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Neuroestrogen, Rapid Action of Estradiol, and GnRH Neurons

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Abstract

Estradiol plays a pivotal role in the control of GnRH neuronal function, hence female reproduction. A series of recent studies in our laboratory indicate that rapid excitatory actions of estradiol directly modify GnRH neuronal activity in primate GnRH neurons through GPR30 and STX-sensitive receptors. Similar rapid direct actions of estradiol through estrogen receptor beta are also described in mouse GnRH neurons. In this review, we propose two novel hypotheses as a possible physiological role of estradiol in primates. First, while ovarian estradiol initiates the preovulatory GnRH surge through interneurons expressing estrogen receptor alpha, rapid direct membrane-initiated action of estradiol may play a role in sustaining GnRH surge release for many hours. Second, locally produced neuroestrogens may contribute to pulsatile GnRH release. Either way, estradiol synthesized in interneurons in the hypothalamus may play a significant role in the control of the GnRH surge and/or pulsatility of GnRH release.

Keywords

GnRH neurons; rapid action of estradiol; GPR30; GnRH surge; GnRH pulses; neuroestrogen; membrane estrogen receptors

Introduction

The classical endocrine effects of estradiol (E_2) have a long history of study (141). A gradual secretion of E_2 from the ovary into the general circulation reaches a variety of cells, binds to nuclear estrogen receptors (ERs), and causes genomic changes over the time course of several hours to days. One typical example is modification of the activity of neurons in the hypothalamus and gonadotrophs in the pituitary gland forming the negative and positive feedback loops of the reproductive cycle. In addition to these classical long-lasting effects of E_2 in feedback mechanisms, investigation of rapid (or acute) actions of E_2 on the uterus, vasculature, and neurons also has a relatively long history (65,72,140,155).

As early as 1971, E_2 production in the hypothalamus by aromatization of androstendione (100) has been reported. However, despite over 40 years of research on acute/rapid E_2 action and its synthesis in the brain, the progress of research in this area has been slow. Just

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recently, exciting features on the local synthesis of E_2 and the role of rapid action of E_2 in neuronal function have emerged: Locally synthesized E_2 may serve as a neurotransmitter in the bird brain and rat hippocampus (7,57) and E_2 appears to prevent cell death due to acute hypoxia in rodent stroke models (83,95,157).

E_2 action in the brain is quite complex, as E_2 is synthesized and released from the ovary as well as neurons in the brain. E_2 released from the ovary is transported into the brain through the general circulation and causes a long-lasting genomic action in the brain, such as generation of the preovulatory GnRH surge and lordosis behavior. E_2 released from the ovary also induces rapid action in the brain, as a single E_2 injection results in phospho-CREB expression in GnRH neurons within 15 min (3). In contrast, as discussed in section 2, locally synthesized E_2 appears to play a role in neurotransmission. Theoretically, locally synthesized estradiol in the brain may cause genomic action, but presently little direct evidence is available to support this possibility.

Since the discovery of the GnRH molecule (128) and the subsequent identification of GnRH neurons in the preoptic area (POA) and hypothalamus (11,74,131), mechanisms of E_2 action on GnRH neurons have remained elusive. It has been believed for many years that E_2 modifies the activity of GnRH neurons indirectly through interneurons. This is due to an initial study showing that GnRH neurons do not express estrogen receptors (ERs) using combined autoradiography and immunocytochemistry (133), whereas interneurons that synthesize norepinephrine, dopamine, glutamate, GABA, neuropeptide Y (NPY), and kisspeptin do (38,43,81,82,94,127, also reviewed by 49). However, the availability of more sensitive techniques for detection of ERs and the discovery of ERβ (77) have led to additional avenues for the rapid direct action of E_2 in GnRH neurons. Co-localization of ERβ and GnRH has been reported in the POA-hypothalamus of several species (51,58-60,132,134) and novel membrane bound ERs, including the G-protein coupled ER (GPR30, 40), ER-X (149), and a membrane ER sensitive to the diphenylacrylamide compound STX (STX-R, 115) have been described. In fact, direct rapid E_2 action on GnRH neurons has now been reported by several groups including our own (1-3,19,22,103,123,139,142). In this article, we will 1) briefly review recent progress on neurosynthesis of steroids in the brain focusing on neuroestrogen and their potential role as a neurotransmitter, 2) present data of rapid E_2 action on the GnRH neuronal system, highlighting our findings in the nonhuman primate, and 3) discuss potential roles for locally synthesized E_2 in GnRH neuronal function, including the preovulatory surge and pulsatile GnRH release.

1. Synthesis of estradiol in the brain

Discovery of steroid synthesis in the hypothalamus began in the early 1970s. Conversion of progesterone to 5-pregnane-3,20-dione in rat hypothalamic tissue (18,63) and aromatization of androstendione to E_2 in the hypothalamus (101) were both reported within similar time frames. However, despite consistent reports on the presence of neuroestrogens (35,92,130,138), the role of neuroestrogens in brain function has not been studied until recently. This is quite a contrast to that of 3-hydroxy-D5-compounds, such as pregnenolone (PREG) and dehydroepiandrosterone (DHEA), their sulfates, and reduced metabolites such as the tetrahydroderivative of progesterone 3a-hydroxy-5a-pregnane-20-one (3a,5a-TH PROG). The 3-hydroxy-D5-compounds are one of the most prominent allosteric modulators of chloride channels in $GABA_A$ receptors (12,96) and therefore, they became synonymous with the term “Neurosteroids”.

Entering the 21st century, a hypothesis that locally produced E_2 in the brain modulates neuronal function as a neurotransmitter or neuromodulator has been proposed (7,126). This

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hypothesis is based on the rapid timing of E_2 synthesis in the brain, the rapid action of E_2 inducing sex behavior in quails and rats (27-30), and the presence of aromatase in the presynaptic boutons in song birds (109).

Aromatase is expressed in many different brain regions, including the hypothalamus, in most vertebrate species (14,100,124). Not only is aromatase expressed in various regions of the brain in multiple species, but all of the enzymes necessary to synthesize E_2 *de novo* are also expressed in specific brain regions (54,55,84,87-90,152). Aromatase is expressed not only as a cluster of neurons but also as scattered individual neurons in the hypothalamus (62,100,129), high vocal centers, and the caudomedial nidopallium of the bird brain (109), and in the rat hippocampus (55,122). Importantly, aromatase is found in neuronal cell bodies and neuroterminals (100,109,122,129). In zebra finches, 50% of aromatase expressed in the brain is at neuroterminals and the expression and activity of aromatase at the nerve terminal is differentially regulated from that in other cellular compartments (122).

E_2 synthesis in some brain regions is independent of E_2 synthesis in the gonads, whereas other brain regions are dependent upon E_2 from a gonadal origin (see further discussion on this issue in section 4). For example, aromatase activity in the male monkey ventromedial hypothalamic nucleus (VMH), cortical amygdala, and basal nucleus of the stria terminalis (BNST) are unaffected by castration, while aromatase activity in the POA, anterior hypothalamus, and medial amygdala are abrogated (124). In addition to genomic seasonal regulation of aromatase activity in birds and fish (45,48,86,108,111,137,150), evidence suggests that aromatase activity is also rapidly regulated within a time frame of a couple of minutes (13,26,28). In the quail hypothalamus, aromatase activity is inhibited by depolarization with high K^+ as well as by treatment with the glutamate receptor agonists NMDA, AMPA, and kainate within 5 minutes and this rapid inhibition appears to require phosphorylation of the aromatase enzyme (7-10,17). Additionally, in response to acute stress, aromatase activity is rapidly increased within 15 min in the male quail hypothalamus (32). Collectively, these aromatase studies indicate possible rapid changes in *de novo* synthesis of E_2 in the brain.

Indeed, in the past 10 years, the concentration of E_2 in the brain has been directly assessed by RIA, ELISA, and liquid chromatography with single or tandem mass spectrometry (LS/MS or LC/MS/MS analysis). As summarized in Table 1, to date, five groups have reported E_2 levels of homogenized rat brain tissue samples, spent/conditioned culture media from organotypic or dispersed cell cultures of the rat hippocampus, and microdialysate samples obtained from the bird cerebral cortex. Reviewing the published data, we have first noticed that there are distinct differences in E_2 concentration due to differences in species, sampling method, or detection methodology. In fact, Hojo et al. (56) highlights methodological differences between LC/MS/MS and RIA for assessment of E_2 measurements, showing that there are ~10 fold differences in the levels of E_2 detected in brain tissues (LC/MS/MS values are higher than RIA values). However, within the data obtained from individual labs, E_2 concentration differs among sex, age, and brain region studied (4,73): E_2 concentrations in the hypothalamus and hippocampus are highest at late embryonic ages and decrease after birth on postnatal day 4 (P4) to adulthood levels in rats of both sexes (4,54,73). Importantly, E_2 concentrations in the homogenized samples from various brain regions are in the high picomolar (pM) to low nanomolar (nM) range (4,55,64,73), about 10 to 100-fold higher than E_2 concentrations in plasma of male rats (56,64). Apparently, E_2 concentrations equivalent to the levels at the proestrous morning to afternoon are found in spent media of cultured hippocampal neurons (39,76), indicating that locally synthesized E_2 is released into extracellular space. Strikingly, E_2 release from cultured hippocampal neurons is enhanced by GnRH challenge (112).

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In an earlier study, aromatase activity in some brain regions, such the VMH, amygdala, and BNST, is not affected by castration (124). A recent direct E_2 measurement study in neonatal rats further indicates that E_2 levels in the hypothalamus are independent of circulating gonadal steroids, as E_2 levels in the hypothalamus are approximately 100-fold higher than circulating E_2 and developmental changes in E_2 concentrations (reduction in E_2 occurs between P0 and P2) are unaffected by neonatal gonadectomy/adrenalectomy in both sexes (73). However, it is also possible that a small portion of E_2 synthesis in these brain areas is attributable to the conversion of circulating testosterone. In fact, in the neonatal rat hippocampus castration reduced E_2 levels by 17% (56), although this small decrease by castration again suggests that the majority of E_2 synthesis (nM concentrations) in the hippocampus is independent of circulating gonadal steroids. Collectively, locally synthesized E_2 in the brain at high pM to low nM levels appears to be maintained regardless of circulating steroid levels, although there may be subtle modifications by peripheral steroids depending on brain region, sex, or age. Nonetheless, presently, we do not have any information regarding 1) whether the total E_2 concentration in the hypothalamus is important or 2) whether the source of the E_2 (peripheral vs. central) is important for control of GnRH release. While to date abundant data show that peripheral E_2 modifies neuronal activity in the hypothalamus, the concept of neuroestrogen is newly born. Therefore, coordinated interactions caused between central and peripheral E_2 remain to be investigated.

An important question arises as to whether E_2 synthesized in the brain influences circulating E_2 levels. The result that neonatal gonadectomy/adrenalectomy in male and female rats does not alter plasma E_2 levels (73) support this possibility, at least during an early developmental stage. However, the answer to this question requires further investigations, as the authors of that study (73) did not measure the effects of gonadectomy/adrenalectomy on plasma testosterone and neonatal castration of male rats is well known to eliminate sexual differentiation of the brain,

Emerging evidence suggests that synthesis of neuroestrogens is also regulated by neurotransmitters and neuromodulators. In rat hippocampal slices, application of NMDA results in an increase in the production of E_2 within 30 min *in vitro* (55,64). E_2 concentration in the rat hippocampus also increases in response to stress (99). Exposure of male songbirds to females increases E_2 levels in the forebrain cortex within 30 min *in vivo* without changes in testosterone levels (120). Infusion of glutamate inhibits E_2 neurosynthesis, whereas infusion of GABA stimulates testosterone neurosynthesis in the forebrain cortex of birds within 30 min *in vivo* (120). These time resolutions can be refined, whenever a more sensitive method for detecting E_2 becomes available. Nonetheless, these results indicate that E_2 synthesis occurs rapidly when neurons receive excitatory and inhibitory signals from other neurons, such as glutamatergic and/or GABAergic input.

2. E_2 induces a rapid excitatory action on primate GnRH neurons

There are several models to examine the effects of E_2 on GnRH neurons (145). Among them we have been using cultured GnRH neurons derived from the nasal placode of monkeys at embryonic age (E) 35-37, which are obtained from time-mated pregnancies. Because transgenic monkeys with GFP-labeled GnRH neurons are not yet available, this approach is quite useful for studying the cellular physiology of GnRH neurons in primates. Earlier, we have demonstrated that 1) cultured GnRH neurons from monkeys contain almost no non-GnRH neurons or glia, as they are not present in the nasal placode at this specific developmental stage (146), 2) cultured GnRH neurons undergo maturational changes *in vitro* (79,146), and 3) placode derived GnRH neurons are functional, as transplantation of fetal placode into the infundibular recess of the third ventricle of adult female monkeys, in which the GnRH final common pathway has been lesioned, restores cyclic ovulation (125).

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We first examined the effects of E_2 on firing activity. Using cell-attached patch clamp recording we found that application of E_2 (1 nM) to cultured primate GnRH neurons induces a ~250% increase in action potential firing frequency within a minute (Figure 1A). E_2 also increases the number of action potentials per burst and burst duration. However, E_2 does not change the timing of bursts (interburst interval) nor the cluster pattern in primate GnRH neurons (2). Similar rapid stimulatory E_2 effects on action potential firing rate have also been reported in GFP-labeled GnRH neurons of ovariectomized mice: E_2 (100 pM-100 nM) directly enhances the firing rate of GnRH neurons in a dose responsive manner, whereas 10 pM E_2 indirectly reduces firing activity of GnRH neurons via suppressing the excitatory GABA neurotransmission (22). The authors of that study state that the E_2 effects started within 5 min and were completed between 10 and 15 min after the initiation of E_2 treatment (22).

Next, we examined the effects of E_2 on $[Ca^{2+}]_i$ oscillations (1,67,69,103). Exposure of GnRH neurons to 1 nM E_2 for 10 min causes an increase in the frequency of $[Ca^{2+}]_i$ oscillations starting during the E_2 application and lasting for 40-50 min (Figure 1B). E_2 also increases the number of activated GnRH neurons. The E_2 effects on $[Ca^{2+}]_i$ oscillations are dose dependent, as 10 nM E_2 induces $[Ca^{2+}]_i$ oscillations with a higher frequency and a longer period as well as a higher percentage of activated cells (69). In addition, E_2 increases the number of synchronized $[Ca^{2+}]_i$ oscillations/hour from ~1 event/hour to ~2.7 events/hour after the initiation of E_2 treatment (1), although the frequency of synchronized $[Ca^{2+}]_i$ oscillations is not dose dependent (69). Importantly, the E_2-induced increase in $[Ca^{2+}]_i$ oscillations is not blocked by TTX (1), indicating that E_2 causes rapid action directly on GnRH neurons (see more discussion in section 4). Additionally, treatment with the membrane impermeable E_2, E_2-BSA, and the nuclear impermeable estrogen dendrimer conjugate (EDC) also increases the frequency of $[Ca^{2+}]_i$ oscillations, suggesting that E_2-induced $[Ca^{2+}]_i$ oscillations are a membrane initiated event (1,103). Similar stimulatory effects of E_2, including E_2-BSA conjugates, on $[Ca^{2+}]_i$ oscillations have been reported in cultured mouse GnRH neurons. However, because these neurons are exposed to E_2 for 30 min before recording (142,143) the latency of the response is unclear. Another study of $[Ca^{2+}]_i$ changes in Pericam expressing mouse GnRH neurons shows that at minimum 15 min is required for direct stimulatory E_2 action as well as indirect inhibitory E_2 action transsynaptically mediated through GABA neurons (123). The data in Pericam expressing mouse GnRH neurons indicate that the latency in murine GnRH neurons appears to be longer than that in primate GnRH neurons.

Henceforth, as E_2 induces a rapid excitatory action in primate GnRH neurons, E_2 should also stimulate GnRH release. Indeed, exposure of cultures to 1 nM E_2 for 20 min results in a rapid increase of GnRH peptide release (103). Again, the increase occurs within 10 min of E_2 application and lasts for 40 min (Figure 1C). Moreover, the plasma membrane impermeable E_2-BSA and the nuclear membrane impermeable form of E_2, EDC, both stimulated GnRH release within 10 min, although the duration and amplitude is smaller than E_2 alone (103). A rapid release of GnRH from mouse cultured or GFP-labeled GnRH neurons, comparable to ours, has not been reported. Although the suppressed frequency of GnRH release with a 4-hour treatment of E_2 (17 pM) in GT1-7 cells (102) has been reported, a 4-hour treatment period is difficult to categorize as a “rapid” E_2 action, because most “rapid” E_2 actions occur within minutes. In addition, E_2 application to rat median eminence explants rapidly stimulates GnRH release (33), suggesting that E_2 can cause excitatory action at the GnRH neuroterminals and this observation from nearly 30 years ago is consistent with our observation. Importantly, we recently found that this rapid stimulatory E_2 action on GnRH release was seen *in vivo*, when we directly applied E_2 to the MBH in both ovarian intact or ovariectomized adult female monkeys (66).

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3. Rapid E_2 action is mediated through multiple membrane receptors

The consequence of E_2 action is dependent upon several factors including dosage, timing, spatial aspects, and receptor subtypes or signaling molecules involved. A fundamental question to understanding rapid direct E_2 action on GnRH neurons is which ER(s) mediate(s) E_2 effects. The most commonly studied ERs are ERα and ERβ. Initially, we too expected to find a role of ERα and/or ERβ in the rapid E_2 action in primate GnRH neurons. Surprisingly, however, the ER inhibitor, ICI182,780, fails to block the E_2-induced increase in $[Ca^{2+}]_i$ oscillations and GnRH release (1,103), indicating that neither ERα, nor ERβ, is involved. In a follow-up study, we transfected primate GnRH neurons with siRNA specific to human ERα or ERβ and tested the effects of E_2. Again, exposure to siRNA for ERα or ERβ fails to block the E_2-induced $[Ca^{2+}]_i$ oscillations (67), confirming our previous results. By contrast, in mouse GnRH neurons ERβ appears to mediate the majority of rapid direct E_2 effects (3,19,22,139,142) or alternatively, indirect E_2 effects are mediated through ERα involving presynaptic GABA inputs (19,22,123). Although Sun et al. (139) report that in mouse GnRH neurons E_2 causes a minor direct effect through GPR30, in general, receptors involved in E_2 action in mouse GnRH neurons are significantly different from those in primate GnRH neurons.

To date three nonclassical membrane ERs have been proposed: ER-X (149), GPR30 (148), and STX-R (115). We first investigated the role of GPR30 in rapid E_2 action in primate GnRH neurons. To our surprise, treatment of the GPR30 agonist G1 at 10 nM, but not 1 nM, stimulates $[Ca^{2+}]_i$ oscillations similar to E_2 (103). Additionally, GPR30 knockdown with siRNA blocks both E_2- and EDC-induced $[Ca^{2+}]_i$ oscillations (103). Interestingly, a high dose of ICI182,780 (1 μM) alone elicits an increase in $[Ca^{2+}]_i$ oscillations (103). It has been shown that a high dose (1 μM) of ICI182,780 is an agonist for GPR30 in cancer cells (40). Finally GPR30 is expressed in a subset of adult GnRH neurons in the monkey hypothalamus (~30%, 103).

There are two reasons to believe that the rapid excitatory E_2 action in primate GnRH neurons is mediated by more than one receptor subtype. First, 10 nM E_2 effects on $[Ca^{2+}]_i$ oscillations are larger than those of 1 nM E_2 and GPR30 siRNA reduces but does not completely block 10 nM E 2+ 2 effects on [Ca]$_i$ oscillations (69). Second, a higher dose of G1 is required to elicit a response similar to E_2 (103). Because STX has been shown to elicit changes in hypothalamic neurons through a phospholipase C (PLC) mechanism in mutant mice lacking ERα, ERβ, ERα/ERβ, and GPR30 (115-117), we examined the role of STX-R in 10 nM E_2 action in primate GnRH neurons. To our surprise, STX (10 nM) treatment of primate GnRH neurons elicits changes in $[Ca^{2+}]_i$ oscillations, similar to those with 1 nM E_2 and STX (10 or 100 nM) treatment also stimulates GnRH release in a dose dependent manner (69). Moreover, GPR30 siRNA transfection of GnRH neurons fails to block effects of STX, whereas treatment with ICI182,780, an antagonist for STX-R (115), blocks STX-induced $[Ca^{2+}]_i$ oscillations, suggesting that E_2 action through STX-R in primate GnRH neurons is independent of GPR30 (69). Therefore, multiple receptor mechanisms are clearly involved in mediating rapid E_2 action on primate GnRH neurons (Figure 2).

4. The role of rapid E_2 action in the mechanism of GnRH release

A “rapid” timing of E_2 action is a membrane initiated phenomenon, rather than “long term” E_2 action, which requires nuclear transcription after E_2 binding to ERs. Then a question arises as to what is the physiological significance of rapid E_2 action in the hypothalamus, and more specifically within the GnRH system? It has been proposed that locally synthesized E_2 contributes to acute synaptic formation in the hippocampus, as E_2 increases spine density and enhances long-term potentiation (LTP) and long-term depression (LTD) in

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hippocampal neurons within 30 min (42,91,98), whereas spine density decreases by treatment with an aromatase inhibitor (76). Whether there is a similar function of E_2 in GnRH neurons remains unknown.

Is there any role of rapid E_2 action in the negative and positive feedback control of GnRH release? In ovariectomized female rhesus monkeys, injection of E_2 induces suppression of LH/GnRH release with a latency of 2-4 hours (the negative feedback phase, 20,53,97,156), followed by stimulation of LH/GnRH release with a latency of 24-36 hours, lasting for 36-48 hours in a positive feedback phase (53,80,156). Because in primate GnRH neurons E_2 induces membrane initiated excitatory, not inhibitory, action within 10 min, it is unlikely that E_2 is involved in the negative feedback mechanism. Is it then involved in the positive feedback mechanism? We will discuss this further in section 5.

Alternatively, is there any possible role in GnRH pulse generation? As discussed earlier, our observations with $[Ca^{2+}]_i$ dynamics *in vitro* consistently show that E_2 is a potent frequency modulator of GnRH neurons. Before we present our view, however, we need to discuss several issues.

Species differences

There are clear species differences in the preovulatory and E_2-induced GnRH and gonadotropin surges. As discussed in a recent review by Plant (110), in rodents the preovulatory GnRH surge is controlled by the AVPV and circadian signals, whereas in highly evolved primates (old world monkeys and humans) the medial basal hypothalamus is sufficient for cyclic ovulations and the preovulatory LH surge is independent of circadian signals. Moreover, the male rodent brain is sterilized by the perinatal elevation of estrogens aromatized from androgens and thus E_2 is not able to induce a surge in castrated males. In contrast, the capacity of the male primate hypothalamus for the preovulatory LH surge remains, as the GnRH neuronal system is not sterilized by prenatal/perinatal gonadal steroids (104). Finally, while a neuronal signal for GnRH/LH surges in rodents is limited to the pentobarbital sensitive critical period of 2h (36,37) and surges last for 4-6 hours (136), in primates there is no critical period for the GnRH/LH surges and surges last for over 36-48 hours (53,80,153,156). Importantly, as discussed above, direct rapid excitatory E_2 effects on GnRH neurons in non-human primates are mediated by GPR30 and STX-R, whereas in mice ERβ appears to be responsible (19). It is also important to point out the fact that the promoter region of the GnRH gene in humans contain an ERE, whereas the rodent GnRH gene does not (118), indicating that genomic E_2 actions through classical ER in rodent GnRH neurons are likely through interneurons. In primates, however, genomic E_2 actions through classical ERs in GnRH neurons can occur both directly or indirectly.

Concentration

Concern has been cast regarding the doses of 1-100 nM E_2 used in *in vitro* studies examining the effects of E_2 on GnRH neurons (50). In our studies in non-human primates the doses at 1-10 nM E_2 were routinely used (1,2,67,69,103) and in electrophysiological studies with acute brain slices or embryonic GnRH neurons in rodents the doses of E_2 as high as 100-1000 nM were used (22,139,142). Certainly, these doses are higher than circulating E_2 levels during the ovulatory cycle, when E_2 levels fluctuate from pM to low nM (0.1-0.2 nM in the follicular phase and 0.7-1.4 nM at the preovulatory surge) in female monkeys (106,107) and 20 pM in diestrus and 200 pM at the preovulatory surge in female mice and rats (136, also see 25). However, as discussed in section 1 and Table 1, the adult rat hypothalamus, hippocampus, and other brain regions of both sexes contain E_2 at high pM to low nM levels and our preliminary data indicate similar E_2 levels in the female rhesus monkey hypothalamus as well as in microdialysate samples obtained from the stalk-median

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eminence *in vivo* (B.P. Kenealy and E. Terasawa, unpublished data). Therefore, E_2 at 1-10 nM in the hypothalamus is not likely "supraphysiological". How about 100-1000 nM used in rodent electrophysiology and culture studies? Peak concentration of E_2 in the synaptic cleft could reach 100 nM or even higher, when E_2 is released as a neurotransmitter. In fact, it has been shown that calculated peak concentrations of the neurotransmitters glutamate and GABA are much higher than those in tissue concentrations measured by a microdialysis method. That is, in the synaptic cleft, glutamate and GABA concentrations reach 1.1 mM (24) and 30-100 mM (47,61), respectively, whereas concentrations of glutamate and GABA in the hippocampus and striatum measured with *in vivo* microdialysis methods are 1-4 μM (6,85,105) and 25 nM in hippocampal brain slice preparation (52), respectively.

Receptors

The classical positive and negative feedback effects of E_2 are mediated by mechanisms through ERα, requiring nuclear transcription. First, E_2 fails to induce LH surges in ERα knockout mice as well as in mutant mice lacking estrogen response element (ERE)-dependent ERα signaling (21,46). By contrast, in ERβ knockout mice E_2 induces LH surges (154), although these mice had ERβ splice variants (75), therefore, reexamination of the role of ERβ in positive feedback in mice with complete elimination of ERβ splice variants (5) is still needed. Second, whereas convincing evidence for the presence of ERα in GnRH neurons has not been shown, the presence of ERα in interneurons that innervate GnRH neurons, such as kisspeptin, have been consistently reported (43,94,135). Taken together, while it is possible that membrane ERα signaling may be, in part, responsible for negative feedback effects of E_2 on LH release, as E_2 can suppress LH levels in mice lacking ERE-dependent ERα signaling (46), ERα expression in interneurons, such as kisspeptin neurons (94) that directly innervate GnRH neurons, is indispensable for LH/GnRH surges. Little is known about ERs involved in the feedback actions of E_2 in primates. Nonetheless, this does not preclude the role of rapid E_2 action in the positive feedback mechanism, as discussed in section 5.

Role of non-neuronal cells

Because our GnRH cultures contain non-neuronal cells (146) and they are involved in the propagation of the $[Ca^{2+}]_i$ wave of the GnRH neuronal network (121), it is possible that E_2 action to GnRH neurons are also mediated through non-neuronal cells. In fact, E_2 rapidly induces a $[Ca^{2+}]_i$ release from astrocytes, stimulates progesterone synthesis within 5 min in astrocytes of rat hypothalamus which are dependent upon both nonclassical and classical receptors (16,78), and modifies tanycyte morphology (70,113). E_2 also rapidly alters function of epithelial cells in the median eminence (31,114). Moreover, E_2 stimulates release of prostaglandin E_2 (PGE_2) from astroglia (107), which directly depolarizes the membrane of GnRH neurons (23). Although we have previously shown that TTX does not block the E_2-induced rapid changes in $[Ca^{2+}]_i$ oscillations (1), this observation does not exclude input from non-neuronal cells. Thus, we need to address this issue in the future.

Time domain

It is important to point out that the negative feedback effect of E_2 on LH/GnRH release precedes its positive feedback effect (144,156). Also, as we have already discussed, the membrane-initiated E_2 action occurs within min, whereas E_2 induced genomic changes require at least several hours to days. Based on this major difference in the time domain of the two modes of E_2 action, it is clear that the positive feedback effects of E_2 are initiated by "genomic action" of E_2. However, this does not preclude the possible role of the membrane-initiated rapid excitatory E_2 action in augmenting the preovulatory GnRH surge. To extend this issue, while the rapid E_2 action on GnRH release in both *in vitro* and *in vivo* are "brief" (5-20 min exposure of E_2 inducing a brief GnRH increase), E_2 increases from the ovary or

by peripheral administration last several hours to days resulting in suppression (negative feedback) for at least several hours and stimulation (positive feedback) for 24-48 hours of GnRH release (Figure 3A).

5. Two possible hypotheses

As discussed in above, we propose two hypotheses for a physiological role of rapid E_2 action: Hypothesis 1 is a potential role for local E_2 that augments the preovulatory GnRH surge. Hypothesis 2 is that local E_2 contribute to GnRH pulse frequency. At this time, evidence for both hypotheses is circumstantial and direct evidence needs to be shown.

Augmentation of the preovulatory GnRH surge (Figure 3B)

During the preovulatory phase, ovarian E_2 binds interneurons, such as kisspeptin neurons and GABA neurons, in the hypothalamus/ POA, which express ERα (and also ERβ to a certain extent). At the negative feedback phase, the suppression of GnRH release is caused by interneurons. In fact, involvement of both membrane initiated and genomic E_2 action through interneurons during the negative E_2 feedback phase has been reported (46,94). In the initial phase, inhibitory interneurons, such as GABA neurons, may rapidly reduce the activity of GnRH neurons via increasing inhibitory postsynaptic potentials (IPSP, 123) resulting in reduction of GnRH release. E_2 activation of nuclear transcription of GABA neurons leads to an increase in GABAergic activity resulting in a subsequent decrease of GnRH release. At the positive feedback phase, excitatory interneurons, such as kisspeptin neurons, are stimulated by genomic action of ovarian E_2 resulting in facilitation of GnRH neuronal activity and an increase in GnRH release.

Ovarian E_2 may also stimulate E_2 synthesis in interneurons, the phenotypes of which are yet to be determined. Evidence for this stimulation of synthesis is based on E_2 effects on aromatase expression. For example, the promoter region of the aromatase gene contains an ERE and aromatase expression is up-regulated in an *in vitro* culture system (158). In addition, aromatase expression in the rat BST and medial amygdala decreases after castration with adrenalectomy, while treatment with E_2 reverses this decrease (160). Assuming that an E_2-induced increase in E_2 production leads to elevated E_2 release from the neuroterminal/ synaptic junction between an aromatase expressing interneuron and GnRH neuron, the released E_2 could rapidly stimulate GnRH release through GPR30 and/or STX-R in primates and ERβ and/or STX-R in mice. Finally, if the GnRH neuron makes a synaptic connection with the aromatase expressing interneuron, GnRH release at synapses could stimulate E_2 release. Because in the hippocampus GnRH stimulates E_2 release (112), it is not difficult to speculate that a similar GnRH stimulation of E_2 release occurs in the hypothalamus. This hypothetical perpetuating positive feedback loop within the hypothalamus would be quite advantageous in the primate brain, as the preovulatory GnRH surge needs to be sustained for 24-48 hours until the pituitary and ovary properly respond for successful ovulation. Importantly, the termination of the GnRH surge would also be prompt due to the rapid nature of E_2 action. Currently, the mechanism of GnRH surge termination is unknown.

GnRH pulse frequency modulation (Figure 3C)

Considering our knowledge that the frequency modulation of GnRH pulses is essential for proper maintenance of the female reproductive cycle (71), we can extend our hypothesis that local E_2 may modulate pulsatility of GnRH release in a subtle manner. This hypothesis is derived from our observations that E_2 modulates frequency of $[Ca^{2+}]_i$ oscillations and synchronization of $[Ca^{2+}]_i$ oscillations in GnRH neurons *in vitro* (1,69,103). It is also known that E_2 is a potent frequency modulator of LH/GnRH release. Although presently, the

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mechanism of GnRH pulse generation is unclear, coordinated periodical release of GnRH is regarded as synchronized activity among GnRH neurons. In fact, GnRH neurons appear to communicate through dendro-dendritic interactions (15) and recently, we have shown that GnRH is released from the cell body and dendrites (44). Local E_2 could cause its effect through KNDy neurons, kisspeptin, neurokinin B, and ß-dynorphin co-expressing neurons, in the ARC (34,119), a concept that has been proposed as the source of GnRH pulse-generation (93,151). Either way (directly or indirectly), it is possible that locally synthesized E_2 may contribute to the frequency modulation of pulsatile GnRH release *in vivo*.

Conclusion

Estradiol causes direct excitatory action within min in GnRH neurons. This rapid E_2 action is a membrane-initiated phenomenon mediated by non-classical receptors, GPR30 and STX-sensitive receptors in primate GnRH neurons. Surprisingly, preliminary data suggest that the hypothalamus of female monkeys release high pM to low nM levels of E_2, which is significantly higher than circulating E_2 levels and infusion of E_2 at a similar concentration into the medial basal hypothalamus results in stimulation of GnRH release *in vivo* (66). Based on the time course and stimulatory nature, this rapid action of E_2 is not likely involved in the negative feedback phase of the ovulatory cycle. Consequently, we propose two hypotheses that the rapid membrane-initiated E_2 action augments and sustains the positive feedback phase of GnRH release or pulsatility of GnRH release. Specifically, ovarian E_2 initiates the preovulatory GnRH surge through indirect stimulation of interneurons, but neuroestrogens in the hypothalamus augment and sustain GnRH release for a prolonged period by a positive feedback loop between GnRH neurons and E_2 synthesizing neurons in the hypothalamus. Alternatively, neuroestrogens may be profoundly involved in GnRH pulse generation. Certainly, these hypotheses are quite provocative. Nonetheless, if future studies validate these hypotheses, we can make a significant advancement in the field of Reproductive Neuroendocrinology.

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Highlights

* Estradiol induces a rapid excitatory action in primate GnRH neurons
* The rapid estradiol action is mediated by non-classical estrogen receptors
* Possible role of neuroestrogens in control of GnRH release is discussed
* Historical perspective of neuroestrogen in the hypothalamus is reviewed

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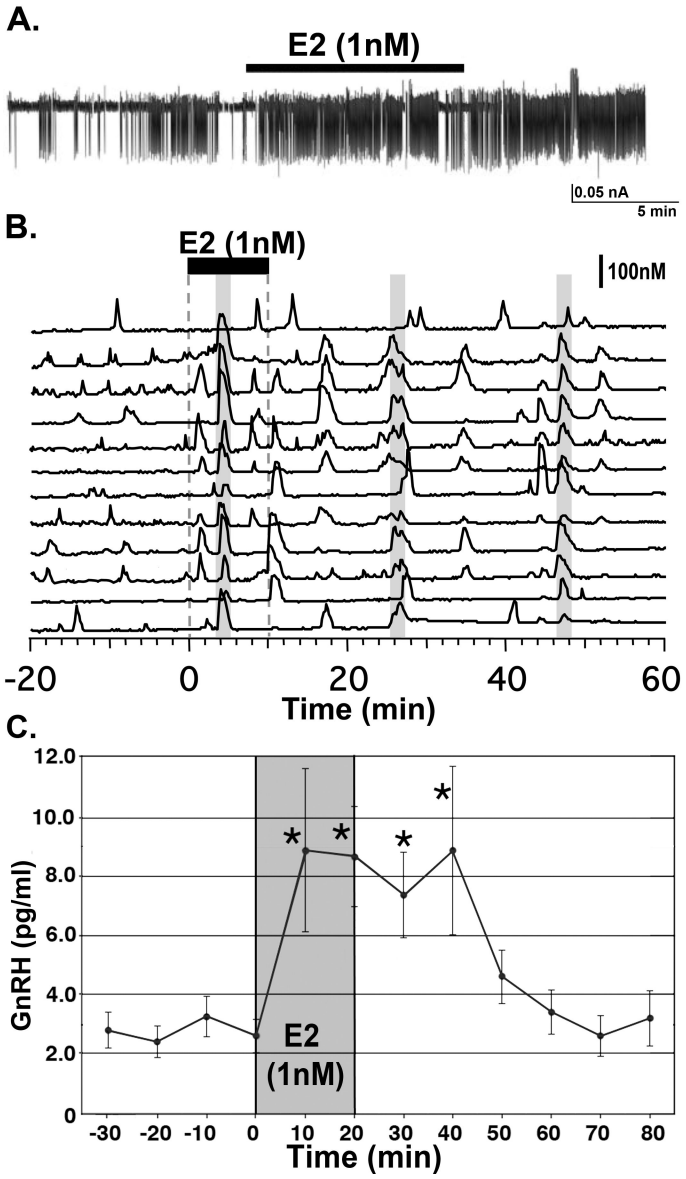

Figure 1.

Application of E_2 induces excitatory firing activity (**A**), intracellular calcium, $[Ca^{2+}]_i$, oscillations (**B**) and GnRH release (**C**) in cultured primate GnRH neurons. E_2 (1nM) was applied to GnRH neurons for 10 or 20 min, as indicated by the black bar (**A**) and (**B**) and gray bar (**C**). Note that in all cases, excitatory effects are induced within 10 min. Gray bars (**B**) indicate synchronization induced by the E_2 treatment. Modified from 1,2,103, permission pending.

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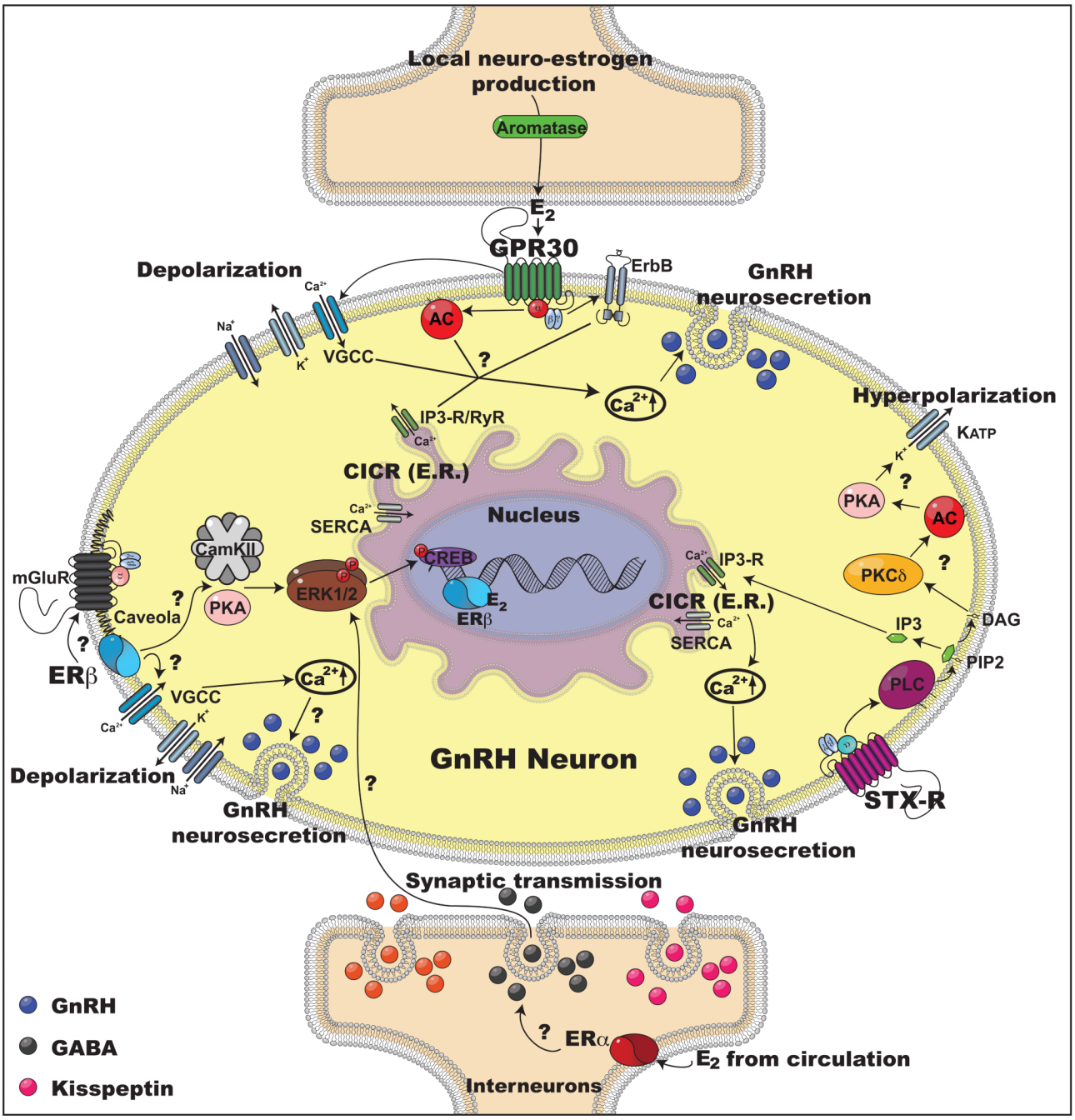

Figure 2.

Schematic illustration showing two modes of rapid E_2 action in GnRH neurons (**middle**) with two synapses (**Top** and **Bottom**). Assuming E_2 is released at the synaptic cleft, there are direct (top) and indirect (bottom) rapid E_2 actions. **Top**: E_2 released at the synaptic cleft **directly** activates GPR30 and STX-R in the primate hypothalamus (and ERβ in the rodent hypothalamus) and modulates activity of GnRH neurons. Exposure of primate GnRH neurons to E_2 rapidly induces $[Ca^{2+}]_i$ oscillations and GnRH peptide release (vesicles in blue color) within 10 min (69,103). Two possible mechanisms for the rapid E_2 action through GPR30 and STX-R are discussed in this article, although many details are yet to be

clarified, which are noted by question marks in the scheme. First, E_2 binding to GPR30 may induce activation of two intracellular pathways: 1) E_2 activation through GPR30 depolarizes the GnRH neuronal membrane via VGCCs (139), which allows $[Ca^{2+}]_e$ entry, resulting in CICR (68) and 2) E_2 transactivates AC and/or ErbB pathways (41), which also results in CICR. Second, E_2 binding to STX-R appears to cause 1) activation of CICR through a PLC and IP3-R mechanism leading to a $[Ca^{2+}]_i$ increase (69) and 2) activation of a PKCδ-AC-PKA mechanism resulting in hyperpolarization of the GnRH neuronal membrane through KATP channels (159), which are essential for burst firing of GnRH neurons, hence neurosecretion. Direct E_2 action through ERß has also been reported in mice GnRH neurons (19,139). **Bottom:** Rapid action of E_2 may be **indirect**, transsynaptically mediated by other neural input, as shown by Romano et al. (123). In this case, E_2 rapidly stimulates GABA release (vesicles in black color), which causes IPSPs in mouse GnRH neurons, resulting in suppression of GnRH release. Alternatively, it is possible that E_2 rapidly stimulates excitatory interneurons (vesicles in magenta color), which would cause EPSPs in GnRH neurons, resulting in stimulation of GnRH release. Abbreviations: AC: adenylyl cyclase; Ca^{2+}: calcium; $[Ca^{2+}]_e$: extracellular Ca^{2+}; $[Ca^{2+}]_{i:}$ intracellular Ca^{2+}; CamKII: Ca^{2+} calmodulin kinase II; CICR: calcium induced calcium release; DAG: diacylglycerol; E_2: estradiol; E.R.: endoplasmic reticulum; ErbB: epidermal growth factor receptor; ERK1/2: Extracellular signal regulated kinase 1 and 2; GnRH: gonadotropin-releasing hormone neuron; GPR30: G protein coupled estrogen receptor; IP3: inositol triphosphate; IP3-R: inositol triphosphate receptor; KATP: ATP sensitive potassium channel; mGluR: metabotropic glutamate receptor; PIP2: phosphatidylinositol biphosphate; PKA: protein kinase A; PKCδ: Protein kinase C delta; PLC: phospholipase C; RyR: ryanodine receptor; SERCA: Sarco/endoplasmic reticulum Ca^{2+} ATPase; STX-R: membrane estrogen receptor sensitive to STX; VGCC: voltage gated calcium channel.

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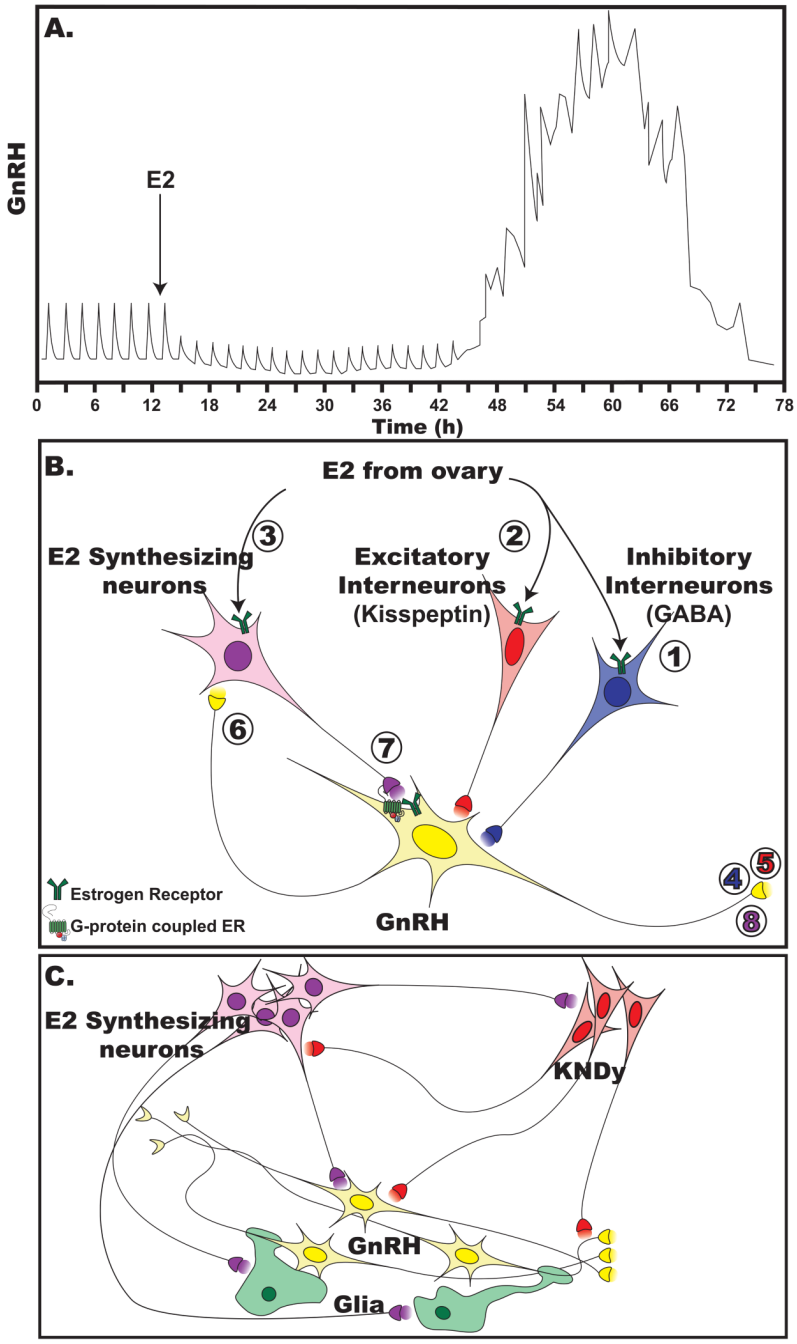

Figure 3.

Schematic illustration showing the effect of ovarian estradiol (E_2) on GnRH release (**A**) and possible roles of neuroestrogens in control of GnRH surge (**B**) and/or pulsatility of GnRH release (**C**) in primates. **A.** E_2 from the ovary suppresses GnRH release (negative feedback phase) with a latency of a couple of hours lasting 24-48 hours. Subsequently, E_2 facilitates GnRH release (positive feedback phase) with a latency of 24-48 hours lasting 36-48 hours. **B.** A possible role of E_2 synthesized in the MBH in the preovulatory GnRH surge. An increase in preovulatory E_2 from the ovary causes action in three types of interneurons to GnRH neurons in the hypothalamus: **(1)** inhibitory interneurons, such as GABA neurons, **(2)**

excitatory interneurons, such as kisspeptin neurons, and **(3)** E_2 synthesizing neurons. Exposure of inhibitory interneurons **(1)** to E_2 inhibits GnRH release **(4)**, initially through a membrane-initiated E_2 mechanism and subsequently through a genomic E_2 mechanism. Over 24 hours after E_2 exposure, activity of excitatory interneurons is stimulated by a genomic action of E_2 **(2)**, leading to the initial increase in GnRH release into the portal circulation **(5)** as well as at the synaptic junction between GnRH neurons and E_2-synthesizing interneurons **(6)**. Although it is possible that neurotransmitter E_2 released from E_2-synthesizing interneurons starts to stimulate GnRH release via the membrane-initiated mechanism, synaptic GnRH release may also feedback to stimulate neurotransmitter E_2 release from E_2-synthesizing interneurons **(7)**. This positive feedback loop between GnRH neurons and E_2-synthesizing interneurons sustains GnRH release to the portal circulation **(8)**. Although this figure illustrates input of interneurons (e.g., GABA and kisspeptin expressing neurons and E_2 synthesizing neurons), to GnRH cell bodies, interaction between GnRH neurons and interneurons could also occur at the neuroterminals. **C.** A possible role of E_2 synthesized in the MBH in pulsatility of GnRH release in primates. E_2 synthesized in the MBH may modulate GnRH neurons directly or indirectly through KNDy (kisspeptin, neurokinin B, ß-dynorphine) neurons and/or glia.

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Table 1

Estradiol (E_2) levels in the brain and plasma

Publications	Species	Brain Regions	Age	Conditions or Challenges	Male E_2 (nM)*	Female E_2	Sex unknown E2	Sample Collection	E_2 Assay
Kawato et al., 2002 Group A	Rat	Hippocampus Hippocampus	Adult	Baseline NMDA (100 μM)	0.67 1.35	- -	- -	Brain slices treated then homogenized tiss	sRIA
		Plasma	Adult	Plasma	0.10	-	-		
Prange-Kiel et al., 2003 Group B	Rat	Hippocampus Hippocampus	Adult, 8 div	Control Letrozole (1 nM)	- -	0.11 0.02	- -	Conditioned medium from primary culture	RIA
Kretz et al., 2004 Group B	Rat	Hippocampus Hippocampus Hippocampus Hippocampus	P4-7, 8 div	Control Letrozole (10 pM) Letrozole (1 nM) Letrozole (100 nM)	- - - - -	- - - - -	0.09 0.06 0.03 0.03	Conditioned medium from brain slice cultur	RIA
		Hippocampus Hippocampus	P4-7, 8 div	Control Letrozole (100 nM)	- -	- -	0.07 0.06	Conditioned medium from primary culture	
Hojo et al., 2004 Group A	Rat	Hippocampus Hippocampus	Adult	Baseline NMDA (100 μM)	0.59 1.27	- -	- -	Brain slices treated then homogenized tissues	RIA
Amateau et al., 2004 Group C	Rat	Frontal cortex Hippocampus Hypothalamus POA Cerebellum Brainstem	P 2h	Intact	6.55 6.26 5.79 5.40 4.39 3.35	4.25 6.37 3.20 3.74 3.60 3.09	- - - - - -	Homogenized tissues	RIA
		Frontal cortex Hippocampus Hypothalamus POA Cerebellum Brainstem	P 32h	Intact	4.71 3.74 5.11 2.84 2.91 2.77	4.07 5.25 2.09 1.73 2.59 2.77	- - - - - -		
Fester et al., 2006 Group B	Rat	Hippocampus Hippocampus	P5, 7 div	Control Letrozole (100 nM)	- -	- -	0.32 0.21	Conditioned medium from primary culture	RIA
		Hippocampus Hippocampus	P5, 7 div	Control siRNA StAR siRNA	- -	- -	0.26 0.20		
Remage-Healey et al., Group D	Bird	NCM NCM NCM NCM	Adult	Baseline Female present Male song Glutamate (10 mM)	1.34** 2.57** 2.20** 0.73**	- - - -	- - - -	Microdialysis	ELISA
		Plasma Plasma Plasma	Adult	Baseline Female present Male song	0.16 0.21 0.15	- - -	- - -		

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Publications	Species	Brain Regions	Age	Conditions or Challenges	Male E_2 (nM)*	Female E_2	Sex unknown E2	Sample Collection	E_2 Assay
Prange-Kiel et al., 2008 Group B	Rat	Hippocampus Hippocampus	P5, 8 div	Control GnRH (10 nM)	- -	- -	0.73 0.88	Conditioned medium from brain slice culture	RIA
		Hippocampus Hippocampus Hippocampus	P5, 8 div	Control GnRH (10 nM) GnRH (100 nM)	- - -	- - -	0.73 1.69 2.50	Conditioned medium from primary culture	
Fester et al., 2009 Group B	Rat	Hippocampus Hippocampus Hippocampus Hippocampus Hippocampus	P5, 7 div	Control Letrozole (100 nM) Testosterone (100 nM) Cholesterol (100 nM) Chol (100nM) +Letr (100 nM)	- - - - -	- - - - -	0.07 0.04 0.31 0.64 0.51	Conditioned medium from primary culture	RIA
Munetsuna et al., 2009 Group A	Rat	Hippocampus Hippocampus	Adult	Pair housed Single housed	8.27 12.38	- -	- -	Homogenized tissues	EIA
Hojo et al., 2009 Group A	Rat	Hippocampus Hippocampus	Adult	Intact Castrated	8.44 6.98	- -	- -	Brain slices treated then homogenized tissu	LeCs/MS/MS
		Plasma Plasma	Adult	Intact Castrated	0.01 0.01	- -	- -		
Konkle and McCarthy, Group C	Rat	Hypothalamus Hypothalamus Hypothalamus	E19 E21 P2-4 P60	Intact	14.39 5.40 7.20 3.60	19.79 1.08 7.20 1.80	- - - -	Homogenized tissues	RIA
		Hippocampus Hippocampus Hippocampus	E19 E21 P2-4 P60	Intact	1.73 0.40 0.54 0.05	1.44 0.36 0.45 0.05	- - - -		
		POA	P 2h	Intact	9.90	1.13	-		
		Hypothalamus Hypothalamus	P3 P3	Intact ADX/GDX	9.00 11.69	8.64 7.20	- -		
		Hippocampus Hippocampus	P3 P3	Intact ADX/GDX	0.40 0.40	0.36 0.36	- -		
		Plasma Plasma	P3 P3	Intact ADX/GDX	0.09 0.12	0.07 0.09	- -		
Remage-Healey et al., Group D	Bird	NCM NCM NCM NCM	Adult	Baseline Male song Female visual stimuli Male visual stimuli	1.70** - 3.06** 1.59**	1.50* 2.32* 1.71* 1.47*	- - - -	Microdialysis	ELISA
		Plasma Plasma	Adult	Baseline Male Song	- -	0.12 0.11	- -		

Group A=Kawato lab; Group B=Rune lab; Group C=McCarthy lab; Group D=Schlinger and his former student's lab

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POA: preoptic area, NCM: caudomedial nidopallium

E: embryonic day, P: postnatal day, div: days in vitro

ADX/GDX: adrenalectomized/gonadectomized

RIA: radio immuno assay, ELISA: enzyme-linked immunosorbent assay, EIA: enzyme immunoassay, LC/MS/MS: liquid chromatagraphy/ tandem mass spectrometry

*E_2 concentrations converted to nM using the estimation that 1 ml≈ 1 g wet weight and 1 nM≈ 0.1 fmol/mg wet weight≈ 10 fmol/mg protein (see Hojo et al., 2004 sup

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