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Neuroprogesterone: key to estrogen positive feedback?

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Abstract

In the cycling female rat, estradiol and progesterone induce reproductive behavior, and the surge of luteinizing hormone (LH) needed for ovulation. Circulating estradiol of ovarian origin induces progesterone receptors in the preoptic area and hypothalamus. Sequential activation of estrogen receptors (ER) and progesterone receptors coordinates reproductive physiology and behavior. In ovariectomized and adrenalectomized (ovx/adx) rats, administration of estradiol alone is sufficient to initiate an LH surge, and central infusion of aminoglutethimide (AGT), a blocker of the P450_{side} chain cleavage enzyme, disrupted the estrous cycle of intact rats without affecting peripheral estradiol levels, suggesting that an endogenous source of progesterone remains in these animals. In ovx/adx rats, progesterone levels in the hypothalamus increase prior to the LH surge, and inhibition of progesterone synthesis prevents the LH surge, suggesting that hypothalamic neuroprogesterone is a necessary for estrogen positive feedback. In support of the idea that estradiol induces neuroprogesterone, estradiol increased expression of the progesterone-synthesizing enzyme 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in the hypothalamus before the LH surge. Further, in vitro experiments demonstrate that estradiol stimulates progesterone synthesis in astrocytes, considered to be the most active steroidogenic cells in the CNS. To stimulate neurosteroidogenesis, estradiol acts through membrane ER and type 1a metabotropic glutamate receptors (mGluR1a) to increase free cytoplasmic calcium ([Ca²⁺]_i) via activation of the PLC-IP₃ pathway. Estradiol-induced progesterone synthesis is mimicked by thapsigargin-induced release of IP₃ receptor-sensitive Ca²⁺ stores in astrocyte cultures. Thus, estradiol induced progesterone synthesis is dependent on membrane ERs that act through mGluR1a to activate the PLC-IP₃ pathway. This neuroprogesterone also facilitated proceptive behavior. Blocking either progesterone synthesis or progesterone receptor in estrogen primed ovx/adx prevented proceptive but not receptive behaviors.

Keywords

Estrogen positive feedback; progesterone receptor; neurosteroids; neurosteroid; lordosis; membrane estrogen receptor; mGluR1a; calcium; brain; luteinizing hormones; astrocytes; glial; LH; reproduction; 3beta-HSD; estradiol

INTRODUCTION

Regulation of reproduction requires the coordinated interaction of peripheral steroids with hypothalamic sites that regulate gonadotrophin secretion and behavior. The prevailing dogma is that the brain is the passive recipient of gonadal steroid signals. That is, ovarian estrogens and progestins act on specific receptors that allow intero- and extero-receptive signals to activate specific circuits in the limbic system and hypothalamus to initiate the coordinate release of GnRH, and stimulation reproductive behaviors. Although the steroidogenic capacity of the brain was demonstrated in the early 1980s by Etienne Baulieu and coworkers (Corpechot et al., 1981); reviews in (Baulieu, 1998), it was not clear how these *de novo* synthesized steroids fit into the CNS regulation of reproduction until relatively recently.

Studies in our laboratory have demonstrated an important interaction between circulating estradiol and the synthesis of progesterone in the hypothalamus that initiates the LH surge. Increased synthesis of neuroprogesterone is a necessary component of estrogen positive feedback that triggers ovulation. Progesterone action in the brain is inextricably linked to estradiol action, since both progesterone and its cognate receptor are stimulated by estradiol. Numerous studies have demonstrated that treating estradiol-primed animals with progesterone initially augments and then inhibits estradiol actions (e.g., receptive behavior; (Sinchak and Micevych, 2003). We will review the evidence for 1) the estradiol regulation of neuroprogesterone synthesis and 2) the potential roles of neuroprogesterone in regulating both positive feedback and reproductive behavior.

Regulation of ovulation

The control of ovulation is dependent on estradiol and progesterone. In the estrous cycle, low levels of estradiol during diestrus have an inhibitory action on the hypothalamus and pituitary, but are important for priming these tissues. In contrast, the peak of estradiol on proestrus induces positive feedback, stimulating the release of GnRH and inducing the LH surge (Chazal et al., 1974). Although estradiol is the primary stimulus coordinating ovulation, progesterone and progesterone receptors have also been implicated in stimulating the surge release of LH. Rising levels of estradiol on the afternoon of proestrus induce expression of progesterone receptors in the hypothalamus that are critical for the estrogen positive feedback and the LH surge (Chappell et al., 1999; Chappell and Levine, 2000; Levine, 1997). Although plasma levels of progesterone remain low prior to the LH surge (Feder et al., 1971; Kalra and Kalra, 1974; Smith et al., 1973; Smith and Davidson, 1974; Smith et al., 1975), blocking progesterone synthesis with a β -HSD inhibitor prevents an estrogen-induced LH surge (DePaolo, 1988; Mahesh and Brann, 1992; Mahesh and Brann, 1998a; Mahesh and Brann, 1998b; Micevych et al., 2003). Thus, both estradiol-induced progesterone receptors in the hypothalamus and progesterone appear critical for the surge release of LH. Circulating progesterone from a peripheral source is not necessary for estrogen positive feedback, since estradiol alone can stimulate an LH surge in ovariectomized and adrenalectomized (ovx/adx) rats (Mann et al., 1976; Micevych et al., 2003). Importantly, in ovx/adx rats, inhibiting progesterone synthesis also prevents the estradiol-induced LH surge (Fig 1). In a parallel group of ovx/adx rats, estradiol treatment increased progesterone levels in the hypothalamus but not in the plasma (Fig 2). To show that hypothalamic steroidogenesis is important for initiating the proestrous LH surge, progesterone synthesis was blocked in intact rats by infusion of aminoglutethimide (AGT) into their III ventricle on the morning of proestrus. The vaginal histology (smears) revealed that the cycle was progressing normally until proestrus. On the day of estrus, the predominately nucleated cells indicated a proestrous cytology, suggesting that an LH surge and ovulation had not occurred (Ogi et al., 2006). The AGT disruption of the estrous cycle was not permanent. On the following cycle, without AGT, rats had normal levels of peripheral estradiol and progesterone. Together these studies suggest that estrogen positive feedback is

dependent on neural progesterone synthesis in proximity to estradiol-induced progesterone receptors.

Steroid Biosynthesis

The first indication that the brain could make steroids *de novo* (i.e., neurosteroids) was the detection of pregnenolone, DHEA and their sulfate esters in the brains of gonadectomized and adrenalectomized rats (Corpechot et al., 1981). Initially, gene expression of P450 side-chain cleavage (P450_{scc}) and aromatase was demonstrated in enriched cultures of astrocytes and oligodendrocytes (Hu et al., 1987; Mellon and Deschepper, 1993; Mellon, 1994; Mellon and Griffin, 2002a; Mellon and Griffin, 2002b). Subsequent studies demonstrated the widespread presence of steroidogenic enzymes and activity *in vivo* (Mellon and Deschepper, 1993).

Much of our knowledge about the synthesis of steroids is a result of work done in the ovary, testis and adrenal gland, and it has been assumed that these processes are similar in the nervous system. All steroid hormones are derived from cholesterol. It has been established that the biosynthesis of steroids requires the expression and regulation of six cytochrome P450 enzymes, four hydroxysteroid dehydrogenases, a reductase, and two sterol carrier proteins (reviewed in (Mellon and Griffin, 2002b). Each of the P450 enzymes are coded for by single genes and can mediate multiple enzymatic steps, while the non-P450 enzymes (e.g., 3 β -HSD) exist as isoforms coded for by multiple genes (Peng et al., 2002).

In the gonads, P450_{scc} converts cholesterol to pregnenolone, which is dehydrogenated by 3 β -HSD to progesterone. (The reader is referred to two excellent reviews of the biochemistry of steroidogenic enzymes: (Mensah-Nyagan et al., 1999; Payne and Hales, 2004). Pregnenolone is successively converted to 17-OH-pregnenolone and dehydroepiandrosterone (DHEA) by 17 α -hydroxylase/17,20-lyase. DHEA is converted to androstenedione by 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β -HSD). Subsequently, 17 β -hydroxysteroid dehydrogenase converts androstenedione to testosterone (Labrie et al., 1997). Estrone and estradiol are synthesized by aromatase from androstenedione and testosterone, respectively. In the ovary, the granulosa cells synthesize testosterone, and theca interna cells synthesize estradiol. Thus, the cells adjacent to the developing follicle share the responsibility of steroidogenesis. After the formation of the corpus luteum, the theca and granulosa cells are luteinized and primarily produce progesterone.

In the peripheral steroidogenic tissues, steroid production is regulated by trophic hormones released from the anterior pituitary. For gonadal steroids, follicle stimulating hormone (FSH) and LH regulate steroid biosynthesis. These trophic hormones activate G protein coupled receptors (GPCRs) that catalyze the formation of cyclic adenosine monophosphate (cAMP), which induces the phosphorylation of protein kinase A and leads to activation of enzyme activity and transcription (Momoi et al., 1992; Waterman, 1994). Two phases of steroidogenesis regulation have been identified: the rapid phase and the long-term phase. The rapid phase does not involve gene transcription, while transcription and translation are hallmarks of the long-term phase (Bose et al., 2002; Johnson et al., 2002; Lehoux et al., 1998; Rossato et al., 2001). The rate-limiting step in steroidogenesis is the delivery of cholesterol to the inner mitochondrial membrane, site of P450_{scc}. This important step is mediated by steroid acute regulatory protein (StAR), which transports cholesterol across the mitochondrial intermembrane space (Granot et al., 2002; Kallen et al., 1998; King et al., 2002; Stocco, 2001).

Synthesis of Neurosteroids

A seminal study by Zwain and Yen (Zwain and Yen, 1999) demonstrated that steroidogenic capacity differs among the cell types of the nervous system. Using neonatal rat cell culture, RT-PCR and activity assays, they demonstrated that the most active steroid-producing cells in

the nervous system are astrocytes. These cells express P450_{scc}, P450_{c17}, 3 β -HSD, 17 β -HSD, and aromatase and produce pregnenolone, progesterone, DHEA, androstenedione, testosterone, estrone and estradiol. The main steroid secreted by astrocytes is progesterone. Although oligodendrocytes also express P450_{scc} and 3 β -HSD, their major steroidal product is pregnenolone. Neurons express all the steroidogenic enzymes and are capable of converting cholesterol all the way to estrogens, but steroidogenesis in neurons appears to be mainly the conversion of circulating androgens to estrogens via aromatization. Indeed, the conversion of circulating testosterone to estradiol is a well established dogma in neuroendocrinology and sexual differentiation. Thus, as in the ovary, steroidogenesis requires the coordinated function of several cell types in the CNS. Astrocytes, neurons and oligodendrocytes all contribute to steroidogenesis.

To study this topic in relation to the control of estrogen positive feedback and the LH surge, we used both *in vivo* and *in vitro* methods. To determine whether peripheral estradiol increases hypothalamic progesterone levels via transcription of carrier proteins and/or steroidogenic enzymes, ovx/adx rats were treated with estradiol benzoate and hypothalamic mRNAs were quantified with real-time RT-PCR (Soma et al., 2005). Estradiol increased hypothalamic 3 β -HSD mRNA levels by 143% after 24 hr, and 3 β -HSD mRNA levels remained elevated at 44 hours (Fig 3). To ascertain whether increased 3 β -HSD mRNA was reflected in increased 3 β -HSD enzyme activity, the conversion of ³H-pregnenolone to ³H-progesterone by hypothalamic homogenates was measured. After estradiol treatment (44 hr), 3 β -HSD activity was increased in the hypothalamus (Fig 3B). The 44 hr time point was chosen because in previous studies, levels of progesterone were elevated in the hypothalamus at 45 hr after EB treatment (Micevych et al., 2003). In contrast, EB treatment had no effect on mRNA for P450_{scc} or the sterol carrier molecules StAR and SCP-2 (Soma et al., 2005). These data suggest that estradiol-induced neuroprogesterone is due, at least in part, to increased transcription of the proximal synthetic enzyme 3 β -HSD. This may constitute long-term regulation of neuroprogesterone synthesis.

Cell-specific neurosteroidogenesis

As reviewed, different cell types are responsible for the synthesis of specific steroids in the CNS (Kabbadj et al., 1993; Zwain and Yen, 1999). Since astrocytes express StAR, P450_{scc} and 3 β -HSD and appear to be the most active steroidogenic cells in the brain, we examined the response of astrocytes to estradiol. Astrocytes from post-pubertal female rat hypothalamus were cultured and challenged with estradiol (Micevych et al., 2007). In response to estradiol stimulation, enriched post-pubertal astrocyte cultures produced greater amounts of progesterone (Fig 4A). However, neonatal hypothalamic astrocytes did not respond to estradiol treatment by increasing progesterone synthesis (Fig 4A).

The estradiol effect was blocked with ICI 182,780, suggesting that an estrogen receptor (ER) with classic ER pharmacology is involved (Fig 4B). Moreover, previous results revealed the expression of ERs in both the nuclear and cell membrane fraction of astrocytes (Chaban et al., 2004). Estradiol treatment induced rapid increases in intracellular free calcium ([Ca²⁺]_i; Chaban et al., 2004). This response was mediated through membrane ER, which activated the PLC-IP₃ pathway and released intracellular stores of calcium (Fig 5A). To determine whether the estradiol signaling at membrane ERs that increases [Ca²⁺]_i flux is associated with estradiol-induced progesterone synthesis, post-pubertal hypothalamic astrocytes were studied (Micevych et al., 2007). We established that estradiol stimulated both [Ca²⁺]_i flux and progesterone synthesis in post-pubertal astrocytes (Fig 4C and 5A).

Increasing [Ca²⁺]_i levels in the absence of estradiol stimulated progesterone synthesis. The sesquiterpene lactone, thapsigargin, was used to rapidly increase [Ca²⁺]_i flux by releasing Ca²⁺ from intracellular stores bypassing the PLC - IP₃ receptor mechanism (Jackson et al., 1988, and references therein). Thapsigargin caused a rapid and transient increase of [Ca²⁺]_i

(Micevych et al., 2007). A one hour treatment of post-pubertal astrocytes with thapsigargin alone (no estradiol) increased progesterone levels in the medium (Fig 4C), which was similar to one hour treatment with thapsigargin and estradiol. These results suggest that estradiol and thapsigargin activate the same intracellular PLC-IP₃ pathway to stimulate progesterone synthesis and implicate a membrane ER initiated pathway. The proximal signaling mechanism of this receptor remains to be elucidated. One possibility is that the membrane ER is itself a G protein-coupled receptor (GPCR) that stimulates PLC. Our results suggest that the membrane receptor is a classic ER, but neither ER α nor ER β have the typical seven membrane-pass structure of known GPCRs. An alternative proposal is that membrane ERs require another protein, mGluR1a, to activate intracellular signaling pathways (Boulware et al., 2005). The mGluR1a is a GPCR protein-coupled receptor that activates the PLC-IP₃ pathway in neurons and astrocytes (Boulware et al., 2005; Zur Nieden and Deitmer, 2006). Thus, the ER/mGluR1a complex may initiate signaling through the PLC pathway stimulating IP₃ production that in turn activates IP₃ receptors causing the release of Ca²⁺ (Chaban et al., 2004). This increase of [Ca²⁺]_i could activate a protein kinase, either PKA or PKC, leading to protein phosphorylation and activation of StAR (Stocco and Clark, 1996; Stocco et al., 2005). Blocking the mGluR1a receptor prevented the normal estradiol-induced increase in [Ca²⁺]_i levels (Fig 5B).

The results from enriched astrocyte cultures were different from those obtained *in vivo*. The astrocyte data demonstrated that steroidogenesis can be rapidly activated without involving gene expression, since mRNA levels for steroidogenic enzymes and steroid carrier proteins were not altered, as measured by quantitative RT-PCR. Thus, although astrocytes in culture synthesize progesterone and respond to estradiol, the mechanism may be different from that found *in vivo*, where 24 hours after estradiol treatment the expression of 3 β -HSD mRNA increased in the hypothalamus (Soma et al., 2005). Currently, we do not understand what underlies the differences between the *in vitro* and the *in vivo* results. It is possible that in an enriched astrocyte culture lacking neurons, important neuron-glia interactions are missing (de Sampaio e Spohr et al., 2002; Sepp and Auld, 2003). Such interactions may be necessary for estradiol to increase transcription of 3 β -HSD as observed *in vivo* (Soma et al., 2005).

These results suggest that an aspect of estrogen positive feedback requires hypothalamic astrocytes to synthesize progesterone. This progesterone diffuses to local estradiol-induced progesterone receptors in neurons that allow for elevated release of GnRH initiating the LH surge. The trigger for this change from negative to positive feedback is the level of circulating estradiol, which when low produces negative feedback, but when as on proestrus, estradiol levels spike – they stimulate the synthesis of progesterone in the hypothalamus (Fig 6).

Female reproductive behaviors

In the female rat there are two types of sexual behaviors, proceptive and receptive. Proceptive behaviors are sometimes referred to as solicitation behaviors. These include hopping, darting and ear wiggling with which the female signals her receptivity (Tennent et al., 1980). Receptive behavior includes the lordosis reflex in which the female dorsiflexes her back (lordosis), exposing her perineum—allowing the male to intromit. Both behaviors are readily quantifiable. Classically, proceptive behaviors are thought to be dependent on estradiol + progesterone, whereas estradiol is necessary and sufficient to induce receptivity.

The primary stimuli regulating reproductive behavior and ovulation are the same, increasing levels of estradiol that peak on proestrus, and estradiol-induced progesterone and its cognate receptors in the hypothalamus. We hypothesized that since neurosteroidal progesterone was important for initiating the LH surge, neuroprogesterone may also regulate proceptive and receptive behaviors in the rodent. It is well known that estradiol will induce lordosis in both ovx and ovx/adx rats (Davidson et al., 1968; Sodersten and Eneroth, 1981). For example,

repeated injections of levels of 3–5 µg estradiol induce successively higher levels of lordosis, eventually achieving a lordosis response similar to estradiol + progesterone priming conditions (Babcock et al., 1988; Bloch et al., 1987; Butler et al., 2001). In our hands, 2 µg estradiol given every 4 days will produce non-receptive animals *ad infinitum*, but adding progesterone will result in a receptive females with normal, intact levels of lordosis (Sinchak and Micevych, 2001) demonstrating the important physiological interaction of these two steroids.

Progesterone has another important function *vis a vis* reproductive behavior, it resets the lordosis regulating circuits in the brain. Sequential treatment with estradiol and progesterone facilitates receptivity in ovx rats, but then terminates the behavior (Zucker, 1967). For example, rats made receptive with 2 µg estradiol + progesterone ($LQ = 98.8\% \pm 1.3$), subsequently treated with estradiol were non-receptive ($LQ = 33.75\% \pm 7.8$). A LQ that was similar to ovx rats treated with estradiol for the first time. This represents the resetting of the lordosis circuit and is not seen in ovx animals made receptive by estradiol alone (Bloch et al., 1987; Davidson et al., 1968).

While it is well recognized that lordosis behavior is maximally stimulated by estradiol plus progesterone, the mechanism by which estradiol induces lordosis in the absence of progesterone is not well understood. In the context of our hypothesis, treatment of ovx/adx females with estrogen increases neurosteroidal progesterone synthesis and this neurosteroidal progesterone may coordinate reproductive behaviors with ovulation.

Proceptive and receptive behaviors

To test for behavior, ovx females were given high doses of 17β-estradiol benzoate (EB; 10 µg) every 4 days for 4 sessions. On the third and fourth tests, 50 µg 17β-estradiol (unconjugated) was injected (sc) 4–6 hours prior to testing to produce a full constellation of receptive and proceptive behaviors (Fig 7). Interestingly, free estradiol given 4 hrs prior to testing (48 hrs after EB) on the fourth treatment produced maximum receptivity and near maximum proceptivity (Fig 7). Thus, our data are consistent with earlier studies that showed estradiol not conjugated with benzoate can substitute for progesterone to produce a full constellation of proceptive and receptive behaviors (Parsons et al., 1984; Pfaff et al., 1994). To test whether estrogen-induced hypothalamic progesterone synthesis facilitates receptivity, we treated estrogen primed ovx/adx animals with either the progesterone receptor antagonist RU486 or enzyme antagonists AGT or trilostane. None of these treatments blocked the estrogen-induced lordosis (Fig 8 A, B and C) suggesting that neither progesterone synthesis nor activation of progesterone receptors is necessary for the estrogen-induced expression of receptive behavior. These results are consistent with earlier studies with RU486 (Blaustein et al., 1987) and with observations in progesterone receptor null mutant mice (PRKO) that indicate progesterone receptors may not be involved in an estrogen-only initiation of lordosis (Mani et al., 1997).

In contrast, proceptive behaviors in ovx and ovx/adx rats treated with estradiol-alone were blocked by RU486, indicating dependence on progesterone receptors (Fig 8A). Since these animals did not have peripheral steroidogenic tissues, progesterone receptors could be activated by estradiol-induced neurosteroidal progesterone or by ligand independent mechanisms (Mani et al., 1994). To further test whether *de novo* progesterone synthesis is required for estradiol induced proceptive behaviors, estrogen primed ovx/adx rats were treated with either AGT (10 mg), a blocker of P450_{scc} immediately following estrogen or trilostane that blocks the conversion of pregnenolone to progesterone. The advantage to blocking P450_{scc} is that steroidogenesis is inhibited. Moreover, AGT does not block the progesterone receptor allowing for ligand-independent activation. Estradiol-induced lordosis behavior was not attenuated by blocking either progesterone receptors (RU486), or progesterone synthesis (Fig 8A, B and C, respectively). Proceptive behavior, however, was significantly decreased by both these treatments indicating the activation of progesterone receptors is through estradiol-induced

neurosteroidal progesterone synthesis and not through a ligand-independent mechanism. Thus, the LH surge and estradiol-induced proceptivity require the synthesis of neurosteroidal progesterone, but estradiol-induced receptive behavior does not.

It is obvious in the cycling female that progesterone is necessary for sexual receptivity, however, the need for progesterone stimulation can be bypassed in ovx/adx females by estradiol-alone stimulation suggesting two mechanisms for facilitating sexual receptivity. It is possible both systems are actually at work in the female but are age dependent. In the young rat, although it is possible to induce sexual receptivity with estrogen alone, she is not exposed to this hormonal situation. Her cycle exposes the reproductive physiological and behavioral circuits to sequential estradiol and progesterone. As the female rat ages, however, cycles become irregular and eventually cease. It is only during middle-age when peak levels of progesterone are greatly attenuated (Lu et al., 1979) that estradiol is the primary stimulus for reproductive behavior. Reproductively aging females in persistent or constant estrus do not have positive feedback, and estradiol does not induce hypothalamic progesterone synthesis (Fig 9; (Mills et al., 2002) even though circulating estradiol levels are elevated. Thus, it is possible that estrogen (only) and estrogen + progesterone induced sexual receptivity are genuine mechanisms that are important for reproductive success in the rat at different ages.

Summary

These studies demonstrate the interaction between circulating levels of estradiol and hypothalamic levels of progesterone. These interactions are critical for the regulation of reproduction. *In vivo*, estradiol stimulates the synthesis of neuroprogesterone by increasing hypothalamic 3β -HSD mRNA and activity after 24 hr. *In vitro*, estradiol rapidly stimulated $[Ca^{2+}]_i$ flux in post-pubertal hypothalamic astrocytes. The estradiol-induced $[Ca^{2+}]_i$ flux requires an interaction between a membrane ER and mGluR1a, which activates the PLC-IP3 pathway and the release of intracellular Ca^{2+} stores. Elevating $[Ca^{2+}]_i$ in astrocytes stimulates progesterone levels in the medium, perhaps by rapidly increasing StAR or 3β -HSD activity. This locally produced astrocrine neuroprogesterone initiates the LH surge by activating estradiol-induced progesterone receptors in neurons that stimulate GnRH neurons. Blocking neuroprogesterone synthesis in gonadally intact rats disrupts their estrous cycle at proestrus, suggesting that peripheral progesterone is insufficient by itself to initiate the LH surge. The same blockade of neuroprogesterone synthesis also prevents the display of proceptive behaviors. These experiments illustrate the importance of astrocyte-synthesized progesterone in estradiol positive feedback stimulating the LH surge and reproductive behavior.

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Figure 1.

Effect of trilostane, a 3β -HSD inhibitor, was measured on the estradiol-induced LH surge in two-month-old OVX/ADX female rats. Basal concentrations of plasma LH (ng/ml) were measured in venous blood serum samples prior to treatment. On the second day following treatment with 50 μ g 17 β -estradiol benzoate (EB), or oil vehicle (VEH), or EB + 16.5 mg trilostane (EB+TRI), serial blood samples (250 μ l) were collected once every 90 min from 1400 to 2130 hr. Plasma was assayed for LH. Data were expressed in terms of NIDDK Rat LHRP-2. EB-treated females showed a significant increase in plasma LH levels compared with those of VEH control. Pretreatment with trilostane blocked the EB-induced LH surge, and attenuated basal LH release. Data are means $\pm$ SEM of at least 7 samples. * $p < 0.05$ versus EB+TRI-treated females. † $p < 0.05$ versus VEH-treated females. (From (Micevych et al., 2003).

Figure 2.

Progesterone concentrations in the hypothalamus (HYP), cerebellum (CRB), parietal cortex (CTX) were measured in OVX/ADX rats following 50 μ g estradiol benzoate (EB) or vehicle (VEH) treatment. Data are means $\pm$ SEM of 4–6 samples. * = $p < 0.05$ significantly greater than VEH group within brain regions SNK. (Modified from (Micevych et al., 2003).

Figure 3.

A. Effects of estradiol treatment on mRNA levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) in the hypothalamus of OVX-ADX rats. Subjects were treated with 50 μg 17β-estradiol benzoate (EB) for 0, 12, 24 or 44 hr (n=6, 5, 5, 5). The mRNA levels were measured by quantitative RT-PCR and expressed as a percent of baseline (0 hr group). EB treatment significantly increased 3β-HSD mRNA levels ($F = 6.075$, $p = 0.003$). * = significantly greater than 0 and 12 hr groups ($p < 0.05$, Fisher's PLSD). **Figure 3B.** Effects of EB treatment on 3β-HSD activity in the hypothalamus and amygdala of OVX-ADX rats. Subjects were treated with EB (n=6) or oil vehicle (n=5) 44 hr before the hypothalami were harvested. 3β-HSD activity was determined by measuring the *in vitro* conversion of [3 H]-pregnenolone to [3 H]-PROG. *

= significantly greater than oil-treated controls within brain region (t-test, $t = 2.349$, $p = 0.04$; from (Soma et al., 2005).

Figure 4.

A. Effect of estradiol treatment of neonatal and post-pubertal hypothalamic astrocytes *in vitro*. The cells were steroid-starved for 24 hours and then incubated for 48 hours with indicated concentrations of 17 β -estradiol or steroid-free media (0). Levels of progesterone in the supernatants were measured by radioimmunoassay. The neonatal astrocytes did not increase progesterone levels in the media in response to any estradiol dose tested. Post-pubertal astrocytes increased progesterone levels after treatment with estradiol. This increase was statistically significant at 10⁻⁶ M 17 β -estradiol. Values are reported as mean $\pm$ SEM (n=8). * indicates significantly greater within developmental age group compared with 17 β -estradiol free group (0) $p < 0.05$ (SNK). **Figure 4B.** Antagonism of 17 β -estradiol (E₂; 10⁻⁶ M) induction

of progesterone levels in media from primary post-pubertal hypothalamic astrocyte cultures from female rats by blocking estrogen receptors. Steroid-starved astrocytes were incubated for 48 hours with either E2-free media with DMSO (DMSO), the vehicle used to dissolve the 10^{-6} M ICI 182,780 (ICI), an estrogen receptor antagonist, E2, ICI, or ICI + E2. Levels of progesterone in the supernatants were measured by radioimmunoassay. Data are means $\pm$ SEM ($n = 4$). * indicates values significantly greater than all other treatment groups ($p < 0.05$, SNK).

Figure 4C. Effect of thapsigargin induced release of internal stores of Ca^{2+} on progesterone synthesis in astrocyte cultures obtained from post-pubertal female rats. Astrocytes were treated with thapsigargin (Thp) or Thp (10^{-7} M) supplemented with 10^{-6} M estradiol (E2/Thp) for one hour. The media was collected and replaced with either estradiol-free DMEM/F12 (DMEM Post Thp) or 10^{-6} M estradiol (E2 48hrs Post Thp). The progesterone concentration in the medium significantly increased following treatment with either E2 for 48 hr, Thp for one hr or Thp+ E2 for one hr. When the media was replaced with DMEM/F12 (DMEM) following Thp treatment there was no increase of progesterone concentration above baseline. Following one hour Thp treatment, subsequent exposure to E2 for 48 hr did not statistically increase the concentration of progesterone in the supernatant. Data are mean $\pm$ SEM ($n = 4$). * = indicates values significantly greater than control media, DMEM + DMSO (DMSO; $p < 0.05$ SNK; modified from (Micevych et al., 2007).

Figure 5.

A. Pharmacological profile of the effect of estradiol on internal calcium concentration $[Ca^{2+}]_i$ in neonatal cortical astrocytes. Stimulation of astrocytes with estradiol (E₂, 10⁻⁶ M) in the presence or absence of extracellular Ca²⁺ or with E-6-BSA (estradiol conjugated to bovine serum albumin) produced a similar $[Ca^{2+}]_i$ increase. Inhibition of estrogen receptors (ER) with ICI 182,780 (E₂ + ICI) blocked E₂-induced increases in $[Ca^{2+}]_i$. Removing Ca²⁺ from the media and replacing with membrane impermeable BAPTA (E₂ + BAPTA) did not block the E₂ induced $[Ca^{2+}]_i$ flux. However, blocking the IP₃-regulated smooth endoplasmic reticulum Ca²⁺ channel with 2-APB (E₂ + APB) prevented the E₂-induced $[Ca^{2+}]_i$ flux. Similarly, blocking phospholipase C (PLC) signaling pathway with U73122 (E₂ + U73122)

blocked the estradiol induced $[Ca^{2+}]_i$ flux. These results indicate that the E2 stimulation of $[Ca^{2+}]_i$ is via activation of an ER associated with the plasma membrane that stimulates the PLC-IP₃ signaling pathway. The number of experiments is indicated in each *bar*. * indicates significantly less than E2 treatment group ($p < 0.05$; modified from (Chaban and Micevych, 2005). **Figure 5B.** Estradiol induced $[Ca^{2+}]_i$ flux in hypothalamic astrocytes is attenuated by mGluR1a antagonism. Estradiol (4 nM) was bath applied to post-pubertal hypothalamic astrocytes to determine that they responded to estradiol stimulation. After a 3 min washout, mGluR1a antagonist, LY367385 (50 nM), was applied for 6 mins and then were treated with estradiol again. The number of experiments is indicated in each *bar*. * indicates significantly different from E2 stimulation at $p > 0.05$; from (Hariri et al., 2006).

Figure 6.

A model of estradiol action on hypothalamic cells involved in the regulation of the LH surge. Circulating estradiol acts on both neurons and astrocytes. The astrocyte has been expanded to illustrate the potential intracellular pathway of estradiol signaling that regulates progesterone (PROG) synthesis. Estradiol acts on membrane estrogen receptors (ER); both ERα and ERβ have been reported in astrocyte membranes (Chaban and Micevych, 2005). Activated ER increase [Ca²⁺]_i by interacting with metabotropic glutamate receptor type 1a (mGluR1a) to initiate the phospholipase C (PLC)/inositol triphosphate receptor (IP₃R) mediated increase in [Ca²⁺]_i. The source of the elevated [Ca²⁺]_i is the smooth endoplasmic reticulum (sER). Estradiol-induced release of Ca²⁺ was mimicked with thapsigargin. Calcium may activate protein kinase A (PKA) and/or protein kinase C (PKC) phosphorylate StAR. StAR mediated transport of cholesterol (CHOL) through the mitochondrial intermembrane space is the rate

limiting step of steroidogenesis. In the inner mitochondrial membrane, cholesterol (CHOL) is converted to pregnenolone (PREG) by cytochrome P450side chain cleavage enzyme (P450_{scc}). PREG is converted to PROG in the sER via the action of 3 β -hydroxysteroid dehydrogenase isomerase (3 β -HSD) and diffuses out of the astrocyte to encounter estradiol induced PROG receptors (PR) in neurons. PR expressing neurons mediate the environmental and circadian signals to neurons stimulating GnRH. GnRH release in the median eminence in turn initiates the LH surge release from anterior pituitary gonadotrophs (modified from (Micevych et al., 2007)).

Figure 7.

Facilitation of sexually receptive and proceptive behaviors in ovariectomized rats treated with 17- β estradiol benzoate (10 μ g) once every four days followed by free estradiol (50 μ g) on injection 4. A near maximal LQ score and a low levels of proceptive behaviors (e.g., ear wiggling, darts, and hops) were seen following the third treatment with EB (injection 3). Animals treated with free estradiol (50 μ g) four hours before testing reached maximal receptivity and high proceptive behavior (injection 4). The LQ was significantly higher after injections 3 and 4 compared to injection 2 (repeated measures ANOVA; $p < 0.05$). Proceptive behavior was significantly higher by the injection 4 compared with tests 2 and 3 (Friedman repeated measures ANOVA on Ranks; $p < 0.05$). Data are means $\pm$ SEM of 12 animals. * $p < 0.05$ significantly greater than proceptive scale score for injections 2 and 3 (Dunnett's post hoc). † $p < 0.05$ versus LQ for injection 2 (Newman-Keuls post hoc).

Figure 8.

Progesterone receptor activity and neurosteroid synthesis are needed for proceptive, but not receptive sexual behaviors. In OVX/ADX rats, EB (10 μ g) was given every 4 days and animals were tested for receptive and proceptive behaviors at 52–54 hours after treatment. **Figure 8A.** animals were treated with EB and then with either RU486 (EB + RU486; 5 mg) or EB 1 hour prior to testing. Antagonism of progesterone receptors with RU486 had no effect on LQ compare to the EB + VEH treated animals (Mann-Whitney, $p > 0.05$). However, proceptivity was significantly decreased in the EB + RU486 treated animals compared with EB animals (Mann-Whitney; $p < 0.05$). Data are means $\pm$ SEM of 12 animals. * indicates significantly less than the EB treated animals, $p < 0.05$. **Figure 8B.** Aminoglutethimide (EB + AGT; 10 mg), an

inhibitor of P450 side chain cleavage enzyme activity, or EB was administered every 24 hours for 3 days prior to testing in EB (10 µg) treated rats. There was no significant change in LQ score between EB + AGT and EB (Mann-Whitney, $p < 0.05$). In contrast to the receptive behavior, AGT significantly decreased the proceptive scale score when compared with EB (Mann-Whitney, $p < 0.05$). Data are means $\pm$ SEM of 12 animals. * $p < 0.05$ versus EB treated control group. **Figure 8C.** Trilostane (EB + Trilostane 16.5 mg) or EB was administered every 24 hours for 3 days prior to testing. Similar to AGT, LQ score between EB + trilostane and EB was not affected ($p > 0.05$), but receptive behavior was significantly decreased in the EB + trilostane treated animals compared with EB treated (Mann-Whitney, $p < 0.05$). Data are means $\pm$ SEM of 12 animals. * = indicates less than EB-only treated control animals $p < 0.05$.

Figure 9.

Progesterone concentrations in the cerebellum (CRB), hypothalamus (HYP), and parietal cortex (CTX) were measured in OVX/ADX, nine months old, acyclic female rats treated with estradiol (50 μ g). Approximately 56 hrs later, when young female rats would produce an LH surge and increased neuroprogesterone levels, these persistently estrous rats still had measurable levels of progesterone in all the regions examined. However, estradiol did not increase progesterone levels compared with vehicle (VEH) controls as seen in the young rats (see Figure 2). ($p > 0.05$; ANOVA; data are means $\pm$ SEM of 4–6 samples). These results indicate that in female rats that have lost the estrogen-positive feedback of the LH, do not have an estradiol-induced increase in progesterone levels.