



The neurosteroid progesterone underlies estrogen positive feedback of the LH surge

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Our understanding the steroid regulation of neural function has rapidly evolved in the past decades. Not long ago the prevailing thoughts were that peripheral steroid hormones carried information to the brain which passively responded to these steroids. These steroid actions were slow, taking hours to days to be realized because they regulated gene expression. Over the past three decades, discoveries of new steroid receptors, rapid membrane-initiated signaling mechanisms, and *de novo* neurosteroidogenesis have shed new light on the complexity of steroids actions within the nervous system. Sexual differentiation of the brain during development occurs predominately through timed steroid-mediated expression of proteins and long term epigenetic modifications. In contrast across the estrous cycle, estradiol release from developing ovarian follicles initially increases slowly and then at proestrus increases rapidly. This pattern of estradiol release acts through both classical genomic mechanisms and rapid membrane-initiated signaling in the brain to coordinate reproductive behavior and physiology. This review focuses on recently discovered estrogen receptor- α membrane signaling mechanisms that estradiol utilizes during estrogen positive feedback to stimulate *de novo* progesterone synthesis within the hypothalamus to trigger the luteinizing hormone (LH) surge important for ovulation and estrous cyclicity. The activation of these signaling pathways appears to be coordinated by the rising and waning of estradiol throughout the estrous cycle and integral to the negative and positive feedback mechanisms of estradiol. This differential responsiveness is part of the timing mechanism triggering the LH surge.

Keywords: estrogen receptor, ER α Δ 4, membrane estradiol signaling, receptor trafficking

INTRODUCTION

For decades the prevailing concept was that the brain passively responded to steroids originating from the periphery. These hormones were thought to mediate feedback mechanisms and regulate reproductive circuits. Although the brain does respond directly to ovarian and adrenal steroids, over the years this straight-forward notion has been drastically modified. The nervous system does not just passively respond to peripheral steroids. It actively metabolizes peripheral steroids to biologically active steroids that regulate numerous brain functions. For example, circulating testosterone can be aromatized to estradiol, or reduced to dihydrotestosterone (DHT) and further to 3β - or 3α -diols that are important in regulating behavior, negative feedback, and sexual differentiation (Lavaque et al., 2006a; Higo et al., 2009). Further, peripheral progesterone can be metabolized to allopregnanolone (3β -hydroxy- 5α -pregnan-20-one). Many of these neurally converted metabolites are ligands for nuclear receptors that are transcription factors that modulate gene expression (Roselli et al., 2009). In contrast, other steroid metabolites allosterically bind to and modulate membrane receptor activity. The most notable of this type is allopregnanolone, which activates the GABA_A receptor (Majewska et al., 1986; Puia et al., 1990; Hosie et al., 2006; Herd et al., 2007; Mitchell et al., 2008).

A surprising discovery was that nervous tissue synthesizes steroids *de novo* to regulate neural functions and behavior (Baulieu, 1981, 1991, 1998; Mellon, 1994; Schumacher et al., 2003). The steroids synthesized in the nervous system are referred to as neurosteroids (Baulieu, 1981, 1991), and are produced in astrocytes, oligodendrocytes, and neurons. The proteins and enzymes required for *de novo* steroidogenesis of estradiol from cholesterol are present in the brain. As in the ovary, multiple cell types cooperate in steroidogenesis. The most prevalent neurosteroids are pregnenolone, progesterone, and allopregnanolone (Corpechot et al., 1993), which are synthesized in astrocytes (Zwain and Yen, 1999).

Neurosteroidogenesis studies localized steroidogenic enzymes, enzymatic activity, and transporter proteins in expected and unexpected regions of the nervous system (Follesa et al., 2000; Wehrenberg et al., 2001; Lavaque et al., 2006b). Because of their highly localized synthesis and relatively low levels compared to circulating steroids, measuring, and determining the roles of neurosteroids in physiological, behavioral, and feedback systems has been difficult. In peripheral nerves, neurosteroids are implicated in myelination (Schumacher et al., 2000, 2003, 2004), and have therapeutic actions in the treatment of epilepsy and traumatic brain injury (Dubrovsky, 2005; Morrow, 2007; Reddy and Rogawski, 2009).

Recently, roles for neurosteroids in reproductive physiology have been defined. Immortalized murine gonadotropin releasing hormone (GnRH) neurons, GT1-1 cells, converted progesterone to allopregnanolone, and stimulated the release of GnRH by activating the GABA_A receptor (el-Etr et al., 1995). Another important milestone for understanding the physiology of neurosteroids was demonstrating that the actions of peripheral steroids are integrated with the actions of neurosteroids. This review focuses on this integration controlling ovulation.

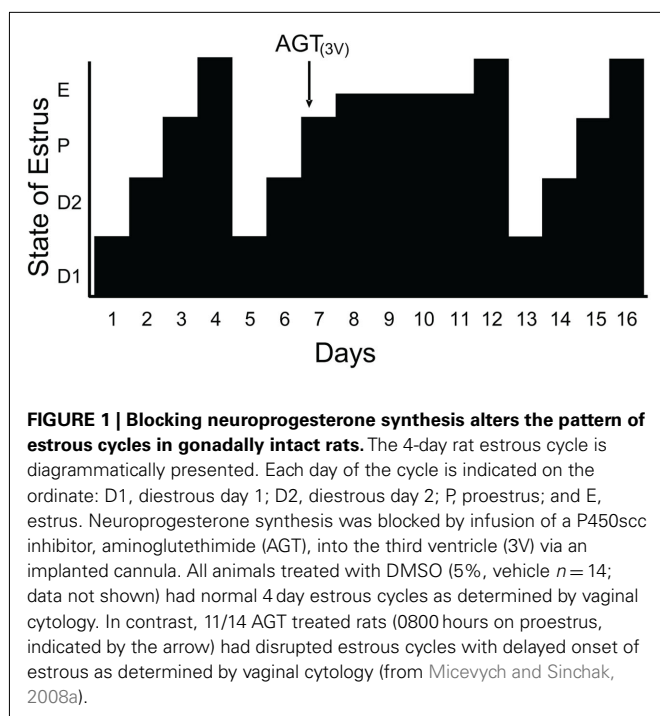
ROLE OF NEUROPROGESTERONE AND ITS METABOLITES IN FEMALE REPRODUCTION

Ovulation, a critical event in mammalian reproduction, is initiated by elevated levels of estradiol released by maturing ovarian follicles which act on the hypothalamus and pituitary. This event, known as estrogen positive feedback, produces a surge of GnRH from the hypothalamus that stimulates the secretion of luteinizing hormone (LH) from the pituitary. A puzzling aspect of estrogen positive feedback is that estrogens which are inhibitory to hormone release from the hypothalamus and pituitary gland during most of the cycle (negative feedback) now stimulate these tissues to induce a surge of hormone release, especially the neural network that controls the GnRH neurons (Chazal et al., 1974). Sequential estradiol and progesterone stimulation of the hypothalamus is essential for estrogen positive feedback to culminate in the LH surge (Chappell and Levine, 2000; Micevych et al., 2003, 2008; Micevych and Sinchak, 2008b). A key step in positive feedback is the estradiol-induced expression of hypothalamic progesterone receptors (PRs; Brom and Schwartz, 1968; Ferin et al., 1969; Labsetwar, 1970; Rao and Mahesh, 1986; Mahesh and Brann, 1998b). Specifically, transcription and activation of PR-A in the hypothalamus are obligatory events in the induction of the GnRH and LH surges in estradiol-primed, ovariectomized (OVX) rats (Chappell and Levine, 2000).

For several years, it was not clear how PR-A was activated. One proposed mechanism was ligand-independent activation of PR that did not require progesterone (Mani et al., 1994, 1996; Mani, 2006). In place of progesterone, dopamine acting through the DA₁ receptor would activate PR. Another hypothesized mechanism was that pre-ovulatory progesterone of adrenal origin activated PR. Both the ovary and the adrenal cortex, highly steroidogenic organs, are capable of producing progesterone needed for the LH surge (Mahesh and Brann, 1998a). However, evidence for a peripheral source of progesterone is lacking. A significant rise in progesterone has not been consistently seen in the systemic circulation prior to the LH surge indicating that the progesterone required for the LH surge may not be synthesized peripherally (Feder et al., 1971; Kalra and Kalra, 1974; Smith et al., 1975). Consistent with this idea is that neither the adrenals nor the ovaries are necessary for an estrogen-induced LH surge (Mann et al., 1976; Sridaran and Blake, 1980). Indeed, OVX and adrenalectomized (ADX) rats primed with only 17 β -estradiol have a robust LH surge (Mann et al., 1976; Micevych et al., 2003). However, inhibiting the enzyme 3 β -hydroxysteroid dehydrogenase (3 β -HSD) that converts pregnenolone to progesterone in ADX/OVX rats prevents estradiol induction of the LH surge, indicating that progesterone is needed for estrogen positive feedback (Micevych et al., 2003).

Since estradiol induces an LH surge in OVX/ADX rats and blocking progesterone signaling inhibits estradiol-induced LH surges, we hypothesized that the source of this progesterone was the brain. The steroidogenic capacity of the brain has been well-established (Baulieu, 1981, 1991; Corpechot et al., 1981; Le Goascogne et al., 1987; Jung-Testas et al., 1989a,b; Mellon and Deschepper, 1993; Mellon, 1994; Guennoun et al., 1995; Koenig et al., 1995; Sanne and Krueger, 1995). Our investigations demonstrate that the enzymes and carrier proteins are present and that estradiol regulates them in a dose and time dependent manner that is congruent with estradiol levels across the estrous cycle. PCR analysis confirmed that hypothalamic tissue *in vivo* expresses mRNA for cytochrome P-450 side-chain cleavage (P450_{scc}) and 3 β -HSD, as well as the cholesterol transport protein steroidogenic acute regulatory protein (StAR) – all of the proteins needed to synthesize progesterone from cholesterol (Soma et al., 2005). Like PR, estradiol primes the neurosteroidogenic pathways. Estradiol increased hypothalamic expression 3 β -HSD with a time course that would allow for neuroprogesterone synthesis to occur hours prior to the LH surge and act through estradiol-induced PRs (Soma et al., 2005). The importance of the steroidogenesis in the brain for the LH surge and estrous cycle was demonstrated in gonadally intact cycling rats. On the morning of proestrus, steroidogenesis in the hypothalamus was blocked by infusing aminoglutethimide (AGT), a P-450 side-chain cleavage (P450_{scc}) enzyme inhibitor into the lateral ventricle. Although peripheral levels of estradiol in vehicle and AGT treated rats were similar, the estrous cycle of AGT female was arrested in proestrus and levels of progesterone in the hypothalamus and circulation remained low compared to controls. In AGT treated rats, the uterus was swollen and fluid filled, the ovaries had numerous developing follicles but no forming corpus luteum – all indications that peripheral steroidogenesis was intact. In spite of the typical proestrus rise in circulating estradiol, blocking neurosteroidogenesis prevented the LH surge, ovulation, and luteinization and arrested progression of the estrous cycle (Micevych and Sinchak, 2008a; **Figure 1**). After several days the effects of AGT wore off and the rats exhibited vaginal cytology of estrus, indicative of the LH surge, and resumed their regular 4–5 day estrous cycles. These data strongly suggest that estradiol stimulates hypothalamic neuroprogesterone synthesis, which is essential in mediating the positive feedback regulation of the LH surge.

It is always interesting to speculate how results in model species, especially rodents, illuminate the mechanism regulating the LH surge and ovulation in women. Typically, circulating levels of progesterone are low to undetectable in most primates and it is only after ovulation and luteinization that circulating levels of progesterone rise. In primates, as in rodents, pre-ovulatory progesterone regulates the LH surge. In women, although estradiol alone can induce a LH surge (Leyendecker et al., 1972; Monroe et al., 1972; Yen and Tsai, 1972), treatment with the PR antagonist, RU 486, or inhibitors of progesterone synthesis attenuate the LH surge and prevent ovulation (Collins and Hodgen, 1986; Liu et al., 1987; Shoupe et al., 1987; Batista et al., 1992; Croxatto et al., 1993). These responses in women and non-human primates are consistent with our findings that estrogen positive feedback induces a hypothalamic source of pre-ovulatory progesterone that



activates neural networks that regulate GnRH neuronal activity triggering the LH surge. In numerous species progesterone also positively regulates pituitary responsiveness to gonadotropes during estrogen positive feedback (Liu and Yen, 1983; Messinis and Templeton, 1990). In line with this idea, women treated with RU 486 exhibit an attenuated release of LH in response to exogenous GnRH (Kazem et al., 1996). Together these results indicate that both central and pituitary sites of progesterone action to facilitate estrogen positive feedback. It is likely that the neuroprogesterone synthesized in the hypothalamus could access the pituitary via the hypothalamo-pituitary portal circulation to modulate coordinate pituitary responsiveness with neural portion of the feedback loop.

ESTRADIOL ACTS ON ASTROCYTES

Astrocytes are responsive to various neuroactive messengers (Hosli and Hosli, 1992; Hirst et al., 1998; Chaban et al., 2004; Oka et al., 2006). Among these modulators of astrocyte function is estradiol which profoundly influences their morphology and function (Mong and Blutstein, 2006), sexual differentiation (McCarthy et al., 2002), and steroidogenesis (Sinchak et al., 2003; Micevych et al., 2007). Astrocytes in turn regulate numerous hypothalamic processes including regulation of releasing factors (Cavarretta et al., 1999; Galbiati et al., 2002; Zwain et al., 2002; Cashion et al., 2003; Mahesh et al., 2006) and synthesis of neurosteroids (Akwa et al., 1993; Jung-Testas et al., 1999; Zwain and Yen, 1999; Zwain et al., 2002). Astrocytes have all the steroidogenic enzymes required to convert cholesterol directly to progesterone (P450_{sc}c and 3 β -HSD), and the transport proteins StAR and peripheral benzodiazepine receptor/translocator protein (PBR/TSPO; Schumacher et al., 2004), and are the main source of neuroprogesterone produced within the hypothalamus (Zwain and Yen, 1999; Micevych et al., 2003, 2007, 2008). As with granulosa cells of the ovary,

astrocytes express estrogen receptor- α (ER α) and ER β , providing a potential avenue for estradiol regulation of neuroprogesterone steroidogenesis (Azcoitia et al., 1999; Garcia-Segura et al., 1999; Buchanan et al., 2000; Chaban et al., 2004; Pawlak et al., 2005; Quesada et al., 2007).

Both intracellular and membrane-associated ERs have been found in astrocytes. However, the regulation of progesterone synthesis in astrocytes is mediated through estradiol membrane signaling (EMS) that has been described in neurons and peripheral tissues (Szego and Davis, 1967; Kelly et al., 1976; Lagrange et al., 1995; Razandi et al., 1999; Filardo et al., 2000; Ivanova et al., 2001; Wade et al., 2001; Qiu et al., 2003; Chaban et al., 2004; Acconcia et al., 2005; Pawlak et al., 2005; Revankar et al., 2005; Thomas et al., 2005; Mhyre and Dorsa, 2006; Pedram et al., 2006, 2007; Bondar et al., 2009; Hirahara et al., 2009; Kuo et al., 2009). As in neurons, estradiol can influence cell signaling in astrocytes, which express ER α and ER β both intracellularly and on the plasma membrane (Garcia-Segura et al., 1999; Chaban et al., 2004; Pawlak et al., 2005; Quesada et al., 2007). Activation of the membrane ER (mER) with estradiol or a membrane impermeable construct (estradiol-coupled to bovine serum albumin, E-6-BSA) initiates a rapid intracellular calcium concentration ($[Ca^{2+}]_i$) increase via activation of the phospholipase C/inositol trisphosphate (PLC/IP₃) pathway that releases intracellular stores of calcium from the smooth endoplasmic reticulum in neurons and astrocytes (Beyer and Raab, 1998; Chaban et al., 2004; Micevych et al., 2007). This rise in $[Ca^{2+}]_i$ stimulates the *de novo* synthesis of progesterone in post-pubertal hypothalamic astrocytes within 5 min (Micevych et al., 2003, 2007; Kuo et al., 2010). The calcium hypothesis was tested by thapsigargin, a potent Ca²⁺-ATPase inhibitor that rapidly releases IP₃-sensitive Ca²⁺ stores from the smooth endoplasmic reticulum. This thapsigargin-induced massive release of Ca²⁺ was similar in magnitude to estradiol stimulation and within minutes resulted in progesterone synthesis (Micevych et al., 2007). Likewise, subnanomolar doses of estradiol were sufficient to induce rapid $[Ca^{2+}]_i$ release and progesterone synthesis in cultured hypothalamic astrocytes within minutes (Kuo et al., 2009). While the estradiol increase in $[Ca^{2+}]_i$ was dose-dependent, the synthesis of progesterone displayed a “step function” responding to physiological levels of estradiol that are reached during the proestrus surge (Butcher et al., 1974; Hawkins et al., 1975; Shaikh and Shaikh, 1975). The threshold response to estradiol is consistent with the idea that stimulation of neuroprogesterone synthesis is part of an “off-on-off” mechanism regulating the transition from estrogen negative feedback to estrogen positive feedback, and back again – at least in rodents (Kuo et al., 2010). Thus, as estradiol rises with developing ovarian follicles, it gradually increases levels of $[Ca^{2+}]_i$ in astrocytes; however, only when estradiol levels peak, consistent with mature follicles, does the $[Ca^{2+}]_i$ reach a critical threshold for stimulating progesterone synthesis. This response prevents hypothalamic progesterone levels from rising too early, resulting in a premature LH surge before ovarian follicles are fully mature and ready to ovulate.

MEMBRANE ESTROGEN RECEPTOR SIGNALING

Understanding the timing and mechanisms involved in estradiol signaling has been made more difficult by discoveries of several

proteins that act as membrane-associated ERs: ER α , ER β , ER-X (Toran-Allerand, 2000; Toran-Allerand et al., 2002), STX-activated protein called mER-G α q (Qiu et al., 2003, 2006), and GPR30 (Filardo et al., 2000, 2002; Revankar et al., 2005; Thomas et al., 2005). Although neurosteroidogenesis was activated by E-6-BSA, it was blocked by the ER α and ER β antagonist, ICI 182,780, suggesting that this rapid signaling was mediated by these receptors (Chaban et al., 2004; Micevych et al., 2007). In the context of reproductive neuroendocrinology, the evidence is most robust for ER α as the critical membrane-associated ER. Mouse astrocytes from ER α KO mice had a significantly attenuated estradiol-induced [Ca²⁺]_i response (Kuo et al., 2010), which was mirrored *in vivo*. Priming OVX ER α KO mice with estradiol failed to significantly increase hypothalamic progesterone levels as observed in OVX wild-type mice (Kuo et al., 2010). Most dramatically, ER α was shown to be vital for estrogen positive feedback when mice in which the ER α was deleted from neurons failed to have a LH surge and ovulate (Wintermantel et al., 2006).

Classic nuclear receptors ER α (and ER β) have been shown to associate with the plasma membrane through a variety of methods. ER over expression demonstrated that a population of these nuclear proteins are targeted to the plasma membrane (Razandi et al., 1999) where they have been localized with immunohistochemistry, western blotting, and surface biotinylation (Lagrange et al., 1995, 1996; Razandi et al., 1999; Ivanova et al., 2001; Wade et al., 2001; Chaban et al., 2004; Acconcia et al., 2005; Pawlak et al., 2005; Mhyre and Dorsa, 2006; Pedram et al., 2006; Bondar et al., 2009; Dominguez et al., 2009; Hirahara et al., 2009; Kuo et al., 2009; Sakuma et al., 2009; Dominguez and Micevych, 2010).

Membrane ERs were demonstrated in hypothalamic astrocytes and neurons using membrane-impermeable constructs such as E-6-BSA-FITC (1,3,5(10)-estratrien-3,17 α -diol-6-one-bovine serum albumin-fluorescein isothiocyanate) and E-6-biotin (1,3,5(10)-estratrien-3,17 α -diol-6-one-6-carboxymethoxime-NH-propyl-biotin; Dominguez et al., 2009; Micevych and Dominguez, 2009; Micevych et al., 2010). EMS activates G protein-dependent cell signaling cascades (Hammes and Levin, 2007), including activation of the mitogen-activated protein kinase (MAPK) pathway, activation of protein kinase C (PKC), increasing [Ca²⁺]_i, and phosphorylation of cAMP-responsive element binding protein (CREB; Dewing et al., 2007, 2008; reviewed by Kelly and Ronnekleiv, 2008). An issue with ER α mediating EMS is that ER α is a transcription factor and does not have the canonical structure of a G protein-coupled receptor. An elegant solution was the proposed mechanism in which ligand-activated mER α or mER β transactivated metabotropic glutamate receptors (mGluRs) to stimulate PLC/IP₃ – MAPK pathways leading to the activation of CREB (Boulware et al., 2005; Dewing et al., 2007). These signaling cascades have been implicated in the estradiol activation of lordosis behavior through neuropeptide expression and receptor activation (Watters and Dorsa, 1998). In astrocytes the interaction of mER α and mGluR1a was necessary to mediate estradiol signaling at the membrane. The increase in [Ca²⁺]_i and progesterone synthesis were blocked with the mGluR1a antagonist LY 367,385 (Kuo et al., 2009, 2010). This physiologic evidence was reinforced by the observation that ER α and mGluR1a co-immunoprecipitate in hypothalamic astrocytes (Kuo et al., 2009, 2010). Significantly,

ER β did not co-immunoprecipitate with mGluR1a, supporting the idea that of ER α is the mER mediating estradiol induction of progesterone synthesis in hypothalamic astrocytes.

In these *in vitro* experiments, we observed an interesting phenomenon. Although glutamate was not needed for estradiol transactivation of mGluR1a, activation of mGluR1a augmented the estradiol response (Kuo et al., 2009, 2010). These results demonstrated that estradiol would be maximally effective at stimulating astrocytes (and progesterone synthesis) when extracellular glutamate was elevated implying that estradiol signaling is strengthened in areas undergoing excitatory neural activity involving glutamate. Under such circumstances, astrocytes are a site of neural–hormonal integration. Indeed, middle-aged females exhibit reduced excitation of GnRH neurons and attenuated LH surges under estrogen positive feedback conditions, in part, due to decreased glutamate neurotransmission in the medial preoptic area, demonstrating the importance of local modulatory effects on estrogen positive feedback, the LH surge, and reproductive function (Neal-Perry et al., 2005, 2009). Further, it appears that menopause may be induced by the loss of estradiol-induced neuroprogesterone synthesis. As rats enter menopause, estradiol levels increase to intermediate level, though not reaching the estrogen positive feedback levels. However, the elevated estradiol levels are maintained for an extended period of time. The rat is in an extended proestrus state, similar to that induced by AGT infused into the lateral ventricle. Treating the menopausal rats with progesterone induces the LH surge, indicating that the brain is not synthesizing progesterone in response to elevated estradiol but remains responsive to progesterone (Lu et al., 1994; Mills et al., 2002). Interestingly, these females are in constant behavioral estrus and the LH surge can be induced by copulating with a male. Thus, female rats appear to become reflex ovulators as they reach their final stages of reproduction. Then after several months of acyclicity, exogenous progesterone can no longer induce the LH surge because estradiol is incapable of inducing PR expression as well as neuroprogesterone synthesis (Mills et al., 2002).

If ER α is also responsible for EMS, does it have the characteristics of other membrane receptors: present on the surface, trafficked to the membrane, and internalized after stimulation with its ligand? Surface biotinylation studies with hypothalamic astrocytes demonstrated that ER α is located on the cell surface, trafficked to the membrane, and internalized by estradiol treatment. Moreover, the trafficking and internalization are dependent upon mGluR1a, and blocked by mGluR1a antagonism with LY 367,385 (Bondar et al., 2009). Similarly in hypothalamic neurons, estradiol treatment significantly increased the internalization of mGluR1a in parallel with ER α , and that the trafficking is dependent on caveolin (Boulware et al., 2007; Dominguez and Micevych, 2010) further supporting the idea of an ER α -mGluR1a signaling unit for EMS (Dominguez and Micevych, 2010).

These experiments revealed that estradiol regulates its own membrane-initiated signaling. Such autoregulation of signaling, for example helps to explain observations *in vivo* of the transient effect of EMS that affect sexual receptivity and progesterone synthesis (Sinchak and Micevych, 2001, 2003; Micevych et al., 2003; Dewing et al., 2007; Kuo et al., 2009, 2010). Estradiol rapidly, within 5 min, induces mER α trafficking to the plasma membrane

(Bondar et al., 2009). Peak mER α levels were achieved by 30 min. Once on the membrane, estradiol activates ER α and induces internalization. mER α trafficking and internalization is blocked by either the ER antagonist ICI 182,780, or the mGluR1a antagonist LY 367,385, verifying ER α -mGluR1a signaling (Bondar et al., 2009). As expected, trafficking to the membrane and internalization occurred in parallel, with the peak of internalization also at 30 min. A possible mechanism for insertion is the fusion of mER α -mGluR1a containing vesicles with the cell membrane. This idea is supported by the presence of ER α immunoreactive vesicles in the hippocampus and pituitary (Gonzalez et al., 2008). Internalization, like that of other membrane receptors is probably mediated by clathrin-coated endocytic vesicles, which retrieve ligand-bound receptors (Micevych and Dominguez, 2009). Once receptors are internalized, the vesicles fuse with early endosomes and release their ligand. At this juncture, the receptors can either be recycled to the cell surface or degraded (Sinchak and Micevych, 2003; Micevych and Dewing, 2011). Degradation wins out over recycling after a continuous 1–2 h exposure to estradiol, which leads to a reduction of both surface mER α and internalized mER α . The loss of mER α (i.e., down regulation) is a mechanism by which estradiol can temporally limit EMS (Bondar et al., 2009; Dominguez and Micevych, 2010).

ANOTHER mER α ?

An unexpected finding of the biotinylation studies was the presence of a 52- to 55-kDa ER α immunoreactive membrane protein. This protein was also found in hypothalamic neuronal membranes and is the product of an alternatively spliced ER α mRNA in which exon 4 is deleted (ER $\alpha\Delta 4$; Dominguez et al., submitted). Initially, Crews and colleagues reported such an ER α mRNA (Skipper et al., 1993). They reported that ER $\alpha\Delta 4$ was very abundant in the brain. Since then, ER $\alpha\Delta 4$ mRNA has been reported in the brain, pituitary, and breast (Fuqua et al., 1992; Bollig and Miksicek, 2000; Deecher et al., 2003; Perlman et al., 2005). We confirmed the presence of the ER $\alpha\Delta 4$ mRNA. The resulting protein is missing the nuclear localization signal helping to explain the large quantity seen in the membrane (Pasqualini et al., 2001; Bondar et al., 2009; Dominguez and Micevych, 2010; Roepke et al., 2010). The function of the ER $\alpha\Delta 4$ is unclear since the deletion of exon 4 affects the ligand binding domain, calling into question its estradiol dependence for signaling. Further, only the full-length 66 kDa ER α co-immunoprecipitated with mGluR1a, which in astrocytes is needed for the estradiol-induced $[Ca^{2+}]_i$ release and progesterone synthesis (Kuo et al., 2009, 2010).

OTHER ERs

In addition to mER α , several other candidate mERs have been proposed (Filardo et al., 2000; Toran-Allerand, 2000; Qiu et al., 2003). GPR30 is a G protein-coupled receptor that activates adenylyl cyclase in breast cancer cells lacking ER α and ER β (Filardo et al., 2000, 2002; Revankar et al., 2005; Thomas et al., 2005). FLAG- and hemagglutinin-tagged GPR30 have been reported at the plasma membrane (Funakoshi et al., 2006; Filardo et al., 2007), but GPR30 could not be identified at the plasma membrane or labeled with surface biotinylation in native astrocytes or neurons (Gorosito et al., 2008; Bondar et al., 2009; Kuo et al., 2010). The GPR30

agonist G-1, a substituted dihydroquinoline (Bologa et al., 2006), stimulated $[Ca^{2+}]_i$ release and progesterone synthesis (Kuo et al., 2010); however, G-1 had a dose response curve that was different from estradiol or PPT, the selective ER α agonist. Further, since G-1 ability to stimulate $[Ca^{2+}]_i$ release was not blocked by antagonizing mGluR1a (Kuo et al., 2010). This also indicates that GPR30 uses a different signaling mechanism compared to estradiol. The lack of interaction between GPR30 and mGluR1a was confirmed by the absence of co-immunoprecipitation between these proteins (Kuo et al., 2010). While it is difficult to understand the discrepancy with GPR30 localization on the plasma membrane, our results can be interpreted to support the observation of estradiol activation of intracellular GPR30 on the endoplasmic reticulum (Revankar et al., 2005). Activation of GPR30 may directly induce the release of intracellular stores of Ca^{2+} , which in turn stimulates progesterone synthesis.

Another candidate receptor is a membrane-associated binding protein that is G α_q -coupled and activated by estradiol as well as STX, a diphenylacrylamide selective estrogen receptor modulator (SERM; Qiu et al., 2003). STX activity is retained in double ER α /ER β knock-out mice, but blocked with ICI 182,780 (Qiu et al., 2006). STX does not activate ER α or ER β and has a six order of magnitude lower binding affinity compared with estradiol for these receptors (Qiu et al., 2003). Unfortunately, this mER-G α_q has much lower affinity for estradiol (~20-fold) compared with STX (Qiu et al., 2006). However, this STX-activated protein also activates PLC (Qiu et al., 2003), a signaling pathway similar to that activated by ER α -mGluR1a. In hypothalamic astrocytes, STX increased $[Ca^{2+}]_i$ and progesterone synthesis through transactivation of mGluR1a (Kuo et al., 2010). It has been suggested that STX signals through GPR30, such that small interfering RNA directed against GPR30 abolished the STX-induced transcription (Lin et al., 2009). However, estradiol has been reported to activate the mER-G α_q signaling pathway in GPR30 knock-out mice (Qiu et al., 2008). In our hands, STX and G-1 produced distinctly different responses in hypothalamic astrocytes (Kuo et al., 2010). Although estradiol and STX responses are blocked by mGluR1a antagonism and activate the same PLC pathway, these actions are mediated through different receptors since STX stimulation of Ca^{2+} release remains in astrocytes from ER α KO mice, where estradiol was ineffective (Kuo et al., 2010). Since ER α KO mice do not demonstrate estrogen positive feedback and lack sexual receptivity due to the lack of estradiol signaling, the STX response in ER α KO mice is not consistent with a STX-related signaling mechanism for induction of the LH surge or receptivity in female reproduction (Rissman et al., 1997; Ogawa et al., 1998; Wintermantel et al., 2006; Kuo et al., 2010). Therefore, the physiological relevance of STX in reproduction remains to be elucidated, but STX may have a future as a therapeutic agent since it does not have uterotrophic actions (Roepke et al., 2010).

Lastly, ER-X has been proposed as an ER during development and following injury, especially in the cortex (Toran-Allerand, 2000; Toran-Allerand et al., 2002). This ER is not inhibited by ICI 182,780, but is activated by 17 α -estradiol. ER-X is unique in that it is neither blocked by ICI 182,780 nor is it stereospecific. In fact, it is preferentially activated by 17 α -estradiol (Toran-Allerand et al., 2002). However, in astrocytes the estradiol action on $[Ca^{2+}]_i$ is

stereospecific (Chaban et al., 2004), and the 17β -estradiol-induced $[Ca^{2+}]_i$ release and progesterone synthesis are inhibited by ICI 182,780, which is not consistent with an ER-X mediated action (Micevych et al., 2007; Kuo et al., 2009).

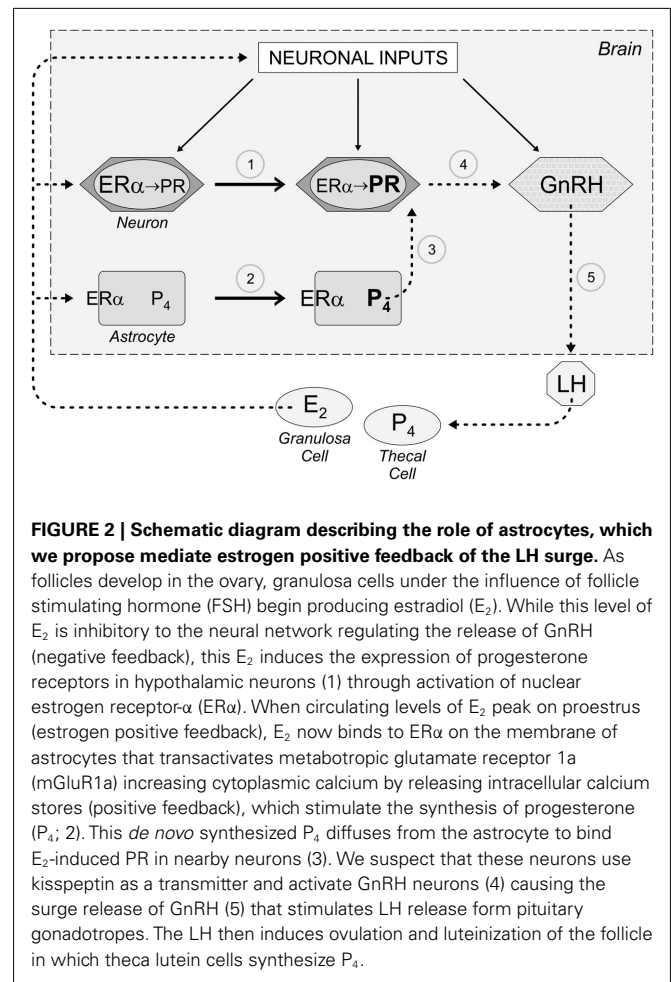
In summary, several putative mERs are involved in regulation of $[Ca^{2+}]_i$ and progesterone synthesis in hypothalamic astrocytes (Kuo et al., 2010). ER α and the STX-activated G α_q -mER had a similar pharmacology including their dependence on mGluR1a to initiate cell signaling. From a reproductive vantage point as well as evidence from wild-type and ERKO hypothalamic astrocytes, ER α appears to be the primary mER responsible for the rapid cell signaling that leads to an increase in hypothalamic neuroprogesterone (Micevych et al., 2007; Bondar et al., 2009; Kuo et al., 2009, 2010).

IS THE LH SURGE STIMULATED BY PROGESTERONE OR A METABOLITE?

Overall, there is firm evidence that estradiol stimulates the synthesis of progesterone in the hypothalamus. Progesterone in the brain can be rapidly converted to dihydroprogesterone by neuronal and glial 5 α -reductases and further to allopregnenolone by astrocytic 3 α -hydroxysteroid oxidase (Corpechot et al., 1993; Mensah-Nyagan et al., 1999). As mentioned earlier, allopregnenolone primarily interacts with the GABA_A receptor. Classically, the GABA receptors are inhibitory. One hypothesis suggests that GnRH neurons have greater intracellular vs. extracellular chloride concentration, which results in an efflux of chloride rather than an influx – depolarizing the neurons and stimulating GnRH release (DeFazio and Moenter, 2002; Sullivan and Moenter, 2003). Indifferent strains of GT1 neurons, allopregnenolone in one case stimulated the release of GnRH (el Etr et al., 1995) and in another did not affect GnRH release (Sleiter et al., 2009). Interestingly, in the latter study progesterone did inhibit GnRH release through an action on membrane PRs: mPR α and mPR β . The *in vivo* data are equally inconsistent. *In vivo*, allopregnenolone inhibited LH release in OVX, estradiol + progesterone primed rats, an effect that was reversed in the presence of bicuculline (Laconi and Cabrera, 2002). However, bicuculline facilitated GnRH release from acute hypothalamic slices (Giuliani et al., 2001). Further confusing the situation is the observation that progesterone can enhance LH secretion independently of GABA_A receptor involvement (Brann et al., 1990). Thus, the role of progesterone in mediating estrogen positive feedback is well-established (Kim and Ramirez, 1982; Drouva et al., 1985; Ke and Ramirez, 1987), but evidence for a role of allopregnenolone is not as robust.

CONCLUSION

The quest to understand the CNS control of the LH surge and the mechanism of estrogen positive feedback has yielded novel insights about the role of astrocytes, progesterone, and EMS. Specifically, estradiol acting through EMS in astrocytes to induce progesterone synthesis must now be considered when describing the mechanism for estrogen positive feedback in reproductive physiology (Figure 2). Preliminary studies suggest that estradiol produced in the ovary also induces the synthesis of PR-A in kisspeptin (KISS) neurons of the GnRH-control network. KISS neurons, of the anteroventral periventricular nucleus (AVPV) and rostral extent of



the periventricular nucleus along the III ventricle, project directly to GnRH neurons (Clarkson and Herbison, 2006; Herbison, 2007). KISS is a potent stimulator of GnRH neurons (Messenger et al., 2005; d'Anglemont de Tassigny et al., 2008; Herbison, 2008; Liu et al., 2008; Clarkson et al., 2010). Local astrocytes, stimulated by peaking estradiol concentrations, increase progesterone synthesis that activates the PR in KISS neurons to stimulate GnRH release (Figure 2). While a role for membrane PRs has not been elucidated in positive feedback, evidence from GT1 cells suggests that neuroprogesterone from astrocytes can also stimulate membrane PRs, perhaps on native GnRH neurons.

These studies bring into sharp focus that understanding brain function should also involve transmitters regulated at the point of their synthesis. These include the known endocannabinoids, prostaglandins, nitric oxide, and the neurosteroids. In terms estrogen positive feedback regulation of the LH surge and ovulation, neuroprogesterone rather than the metabolite, allopregnenolone that binds to the GABA_A receptor, appears to be key.

Finally, astrocytes are another point of convergence and integration of hormonal and transmitter signaling (Figure 2). Estradiol, from the periphery binds to mER, which transactivates a mGluR1a receptor to initiate cell signaling that results in a release of intracellular stores Ca^{2+} and a stimulation of progesterone

synthesis. Glutamate is not needed for the estradiol-induced $[Ca^{2+}]_i$ increase, but in the presence of glutamate, the efficacy of estradiol is increased. Interestingly, in spite of the necessity of ER α for reproduction, including estrogen positive feedback, STX appears to activate a novel protein that mobilizes Ca^{2+} , stimulates progesterone synthesis but whose actions are blocked by antagonism of the mGluR1a. This suggests a model for EMS in which

mERs, be they ER α , ER β , or the STX-activated G α_q -mER need to transactivate mGluRs. This holds the promise of novel therapeutic approaches for any number of pathologies influenced by estradiol.

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