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Estradiol Membrane-Initiated Signaling and Female Reproduction

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Abstract

The discoveries of rapid, membrane-initiated steroid actions and central nervous system steroidogenesis have changed our understanding of the neuroendocrinology of reproduction. Classical nuclear actions of estradiol and progesterone steroids affecting transcription are essential. However, with the discoveries of membrane-associated steroid receptors, it is becoming clear that estradiol and progesterone have neurotransmitter-like actions activating intracellular events. Ultimately, membrane-initiated actions can influence transcription. Estradiol membrane-initiated signaling (EMS) modulates female sexual receptivity and estrogen feedback regulating the luteinizing hormone (LH) surge. In the arcuate nucleus, EMS activates a lordosis-regulating circuit that extends to the medial preoptic nucleus and subsequently to the ventromedial nucleus (VMH)—the output from the limbic and hypothalamic regions. Here, we discuss how EMS leads to an active inhibition of lordosis behavior. To stimulate ovulation, EMS facilitates astrocyte synthesis of progesterone (neuroP) in the hypothalamus. Regulation of GnRH release driving the LH surge is dependent on estradiol-sensitive kisspeptin (Kiss1) expression in the rostral periventricular nucleus of the third ventricle (RP3V). NeuroP activation of the LH surge depends on Kiss1, but the specifics of signaling have not been well elucidated. RP3V Kiss1 neurons appear to integrate estradiol and progesterone information which feeds back onto GnRH neurons to stimulate the LH surge. In a second population of Kiss1 neurons, estradiol suppresses the surge but maintains tonic LH release, another critical component of the estrous cycle. Together, evidence suggests that regulation of reproduction involves membrane action of steroids, some of which are synthesized in the brain.

Introduction

Over the years, it has become clear that steroids have global effects and that the brain produces neurosteroids synthesized *de novo* in both glia and neurons [reviewed in (99, 126, 181)]. Coincident with these discoveries was the finding that steroid signaling is not restricted to the regulation of transcription, but steroids can also rapidly elicit intracellular changes [e.g., (18, 24, 115, 161)]. These observations have radically changed our understanding of the sex steroid modulation of central nervous system circuits including those that that mediate reproductive physiology and behavior. While there is no doubt that sex steroids can and do behave like traditional steroid hormones, the ability of the brain to

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synthesize and rapidly respond to steroids strongly suggests that sex steroids can also signal like neurotransmitters. Conceptually, neurosteroids may be considered fourth generation (fourth Gen) transmitters (74, 120), whose members include nitric oxide (NO), prostaglandins, and endocanabinoids [reviewed in (6, 95, 157)]. A hallmark of fourth Gen transmitters is that unlike cholinergic, amino acid, adrenergic, or neuropeptide transmitters, they are regulated at the point of synthesis rather than release. Once synthesized, these molecules diffuse out of the cell to activate nearby receptors, which couple to a wide array of signaling cascades (e.g., cAMP, MAPK, Akt, Src, and Ca^{2+}), and lead to either excitation or inhibition of "postsynaptic" neurons.

Sex steroids, for decades, were considered to be classic hormones whose receptors acted as ligand-gated transcription factors at specific sites on DNA [estrogen response element, ERE, the AP-1 site (209) or at the Sp1 site (82, 175)]. Both physiological and anatomical studies were tailored to examine this nuclear role of steroid hormones in the brain. For example, when examining the expression of neuropeptides or receptors, steroids were given for an extended time, from 48 h to weeks. Similarly, autoradiographic studies to identify steroid-receptive cells were quantified by examining the accumulation of exposed silver grains marking the location of radiolabeled estradiol (or testosterone or progesterone). A steroid-receptive cell was identified as a cell with silver grains concentrated over the nucleus—the apparent site of steroid action (152, 206). Interestingly, sites in the brain that had accumulations of silver grains over cell nuclei also had higher "background" compared with nonsteroid sensitive regions (Fig. 1). This was usually not discussed in formal reports, but it was debated among autoradiographers who proposed that high "background" might imply the presence of steroid receptors on the membrane.

In fact, over the years, there had been indications that not all steroid action could be explained by nuclear receptors. In the periphery, the seminal work of Clair Szego and coworkers demonstrated the accumulation of cAMP within the uterus just minutes after exposure to estradiol (200). In the brain, initial clues about the rapid action of estradiol were reported by Martin Kelly and co-workers [(76, 77); reviewed in (75)]; however, the prevailing idea at the time was that steroids (estradiol in particular) acted in the nucleus to modulate transcription of genes.

Indeed, it is a well-established fact that estradiol, acting as a hormone, affects gene transcription, and can thus affect physiology and behavior. For example, the expression of a large number of neuropeptides and receptors implicated in reproductive function are regulated by estradiol [reviewed in (119)]. However, in some cases, colocalization of nuclear ER and estrogen-sensitive neuropeptides could not be demonstrated (5), suggesting that other mechanisms were involved. One possibility was that steroids act indirectly, on afferent neurons whose neurotransmitter(s) modulate protein expression in target neurons. The most famous example of this is the GnRH neuron, which does not express the steroid receptors critical for neural control of ovulation, but whose activity and GnRH expression are regulated by estradiol and progesterone [(187); reviewed in (34)].

Reports of membrane actions of estradiol increased in the 1990s [e.g., (115)], but it was the demonstration that nuclear ERs were trafficked to the plasma membrane by Ellis Levin's

group (166) that changed the way we viewed and understood membrane-initiated steroid signaling. Prior to this discovery, it was assumed that the membrane ER would be a different receptor from the nuclear ER. There are now several such candidates for the cell-surface membrane ERs. One such candidate is GPR30/GPER, a seven transmembrane receptor first described in breast cancer cells [(22,169); reviewed in (159)]. However, in hypothalamic cells, GPR30/GPER has not been localized to the cell surface (15, 48, 58), but appears to be present in another subcellular compartment, the smooth endoplasmic reticulum. As estradiol can freely diffuse into the cell, GPR30/GPER in this compartment may be readily activated (88). The precise role for GPR30/GPER is unknown, as either disruption of the coding region of GPR30/GPER or knockdown of the gene does not appear to influence reproduction (70, 144). It has been proposed that GPR30/GPER interacts with ERα at the membrane to organize downstream signaling although the details of such a role have not been elucidated (96). Another potential membrane ER is Gq-mER, which is activated by a tamoxifen derivation, STX (161). Gq-mER, which is blocked by the universal ER antagonist faslodex (ICI 182,780), activates kinase pathways and retains its activity in ERα, ERβ, and GPR30/GPER knockout mice [reviewed in (128,170)]. STX activation of Gq-mER in the arcuate nucleus (ARH) potentiates sexual receptivity, suggesting a role in mediating reproductive behavior (31). At present, it is difficult to sort which one (or more) of these putative receptors are responsible for estradiol actions at the membrane. The predominance of evidence suggests that ERα mediates the critical actions of estradiol for the central control of reproduction (128,129). Found in the cell nucleus and membrane, the same receptor, ERα, mediates estradiol's slow, hormone-like actions, and more rapid, neurotransmitter-like effects.

This review discusses the estradiol activation of female sexual receptive behavior and the control of gonadotropin release by the hypothalamus. Both of these events require the hormonal (direct nuclear) and the neurotransmitter (membrane) actions for a coordinated response.

Sexual Receptivity

Estradiol is essential for the eventual induction of sexual receptivity, which in rodents is expressed as lordosis behavior [reviewed in (116)]. However, immediately after systemic estradiol treatment, ovariectomized (ovx) rats are not sexually receptive (11). Explanations have ranged from the ideas that transcription and the formation of active dendritic spines take days (29) to the idea that estradiol activates inhibitory circuits (192, 194). In fact, all of these processes have been shown to be necessary. Under the rubric of longer timescale events, estradiol induces a plethora of protein expression throughout the nuclei of the limbic-hypothalamic circuit including the medial preoptic area (MPN), the bed nucleus of the stria terminalis (BST), posteriodorsal part of the medial nucleus of the amygdala (MeApd) and ventromedial nucleus of the hypothalamus (VMH). Indeed, this was convincingly demonstrated in a classic study, in which blocking protein synthesis in the hypothalamus prevented the display of lordosis behavior (150). This estrogenic transcriptional regulation is widespread including neuropeptides [e.g., CCK, enkephalins (123,127,158)], transmitter-synthesizing enzymes [e.g., GAD (139)] and receptors [e.g., opioid receptors, CCK receptors (151, 156, 162)]. There are also complexities in estradiol's

influence on peptide expression that include the opioid regulation of estrogen-induced CCK expression (52). Arguably the most critical expression for regulation of sexual receptivity (and perhaps reproduction) is the estradiol-induction of progesterone receptors [(12, 13); reviewed in (107)]. While estradiol-only induced sexual receptivity is not dependent on progesterone receptors, estradiol + progesterone induced behavior (the natural *in vivo* condition) requires activation of progesterone receptors (108). In addition to these more classically hormonal actions, lordosis behavior is also dependent on estrogen membrane-initiated signaling (EMS).

Estradiol membrane-initiated signaling

Within minutes of treatment with estradiol, an active inhibition of lordosis is initiated in the ARH (Fig. 2). Here, estradiol induces the release of neuropeptide Y (NPY) onto proopiomelanocortin (POMC) neurons expressing NPY-Y1 receptors and GABA$_B$ receptors (134) (190). These neurons project to the MPN where released β-endorphin (β-END) activates and induces internalization of μ-opioid receptors (MOR) (53). The MOR neurons in turn innervate VMH neurons regulating the descending output of the hypothalamus that controls lordosis behavior [for review, see (126)]. For approximately 20 h, this activation of MOR prevents the display of lordosis behavior. In the gonadally intact female, progesterone reverses the estradiol inhibition—allowing for lordosis (191).

The time course of estradiol-induced MOR activation along with the ability of a membrane-impermeable estradiol (E-biotin) introduced into the ARH to activate MOR indicated an EMS action (44). These results are consistent with the rapid estradiol inhibition of MPN neuronal firing (83) and the necessity of an opioid inhibition for full lordosis behavior (194, 207, 208). Thus, the induction of sexual receptivity requires both relatively rapid EMS and nuclear-initiated increase in protein expression. Such cooperation between EMS and nuclear estradiol signaling was demonstrated using estradiol in conjunction with E-BSA, another membrane-impermeable estradiol (84). In these experiments, pretreatment with E-BSA augmented the estradiol benzoate (EB)-induced lordosis behavior in ovx rats, which is consistent with the activation of a transient inhibitory circuit that allows for the full display of sexual receptivity behavior.

EMS-mediated lordosis behavior depends on ERα, which induces PKCθ phosphorylation in ARH and increases free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) in immortalized NPY neurons (45, 46, 130). Most putative membrane ERs implicated in membrane-initiated signaling share the ability to active G protein-coupled cascades (117, 128). For ERα on the membrane (mERα), this requires a transactivation of metabotropic glutamate receptor-1a (mGluR1a) (18), which initiates an intracellular signaling cascade involving Ca^{2+} release and a series of kinases, which can also ultimately modulate gene transcription (2, 27, 45, 89, 112, 142, 167, 185). Indeed, ERα transactivation of mGluR1a in the ARH is needed for the estradiol activation of MOR in the MPN and eventual lordosis behavior. Direct stimulation of mGluR1a, without mERα activation also results in MOR internalization and facilitation of lordosis behavior, further supporting a downstream role for mGluR1a in mERα-initiated signaling (44). Similarly, further evidence of this rapid estradiol signaling is that PKC stimulation in the ARH can overcome both ER and mGluR1a antagonism to stimulate lordosis behavior (45).

This set of experiments show that lordosis behavior, a classical assay of estradiol action, has an important EMS component and underscores the fundamental importance of ER-mGluR signaling in explaining estradiol actions in the neuraxis.

Though not within the scope of this review, it is worth noting that estradiol actions in ARH neurons may directly impact energy balance—a hypothalamic function with which NPY and POMC neurons are more commonly associated. Sex steroid modulation of appetite, satiety, and energy expenditure is incredibly complex, though estrogens have been shown to increase activity and depress appetite. Withdrawal of ovarian steroids is associated with increased body weight and early work demonstrated this to be associated with increased meal size (14). Estradiol acting through ERα appears to be the dominant player in ovarian steroid control of body weight, as ERα knockout mice have increased adiposity (65). The critical site for ERα activation in maintenance of body weight has not been determined, but is very likely to involve NPY and POMC neurons [see (20) for an in depth review of sex hormones and energy balance]. However, tract tracing studies indicate that ~10% of the ARH POMC neurons project to any single target (79). Our own observations indicate that the MPN-projecting POMC neurons are not the same as those that project to the paraventricular nucleus and lateral hypothalamus (unpublished observations, Dong and Micevych).

Dynamics of membrane ERα: Trafficking and internalization

Receptor levels on the cell membrane are not static. Levels of mERα are modified by the counteracting processes of trafficking to the membrane and removal from the membrane or internalization. To be trafficked to the cell membrane, ERα, a classic ligand-gated nuclear receptor, must be palmitoylated and complexed with caveolin-1 [CAV1 (111, 165)]. In the ARH, CAV1 knockdown does not affect intracellular levels of ERα, but significantly reduces levels of membrane ERα confirming that CAV1 is important for the trafficking of ERα to the membrane (30). Significantly, in animals with reduced mERα in the ARH, estradiol-induced lordosis behavior is attenuated, confirming that EMS is an important component of the estradiol actions in the ARH to induce sexual receptivity. To further study membrane ERα, we utilized both primary embryonic hypothalamic neuronal cultures and immortalized hypothalamic NPY expressing neurons (N-38). Interestingly, both the primary cultured neurons and the N-38s expressed full length membrane ERα (66 kDa) and a form of ERα translated from an alternatively spliced mRNA that is missing exon 4 (ERαΔ4) (46, 48). While the function of ERαΔ4 remains to be elucidated, it is the full-length ERα that interacts with mGluR1a to induce PKCθ phosphorylation in the N-38s. This is the same PKC isoform that is activated by EMS in the ARH, and on which EB-induced lordosis depends (45). Interestingly, estradiol-induced trafficking of ERα and ERαΔ4 to the membrane requires PKC activation (46). These results demonstrate the ability of estradiol to regulate its receptors on the membrane through which estradiol modulates its own signaling.

Internalization, the process opposing trafficking, can limit cellular responses initiated by agonist stimulation or adaptation to a persistent stimulus (i.e., desensitization). Internalization removes ligand-bound receptors from the cell membrane and sequesters them into early endosomes, where the receptor releases its ligand. We reasoned that such a

mechanism was critical to constrain estradiol signaling in the face of long lasting estradiol stimulation. The internalization mechanism involves activation of G protein-coupled receptor kinases (GRKs), which in turn phosphorylate membrane receptors, including mERα (37, 47). Subsequently, arrestins bind to the phosphorylated receptors, uncouple G proteins and link receptors to clathrin-dependent internalization pathways (32,101,132,217). Estradiol treatment of N-38 neurons results in rapid ERα and ERα 4 internalization that is counterbalanced by their trafficking to the membrane, but within several hours, mERα levels are depleted indicating a downregulation of this receptor. To establish that the initial internalization is due to arrestins, we first determined that both N-38 and the ARH have β-arrestin-1 (Arrb1) as the predominate arrestin. Then, we used siRNA to knock down Arrb1 and found that mERα and ERα 4 internalization was significantly attenuated. In fact, membrane levels were increased indicating that trafficking to the membrane was not compromised, only internalization, which we measured by tracking surface-biotinylated mERs (211).

In vitro, cell surface ERα and ERα 4 are downregulated by 2 h of estradiol treatment while *in vivo*, EMS is prolonged, lasting for more than 30 h. If internalization abrogates EMS, then the time course of internalization does not appear to match action of estradiol in the ARH to MPN circuit (191). A possible explanation is that internalized ERs continue signaling. This has been described for other sequestered membrane receptors where arrestins organize intracellular signaling through a scaffolding action to bind and organize downstream signaling molecules (e.g., Ras/Raf/MEK) (57, 92, 113, 132). To test whether EMS involved Arrb1 and whether internalized ERs continue to signal we tracked estradiol induced MAPK (ERK1/2) phosphorylation, *in vitro*. As suggested by the *in vivo* results, this signaling continued after mERα was internalized (211). Direct demonstration of the role of Arrb1 was obtained by siRNA knockdown that prevented both the initial EMS and endosomal signaling *in vitro*, and *in vivo* where Arrb1 knockdown in the ARH prevented estradiol-induced lordosis behavior (211). These results provide a mechanistic explanation of estradiol-only activation of female sexual receptivity. Physiological doses of EB require progesterone treatment to stimulate lordosis (103). These levels of EB maintain EMS, even after membrane levels of ERs are reduced. However, our results that internalized mER continues to signal suggests that estradiol continues to activate the opioid circuitry inhibiting sexual receptivity even after the receptors are missing from the cell membrane.

We still do not understand precisely how progesterone activation of neural signaling interacts with direct nuclear estradiol action and EMS, to modulate sexual receptivity. The importance of progesterone to sexual receptivity has been known for decades, and this was formally established by breeding mice with a null mutation in the progesterone receptor gene (100). These null PR mice were not able to show lordosis behavior following estradiol plus progesterone stimulation. Within the brain, progesterone actions are complex including their activation by dopamine through the D1 receptor (106). There is now intriguing evidence that progesterone activates a separate neural circuit to facilitate lordosis behavior in distinction to the circuit activated by estradiol-only facilitation of lordosis behavior. Following the initial inhibition, estradiol activates an opioid circuit that involves orphanin FQ/nociceptin (193). However, in estradiol primed females, progesterone activates another

circuit that coexists in the ARH and interacts with the lordosis inhibitory elements (176, 177, 189). Interestingly, progesterone facilitates sexual receptivity by suppressing activity of β-END neurons (183), but whether progesterone acts presynaptically or directly on these neurons has not been resolved. What is clear is that the progesterone activation of lordosis behavior requires activation of progesterone receptor isoform B and Src kinase, a member of the nonreceptor tyrosine kinase family. Within 30 minutes, progesterone infusions into the ARH facilitate lordosis behavior. These relatively rapid actions are blocked by antagonism of Src family kinases (183). At present, coimmunoprecipitation suggests a direct interaction of membrane-associated progesterone receptor-B with Src (153). Thus, the actions of estradiol and progesterone are relatively rapid, involving membrane-initiated signaling in different ARH circuits that converge on β-END neurons.

Control of Estrogen Negative Feedback

In addition to its role in female sexual receptivity, estradiol signaling is also important in regulation of gonadotropin release. During much of the estrous cycle, estradiol inhibits surge release of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) and maintains a tonic pattern of release from the hypothalamus and pituitary. Throughout most of the estrous cycle, LH levels are low but have an oscillatory pattern [e.g., (213)], due to the negative feedback of estrogens. Withdrawal of this feedback (e.g., following ovx) is marked by high LH levels. Estradiol negative feedback is dependent on ERα (49, 214), which is not expressed in GnRH neurons. Mounting evidence implicates neurons in the ARH, coexpressing ERα, kisspeptin (Kiss1), neurokinin B, and dynorphin [known as kisspeptin/neurokinin B/dynorphin (KNDy) neurons], as mediators of estrogen negative feedback [(28,114,135,137); reviewed in (93)].

Kisspeptin/neurokinin B/dynorphin neurons and estrogen negative feedback

KNDy neurons exhibit many features consistent with a role in mediating estrogen negative feedback. Importantly, KNDy neurons express ERα and project to GnRH neurons, criteria that are necessary for a population of neurons mediating estrogen negative feedback. GnRH neurons express GPR54 and NK_3R (85,204), the dominant receptors for kisspeptin and neurokinin B, respectively, providing a framework for communication between KNDy and GnRH neuronal populations. Two of the KNDy peptides, Kiss1 and neurokinin B, are critical for reproduction. In humans, mutations in the genes encoding Kiss1, neurokinin B, or their respective receptors, lead to hypogonadotrophic hypogonadism (41,182,205). This disorder is marked by infertility and failure to reach sexual maturation. Animals with impaired Kiss1 or neurokinin B signaling are also subfertile [(212); reviewed in (51)]. KNDy neurons are the only cells that coexpress these two neuropeptides, further implicating these neurons in reproductive function. Infusions of Kiss1 or NK_3R agonists modulate LH release although the direction of the change is not always consistent: while Kiss1 is reliably stimulatory, NK_3R agonists have variable effects (36, 56, 140, 179). These disparate data suggest that timing and quantities of neuropeptide release are critical factors for central control of reproductive physiology. Timing may be particularly critical in studies examining effects of NK_3R agonism because KNDy neurons, themselves, express NK_3R. Therefore, NK_3R agonists may affect inter-KNDy neuron communication in addition to their effects on

GnRH neurons, which may explain why Kiss1 (whose receptor has not been demonstrated in KNDy neurons) seems to consistently stimulate LH release: Kiss1 does not appear to affect KNDy neurons, whereas NK_3R agonists do (39, 62, 174). Indeed, factors such as physiological concentration, location, and precise timing of steroid or neuropeptide release are difficult to mirror using pharmacological approaches. Regardless, it is clear that Kiss1 and NKB play important roles in both tonic and surge LH release.

Congruent with their role as negative feedback mediators, estradiol suppresses Kiss1 and NKB gene expression in ARH KNDy neurons (1, 3, 38, 163, 164, 171, 196). Currently, the type of estradiol signaling (nuclear or EMS) regulating KNDy neurons has not been resolved, though evidence indicates that EMS may also be responsible for estrogen feedback in KNDy neurons (61). Previous studies have examined tissue from pre- versus postmenopausal women or ovx animals with prolonged exposure to estradiol. The long time course and the effect on transcription might lead one to assume that actions on Kiss1 and NKB are through classic nuclear signaling. However, these results do not exclude the possibility of membrane-to-nuclear estrogen signaling, since EMS activates CREB (2, 18). Clearly, more studies are needed to uncover these signaling pathways. The electrophysiological effects of steroids on KNDy neurons have provided some clues [reviewed in (155)]; however, the results are inconsistent. Some data show no increase in spontaneous firing rates in KNDy neurons following gonadectomy (28, 55, 174), while others do report a change (40). In ovx females, estradiol appears to suppress evoked excitability of KNDy neurons [(28) but see (7)]. The source of the variability between studies may be due to sex differences or the hormonal environment (ovx and ovx + estradiol vs. gonadally intact mice). Studies to date have examined the effects of long-term steroid exposure (intact or replaced) as opposed to acute stimulation with steroids. Whether estradiol has more acute, membrane-initiated effects in these neurons remains to be shown. Therefore, while it seems likely that KNDy neurons mediate estradiol negative feedback, much needs to be worked out regarding the nature of estradiol signaling in these neurons.

Control of Estrogen Positive Feedback

As reviewed recently, EMS plays an important role in the CNS synthesis of progesterone (neuroP) needed for estrogen positive feedback of the LH surge (90, 131). The preovulatory rise in circulating estradiol is essential for stimulating gonadotropin release (19, 54, 91), but preovulatory progesterone is also necessary for the LH surge (42,68,104,121,168). This is contrasted with the high levels of ovarian progesterone that follow ovulation, which participate in negative feedback (72). In ovx rats, exogenous estradiol alone will cause LH release (43). However, blocking progesterone receptors (PR) or progesterone synthesis prevents the estradiol-induced GnRH and LH surges in ovx rats (25), and arrests the estrous cycle in intact female rats [reviewed in (131)]. Therefore, it is clear that both estradiol and progesterone are necessary for surge release of LH.

Although the adrenal has been proposed as the source of preovulatory progesterone (21, 105, 160, 184), it is becoming clear that it is the locally synthesized progesterone that affects hypothalamic control of LH release. In ovx and adrenalectomized (ovx/adx) rats, the estradiol-induced LH surge is blunted but not blocked [(110, 121), but see (109)], supporting

a role for alternate sources of progesterone. Treatment with PR antagonist RU486 or P4 synthesis inhibitors (trilostane or epostane) blocks the estradiol-induced LH surge (42,68,104,121,178,198). In gonadally intact rats, hypothalamic neuroP synthesis is critical for initiating the LH surge [(121, 125, 133); reviewed in (129)]. When neuroP synthesis in the hypothalamus is blocked, the estradiol-induced LH surge is eliminated in ovx/adx rats, but the surge can be restored with exogenous progesterone (121, 146), suggesting that neuroP plays an important role downstream of estradiol.

Regulation of neuroP synthesis in astrocytes

Hypothalamic astrocytes locally synthesize neuroP, representing a critical step of estradiol-positive feedback on gonadotropin release. Astrocytes have the steroidogenic enzymes and transport proteins required to convert cholesterol to neuroP (180). In fact, astrocytes are the most steroidogenic cells in the CNS (220). Only in females and female-derived astrocytes will estradiol stimulate neuroP biosynthesis (87, 118). The rapid neuroP synthesis is not regulated by transcription; however, it is dependent on estradiol-induced $[Ca^{2+}]_i$ increase [(87–89, 122) but see (199)]. Using primary cultures of adult female hypothalamic astrocytes, we mapped the signaling pathway through which estradiol acts at the membrane to regulate neuroP synthesis (27). Like neurons, astrocytes express ERα, ERα 4, and mGluR1a on the cell membrane (15, 23, 89). Estradiol stimulation induces transactivation of mGluR1a leading to the production of inositol trisphosphate (IP$_3$), which stimulates the release of intracellular Ca^{2+} stores. Stimulating $[Ca^{2+}]_i$ even without estrogen treatment can stimulate neuroP synthesis (23, 124).

In contrast to the activation of PKC in neurons, estradiol activates protein kinase A (PKA) in astrocytes (27). The estradiol increase of $[Ca^{2+}]_i$ activates a Ca^{2+}-sensitive adenylyl cyclase-1 (AC1) producing cyclic AMP (cAMP) needed for phosphorylation of protein kinase A. This PKA mediates the rate-limiting step of steroid biosynthesis—cholesterol transport into the mitochondrial matrix through the activation of steroid acute regulatory protein (StAR) and translocator protein [TSPO (71,148,188)]. In the mitochondrion, cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage enzyme, P450scc (CYP11A1). The resulting pregnenolone is converted by 3-β-hydroxysteroid dehydrogenase (3-βHSD) to neuroP and secreted. Inhibition of any step of this pathway via antagonists to mERα, mGluR1a, IP$_3$ receptors, AC1, or PKA inhibits neuroprogesterone synthesis (27,88,89) (See Fig. 3).

RP3V Kiss1 neurons: Integrators of steroid input to the GnRH neuron

In addition to the ARH, Kiss1 expressing neurons are also found in the rostral periventricular nucleus, RP3V (an area including the anterior periventricular nucleus and AVPV). These neurons are prime candidates to integrate estradiol and progesterone information that regulates GnRH secretion underlying the LH surge. Although most of the work on steroid regulation of Kiss1 and its gene, KiSS-1 has focused on estradiol (80,197), there are interesting results that suggest estradiol and neuroP may interact to stimulate Kiss1 in the RP3V (136). First, both ERα and PR are needed for positive feedback of the LH surge (25, 26, 210), and both have been localized in RP3V Kiss1 neurons, but neither are found in GnRH neurons (67, 94, 186, 196, 218). A substantial number of Kiss1 neurons in RP3V

express PR after estradiol treatment (33, 195, 197, 218). During proestrus, rising estradiol levels induce neuroP synthesis, and Kiss1 expression in the RP3V. Kiss1 neurons innervate GnRH neurons that express the Kiss1 receptor, GPR54 (17, 35, 63, 66, 69, 81, 97, 143, 149, 172, 210) and Kiss1 is the most potent activator of GnRH neurons (33, 50, 63, 98, 154, 196, 216). Together, these findings indicate that Kiss1 neurons are presynaptic to GnRH neurons and are the primary modulators of GnRH neuronal activity [as reviewed in (33, 78)].

In a continuation of these studies, we showed that neuroP acts though Kiss1 to mediate estrogen positive feedback (see Fig. 3). Preliminary experiments demonstrate that exogenous progesterone rescues the LH surge in females where hypothalamic steroidogenesis was blocked with the CYP11A1 inhibitor aminoglutethimide [AGT (145, 146)]. Further, infusions of Kiss1 into the diagonal band of Broca (DBB) in AGT-treated animals induced an LH surge, confirming that Kiss1 operates downstream of neuroP. Finally, Kiss1 knockdown in the RP3V prevented the estradiol-induced LH surge. The nature of progesterone signaling in Kiss1 neurons remains to be elucidated. In addition to classical nuclear PR, there are intriguing suggestions that Kiss1 neurons may have membrane progesterone receptors, especially mPRβ (136, 219). The mPRs are 7-transmembrane domain proteins that belong to the progestin and adipoQ receptor (PAQR) family, not the classic G protein-coupled receptor (GPCR) family (138,201,203). PAQRs can signal through MAPK activation by increasing $[Ca^{2+}]_i$ [(9, 10, 64, 73, 138, 147, 202, 215); but see (86)]. It will be interesting to determine the role of these mPRs in reproduction in light of the demonstrated importance of classical PRs.

Sexual differentiation of neuroP synthesis in astrocytes

In rodents, the ability to have estrogen positive feedback is highly sexually differentiated, with males losing this ability upon exposure to testosterone during development [(60, 102, 141, 173) reviewed in (59) and (16)]. While both male and female rodents synthesize neuroP, estradiol-stimulated neuroP is specific to females *in vivo* (121). To determine whether hypothalamic astrocytes are sexually differentiated and whether gonadal steroid or chromosomal actions are responsible, astrocytes were isolated and cultured from wild-type male and female mice as well as from "Four Core Genotype" mice in which the chromosomal sex and the gonadal sex are uncoupled through the deletion of the sex determining region of the Y chromosome (*Sry*) from the Y chromosome and insertion onto an autosome [reviewed in (8)]. Regardless of the chromosomal sex, astrocytes from mice with testes did not respond to estradiol with an increase on neuroP synthesis, a finding consistent with our idea that astrocytes provide the necessary neuroP needed for positive feedback of the LH surge. While the mechanism through which testosterone blocks estradiol action in astrocytes has not been characterized, we have observed that only in astrocytes from gonadal females does estradiol increase mERα levels, which results in the $[Ca^{2+}]_i$ signaling required for neuroP synthesis. This indicates that sexual differentiation of astrocytes is mediated by sex steroid hormones and suggests that early exposure to testosterone affects the mechanism responsible for trafficking ERα to the cell membrane.

Conclusion

The studies reviewed here demonstrate that estradiol acts as both a classical steroid hormone and as a neurotransmitter in the CNS to regulate reproduction. It is clear that EMS is responsible for the estradiol stimulation of neuroP synthesis— a step needed for estrogen positive feedback. Interestingly, neuroP does not appear to affect sexually receptive behavior but it does stimulate proceptivity in rats (122). Specifics of estradiol signaling in both positive and negative feedback governing LH release remain to be elucidated. Further, the picture of how estrogens and progestins interact has recently grown more complicated with the discovery of membrane and membrane to nucleus signaling for estradiol and for progesterone. While much has been learned, much remains to be learned, especially about the actions of progesterone both in reproductive behavior and control of the LH surge.

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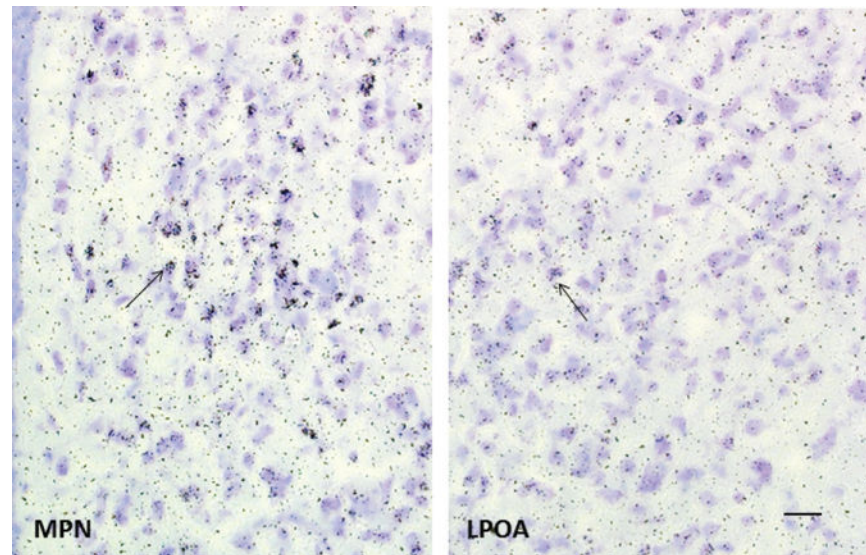
Figure 1.
Female rats were anesthetized with pentobarbital and injection (i.v.) with 100 μCi/100 g body weight of 2,4,6,7,16,17-[3H]estradiol (specific activity 130 Ci/mmol, New England Nuclear). At 2 h after injection of the isotope, animals were killed, brains removed and cryosectioned (10 μm) through the medial preoptic nucleus (MPN) and lateral preoptic area (LPOA). Sections were collected under safe-light conditions and thaw-mounted onto slides which had been coated with nuclear track emulsion and exposed at −70°C for 28 weeks, then photodeveloped. The MPN contains many cells that concentrate 3H-estradiol as indicated by the accumulation of silver grains over nuclei (arrow). In the LPOA, some cells have accumulations of silver grains (arrow) but levels are much lower in both the estradiol-accumulating cells and the surrounding background compared with the MPN. Bar in LPOA is 20 μm and applies to both images. Sections are adapted, with permission, from a study by Akesson TR and Micevych PE (4).
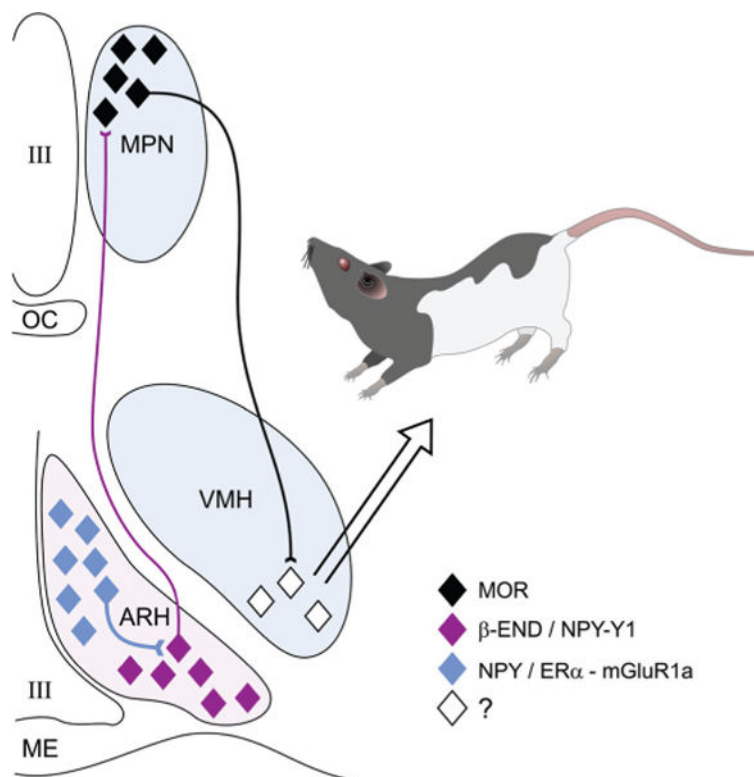
Figure 2.
The estradiol (E2) induction of sexual receptivity in the female rat is indicated by lordosis behavior. The CNS regulation of this global response to hormonal and sensory input is regulated by a diffuse circuit that extends from the limbic system to the spinal cord. Within this lordosis regulating circuit, E2 acts rapidly through estrogen membrane signaling (EMS) to release neuropeptide Y (NPY) in the arcuate nucleus of the hypothalamus (ARH), which activates β-endorphin (β-END) projection neurons that extend to the medial preoptic nucleus (MPN). The MPN is an important integrative node receiving accessory olfactory and limbic input. β-END activates μ-opioid receptors (MOR), producing a transient inhibition of the MPN which is relieved by progesterone in the cycling female. The MPN MOR neurons in turn project to the ventromedial nucleus of the hypothalamus (VMH), the final common output of the hypothalamus. The integrated hypothalamic output is modified by inputs from the periaquaductal gray, and the vestibular complex on its way to the motoneurons mediating lordosis behavior. The EMS that mediates this activation of the circuit requires the transactivation of metabotropic glutamate receptor-1a (mGluR1a), which leads to the phosphorylation of PKCθ and the release of NPY and activation of the Y1 receptor on β-END projection cells. The EMS and resulting transient inhibition is necessary for the full expression of lordosis behavior in the rat [adapted, with permission, from (116)].
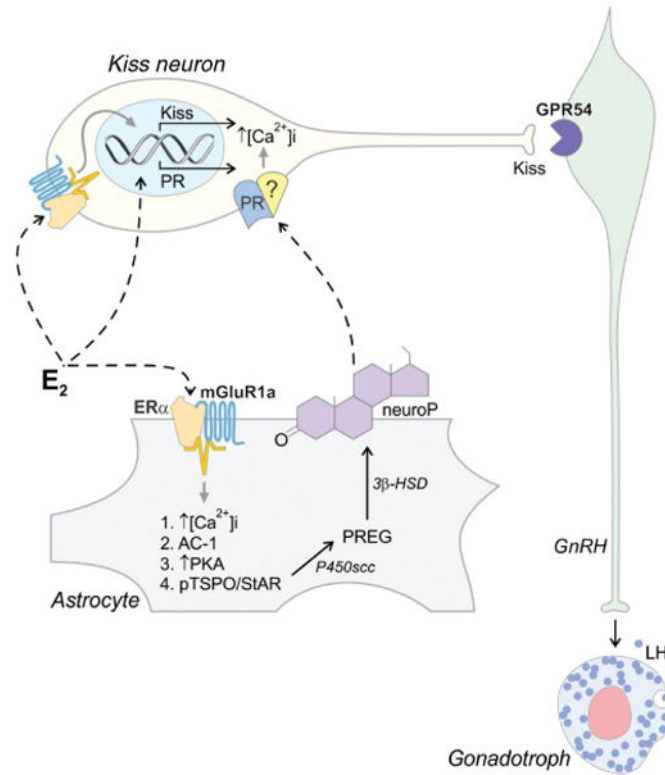
Figure 3.
A model showing proposed estradiol (E2) actions on hypothalamic cells. In Kiss1 neurons, E2 acts at both membrane and nuclear estrogen receptors. During diestrus, classical nuclear E2 signaling induces PR expression in kisspeptin (Kiss1) neurons in the RP3V. On proestrus, rising E2 leads to transactivation of mGluR1a in astrocytes, which increases $[Ca^{2+}]_i$ leading to stimulation of a kinase cascade resulting in the activation of translocator protein (TSPO) and steroid acute regulatory protein (StAR), which results in an increase of cholesterol import into the mitochondrion—the rate limiting step in steroidogenesis. The resulting pregnenolone (PREG) is converted to progesterone (neuroP) by 3β-HSD. Simultaneously, E2 activates an ERα-mGluR1a complex in neurons leading to the expression of Kiss1. Newly synthesized neuroP diffuses out of the astrocytes and activates E2-induced PR, which have been trafficked to the Kiss1 neuronal membrane. This leads to a series of events culminating in Kiss1 secretion onto GPR54 expressing GnRH neurons. We hypothesize that signaling through the membrane PR involves transactivation of another receptor (indicated by [?]) to stimulate $[Ca^{2+}]_i$, and induce Kiss1 release, activating GnRH neurons and triggering the E2-induced LH surge from anterior pituitary gonadotrophs.