brain regions important for reproductive physiology and behavior, and estradiol upregulates ORL-1 expression in the ARH and VMH (Neal et al., 1999a; Neal et al., 1999b; Sanathara et al., 2014; Sinchak et al., 2006).

In addition to regulating expression, estradiol regulates the signaling efficiency of ORL-1 in MPN projecting POMC/ β -End neurons in accordance to the sexual receptive state of the rat (Borgquist et al., 2013; Borgquist et al., 2014; Quesada and Micevych, 2008). ORL-1 is a $G_{i/o}$ -coupled receptor that regulates the G protein-gated, inwardly-rectifying K^+ (GIRK-1). Activation of ORL-1 increases postsynaptic K^+ currents and decreases β -End excitability, leading to facilitation of lordosis (Borgquist et al., 2013; Borgquist et al., 2014; Farhang et al., 2010; Wagner et al., 1998). In ovariectomized rats, OFQ/N induces robust GIRK-1 currents in POMC neurons projecting to the MPN (Borgquist et al., 2013). Interestingly, OFQ/N-induced GIRK-1 currents were reduced in POMC neurons 30 hours after EB (2 μ g) priming, effectively reducing inhibitory tone in β -End neurons, and allowing for increased β -End neurotransmission to inhibit lordosis. Estradiol-induced decoupling of ORL-1 to the GIRK-1 channel is rapidly induced and mediated by a plasma membrane ER. Within seconds, estradiol, E-6-BSA, STX and PPT, an ER α agonist, all reduce OFQ/N-induced GIRK-1 currents in POMC neurons that project to the MPN. This effect is blocked by ICI 182,780. However, GPER and ER β agonists did not alter OFQ/N-induced GIRK-1 (Conde et al., 2016). Gq-mER (STX) appears to signal through the PLC/PKC/PKA pathway and mER α via phosphatidylinositol-3-kinase (PI3K)/neuronal nitric oxide synthase (nNOS) pathways to decouple ORL-1 from GIRK-1 (Conde et al., 2016). Thus, multiple membrane initiated ER signaling pathways mediate initial rapid and maintained actions of estradiol to increase β -End neurotransmission that activates MPN MOR to inhibit sexual receptivity: mER α -mGluR1a induces excitation and mER α and Gq-mER reduce inhibitory tone. The decoupling of ORL-1 from GIRK is reversed by steroid treatments that facilitate lordosis (Borgquist et al., 2014). Subsequent progesterone or 48 hours after a large dose of EB restore OFQ/N-induced GIRK-1 currents (Borgquist et al., 2014). This allows for robust ORL-1-induced GIRK-1 inhibiting β -End release and to MPN MOR activation. The mechanism for this is not understood.

Rapid facilitation of lordosis by progesterone

Progesterone modifies the actions of estradiol and adds a layer of complexity to the estradiol induction of sexual receptivity. This is foreshadowed by the fact that progesterone levels rise after estradiol levels rise in the circulation (reviewed in Weaver and Clemens, 1987). While it was logical to ask whether neuroprogesterone has a role here, we have not been able to tie progesterone synthesized in the brain to sexual receptivity (Micevych et al., 2008). On the other hand, proceptive behavior- hopping, darting, “ear-wiggling”- is disrupted when either PGR or progesterone synthesis in the hypothalamus is blocked (Micevych et al., 2008). Sexually receptive behavior is unaffected by these treatments. Progesterone relieves the estradiol-induced MPN MOR inhibition of lordosis behavior (Sinchak and Micevych, 2001),

but detailed examination of the mechanism of action points to activation of a different circuit than that stimulated by estradiol treatment alone.

Extranuclear PGR mediation of progesterone facilitation of lordosis

Estradiol priming through ER α is sufficient for PGR upregulation in the VMH, ARH and other hypothalamic brain regions for progesterone facilitation of lordosis (Alves et al., 2000; Blaustein and Feder, 1979; MacLusky and McEwen, 1978, 1980; Mazzucco et al., 2008; Moguilewsky and Raynaud, 1979; Sanathara et al., 2011; Sanathara et al., 2014; Shughrue et al., 1997c). This is probable partially dependent upon gene transcription, since an estrogen receptor response element is associated with the PGR gene (Kraus et al., 1994).

However, not all estradiol-induced PGR expression is mediated by direct classical ER signaling via response elements. In ER α KO mice, estradiol increases PGR expression (Moffatt et al., 1998). Moreover, in rat VMH, estradiol-induced up regulation and trafficking of PGR could not be mimicked by individual or simultaneous activation of ER α and ER β (Sa et al., 2015), suggesting another ER such as GPER, which may mediate estradiol effects on PGR expression and trafficking (Anchan et al., 2014).

Progesterone has been shown to have rapid neurotransmitter-like actions through PGR (specifically, PGR-B isoform) to facilitate sexual receptivity (Mani et al., 1994b; Serey et al., 2014a). PGR-B signaling modulates sexual receptivity in the ARH (reviewed in Sinchak et al., 2015; Sinchak and Wagner, 2012). In order to initiate rapid signaling at the level of the plasma membrane, PGR-B complexes with and signals through Src family kinase (Src) (Boonyaratanakornkit et al., 2007; Boonyaratanakornkit et al., 2001; Phan et al., 2014b). ARH infusion of progesterone in estradiol-primed ovariectomized rats facilitates receptivity within 30 minutes by inhibiting ARH β -End neurons that project to the MPN (Huss et al., 2011). This progesterone-induced lordosis is mimicked by activating Src in the ARH. PGR-B-Src signaling is also dependent on D1/D5 dopamine receptors (D1/D5). PGR-B, D1/D5, and Src signaling that facilitates sexual receptivity converge in the ARH and are interdependent. Antagonizing one prevents facilitation of lordosis by the other two (Serey et al., 2014b). Immunohistochemistry showed an estradiol sensitive PGR and D1 colocalization in the ARH (Sinchak et al., 2014). Subpopulations of ARH β -End neurons also contained PGR and D1 immunostaining (Figure 2; (Phan et al., 2015). These results point to a subpopulation of β -End neurons that express both PGR and D1. Co-immunoprecipitation revealed D1 and D5 do not complex with either PGR or Src on the plasma membrane (Phan et al., 2015; Phan et al., 2016). However, D1 and Src are in close proximity (<40 nm) in the plasma membrane as demonstrated by Duolink proximity ligation assay (Figure 2). Although the results indicate that D1 is in close proximity Src, exactly how D1 regulates PGR-B-Src rapid progesterone signaling is unclear. While other mechanisms of dopaminergic-PGR signaling inducing sexual receptivity have been described (Mani, 2006; Mani et al., 1994a; Mani et al., 2000; Power et al., 1991a; Power et al., 1991b), our schema appears to fit the physiology best. Thus, rapid progesterone signaling is initiated through PGR-B transactivation of Src through direct protein-protein interactions at the level of the membrane that are interdependent on D1/D5 signaling.

Extranuclear actions of estradiol alter dendritic spine morphology

Estradiol induction of morphological plasticity is a well known phenomenon in several brain regions including the hypothalamus (Flanagan-Cato, 2011; Matsumoto and Arai, 1981; Woolley and McEwen, 1994). In MOR-KO mice, estradiol and progesterone induced lordosis behavior is reduced ~20% compared with wild type. Pharmacological blockade of mGluR1a virtually abolishes sexually receptivity (Dewing et al., 2007), suggesting that membrane-initiated estradiol signaling may disrupt other estradiol-induced events in the ARH. The formation of new spines in the ARH in response to estradiol treatment was found to be critical for lordosis behavior (Christensen and Micevych, 2012 Christensen, 2011 #3143). Blocking ERs, mGluR1a or spinogenesis in the ARH prevented estradiol induced lordosis, suggesting that indeed membrane-initiated estradiol signaling induced spine formation is needed for lordosis behavior. Estradiol rapidly induces phosphorylation of the actin severing protein, cofilin – deactivating it, and allowing for the formation of new spines. Newly formed spines are filopodial, the morphology of immature, nonfunctional spines. After the initial spinogenesis, the number of spines in the ARH remain stable for 48 hours, but there is an increase in the percentage of mushroom-shaped spines. This suggests that a portion of the newly formed spines mature over these hours and take on a morphology indicative of functional synapses. The time course of the increase in the number of mushroom-shaped spines coincides with the appearance of lordosis behavior.

Newly formed spines are unstable. Another event or stimulus is thought to be needed to stabilize them. After estradiol treatment in vitro, a long-term potentiation (LTP) protocol increases connectivity (Srivastava et al., 2008). We looked to see if markers of functional synapses increased during the time of spine maturation. Estradiol increases the expression of postsynaptic density protein-95 (PSD-95) and the axonal growth associated protein, GAP43, but pretreatment with ICI 182,780 blocks the increase. (Rudolph et al., 2016). Thus in the ARH, estradiol appears to increase behaviorally relevant synapses via ER α -mGluR1a signaling.

Conclusion

It is clear that estrogens and progestins initiate extranuclear signaling pathways via membrane localized classical PGR and ER in addition to nuclear transcriptional regulation. In order for these classical receptors to initiate signaling at the membrane they require another protein to complex with and transactivate (ERs-mGluRs; PGR-Src). In addition to classical receptors, estrogens and progestins also signal through recently discovered extranuclear receptors that are found in the membrane and cytoplasm. Ovarian hormone induction of sexual receptivity is a highly orchestrated series of events utilizing multiple types of ER and PGR signaling pathways. Our findings indicate that initially, estradiol is activating multiple types of ER α signaling that include transcriptional pathways to alter receptor expression, enzyme, and neurotransmitter levels. Simultaneously, ER α -mGluR1a membrane initiated signaling induces neurotransmission that inhibits sexual receptivity, influences ER α trafficking to the membrane, and induces spinogenesis. Following exposure to sufficient dose and duration of estradiol priming, subsequent estradiol and progesterone signal rapidly to reduce β -End neurotransmission and facilitate sexual receptivity. This

occurs through membrane initiated pathways via GPER and PGR-Src, respectively. A future challenge will be to characterize the overlapping but distinct circuits activated by estradiol alone and estradiol + progesterone that induce lordosis behavior (Figure 3). It will also be interesting to determine how membrane-initiated and nuclear-initiated signaling pathways interact to modulate the effects of estradiol and progesterone in a given circuit, since multiple receptor types are expressed in a single cell (Figure 1). An additional level of complexity is how the activity of these steroid receptor signaling pathways change over reproductive states. Understanding these steroid receptor-initiated pathways will allow for better design of therapies that target specific receptors and associated signaling pathways. The MPN-ARH sexual receptivity neurocircuitry remains an excellent model to study temporal actions of ovarian steroid dose and duration on classical nuclear and extranuclear membrane ER and PGR signaling.

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ABBREVIATIONS

ARH	arcuate nucleus of the hypothalamus
β-End	β-endorphin
cAMP	cyclic adenosine monophosphate
E2	nonesterified 17β-estradiol
EB	17β-estradiol-benzoate
ERα	estrogen receptor-α
Era 4	estrogen receptor-α splice variant missing exon 4
Erβ	estrogen receptor-β
GnRH	gonadotropin releasing hormone
GPER	G protein coupled estrogen receptor (GPR30)
Gq-mER	G protein q-coupled membrane estrogen receptor
icv	intracerebral ventricular
mER	membrane estrogen receptor
mGluR	metabotropic glutamate receptor
MPN	medial preoptic nucleus
mPR	membrane progesterone receptor
MOR	μ-opioid receptor

NPY	neuropeptide Y
OFQ/N	orphanin FQ/nociceptin
ORL-1	opioid receptor like-1 receptor
PGR	progesterone receptor
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
POMC	proopiomelanocortin
VMH	ventromedial nucleus of the hypothalamus

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Highlights

Estradiol and progesterone initiate extranuclear receptor signaling important for facilitating sexual receptivity

Estrogen receptor- α (ER α) transactivates metabotropic glutamate receptor-1a (mGluR1a) in the plasma membrane

ER α -mGluR1a signaling initially inhibits the lordosis circuit while inducing dendritic spines critical for the ultimate expression of lordosis.

In estradiol primed rats, subsequent activation of GPER (G protein-coupled estrogen receptor) facilitates lordosis

Membrane progesterone receptor signals through Src tyrosine kinase to rapidly facilitate lordosis

FIGURE 1.

Photomicrographs of immunohistochemical colocalization of A) PGR (green) and OFQ/N (red); B) OFQ/N (red) and GPER (green); C) GPER (green) and PGR (red) and D) GPER (green) and ERα (red) immunopositive neurons in the arcuate nucleus of the hypothalamus (ARH) of ovariectomized rats treated with 2 μg EB and transcardially perfused with chilled physiological saline and 4% paraformaldehyde in Sorensen's buffer 30 hours after treatment. PGR and ERα immunostaining was mainly associated with the nucleus, whereas GPER and OFQ/N staining was mainly observed in the cytoplasm. Arrows indicate double labeled neurons. 3V – third ventricle.

FIGURE 2.

Photomicrographs of immunohistochemical colocalization in the arcuate nucleus of the hypothalamus of ovariectomized rats treated with 2 μ g EB and transcardially perfused with chilled physiological saline and 4% paraformaldehyde in Sorensen's buffer 30 hours after treatment. Row A) photomicrographs of triple labeled neurons. In all panels progesterone receptor (PGR) is labeled with DAPI (blue), Src tyrosine kinase (Src) is labeled in first panel with FITC (green); dopamine receptor type D1 is labeled with FITC in second panel, and β -endorphin (β -End) is labeled with TRITC (red) in both panels. Arrows indicate triple labeled neurons. Row B) Confocal reconstructions of immunostaining for the Duolink Proximity Ligation Assay for PGR-Src and D1-Src counterstained with Hoechst to stain nuclei (blue). This assay allows for visualization of proteins that are in close proximity of each other (<40 nm). These proximity reactions appear as discrete punctate staining (red). Discrete punctate staining (arrows in first panel) was visualized in the ARH when antibodies for PGR and Src were used with the Duolink kit supporting that PGR and Src are in close proximity and likely forming complexes in the plasma membrane as indicated by co-immunoprecipitation studies. More importantly, we observed discrete punctate staining that appeared in the membrane for Src and D1 antibodies in the ARH (arrows in second panel). This indicates that although Src and D1 do not form complexes, they are in close proximity to each other on the plasma membrane, which allows for interdependent pathway signaling to occur.

FIGURE 3.

Different doses and sequences of steroid hormones activate different populations of neurons in the ARH leading to lordosis behavior. Supraphysiological doses of estradiol treatment (top) in ovariectomized rodents lead to a pattern of neural activation that results in lordosis behavior when the animals are mated with a male. Similarly, estrogen followed by progesterone treatment (E2+P4; bottom) in ovariectomized rodents induces a pattern of neuronal activation that is overlapping but distinct - yet produces lordosis behavior. For example, sexual receptivity induced by estradiol, whether by a single large bolus or sequential estradiol treatments requires the activation of the OFQ/N-ORL-1 system in the ARH to be activated to inhibit β -endorphin transmission. In contrast, estradiol followed by progesterone acts directly on β -endorphin neurons and dopamine neurons to inhibit β -endorphin transmission. Thus, rapid actions of multiple estradiol and progesterone signaling pathways acting in different sets of neurons produce the same result, reduction of β -endorphin release in the MPN that ultimately facilitates lordosis.