Chapter

Regulation of GnRH Release by Neuroestrogens in Non-human Primates

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Abstract

This chapter summarizes recent progress in reproductive neuroendocrinology in rhesus monkeys as a model for humans. First, to characterize properties of primate GnRH neurons, GnRH neurons are isolated from rhesus monkey embryos and cultured, and human GnRH neurons are generated from induced pluripotent human stem cells. Results from cultured GnRH neurons indicate that exposure to estradiol rapidly stimulates firing activity, increases Ca^{2+} oscillations, synchronization of Ca^{2+} oscillations among GnRH neurons, and estrogen-induced GnRH release. Second, we describe a series of findings *in vivo*, showing that neuroestrogens, locally synthesized and released within the hypothalamus, play an important role in reproduction: Blocking neuroestrogens synthesis alters the pulsatility of gonadotropin releasing hormone (GnRH) release, and the estradiol-induced GnRH/LH-surge in ovariectomized females. Third, neuroestrogen recapitulates the rapid estrogen action in the brain. Collectively, these findings indicate importance of neuroestrogens in regulation of the HPG-axis.

Keywords: neuroestrogen, GnRH neuron, primates, hypothalamus, cell culture

1. Introduction

Ovarian derived estrogens play a central role in the hypothalamo-pituitary-gonadal (HPG) axis and female reproductive function. During early to mid-follicular phase of the ovulatory cycle, when circulating estrogen levels are low, estrogens inhibit gonadotropin releasing hormone (GnRH) release from the hypothalamus, and thereby luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland (negative feedback effects). During late-follicular phase, however, an increase in circulating estrogens from the matured follicle facilitates release of GnRH, LH and FSH (positive feedback effects), resulting in ovulation. While the importance of ovarian estrogens in regulation of reproductive cycles is well recognized, the role of estrogens synthesized in the brain is less appreciated. In 1971, Naftolin et al. reported aromatase enzyme activity in the human brain by observing conversion of radioactively tagged androstenedione to estrone (E_1) and 17β-estradiol (E_2) in tissue homogenates of the median eminence in the hypothalamus, [1] suggesting the *de novo* synthesis of estrogens in the brain. Since then, the role of neuroestrogens in brain fuction has been studied in a wide variety of animals [2, 3]. The presence of aromatase enzyme that converts

androstendione and testosterone to estrone and estradiol, respectively, is identified in cell bodies, axons, and axo-terminals of neurons in a variety of species including humans and non-human primates [4–6]. Hence, the term *neuroestrogen* was established to represent estrogens produced by neurons, and the role of neuroestrogens in the brain function has been investigated for over 50 years. For example, Lu and Brann et al., reported significant memory deficits and diminished astrocyte mediated neuroprotection in forebrain aromatase knock-out mice [7, 8]. Zhou et al. described that treatment of mice with aromatase inhibitor resulted in impaired hippocampus synaptic plasticity and cognitive deficits [9]. In the hypothalamus, the presence of aromatase mRNA and protein, as well as aromatase activity are described, and the significance of neuroestrogens in the hypothalamo-pituitary-gonadal-axis has been reported [6, 10–14]. We have been studying whether and how neuroestrogens are involving in regulation of GnRH release for some time. In this chapter we summarize our recent findings.

2. E_2 rapidly and directly stimulates GnRH release from GnRH neurons *in vitro*

Until ~25 years ago, it was widely believed that the genomic action of estrogens on GnRH neurons was indirectly mediated through other neurons or glia. This concept was based on the observation that nuclear estrogen receptor expression was absent in GnRH neurons [15]. We have made a breakthrough. Toward investigating GnRH regulation at the molecular and cellular levels we developed a primary cell culture system of GnRH neurons. Knowing GnRH neurons originate from the embryonic olfactory placode and migrate into the pre-optic area and hypothalamus during the early embryonic stage [16], we dissected out olfactory placode and the migratory pathway of GnRH neurons from time-mated monkey fetuses and cultured them [17]. Under the microscope GnRH neurons were easily identifiable, as they exhibited fusiform shape and there were no other type of neurons originating from the olfactory placode at this developmental stage. Using these GnRH neurons, we characterized electrophysiological properties and factors influencing GnRH neuronal activity. As such, it was a huge surprise for us to observe that estradiol induced rapid excitatory action

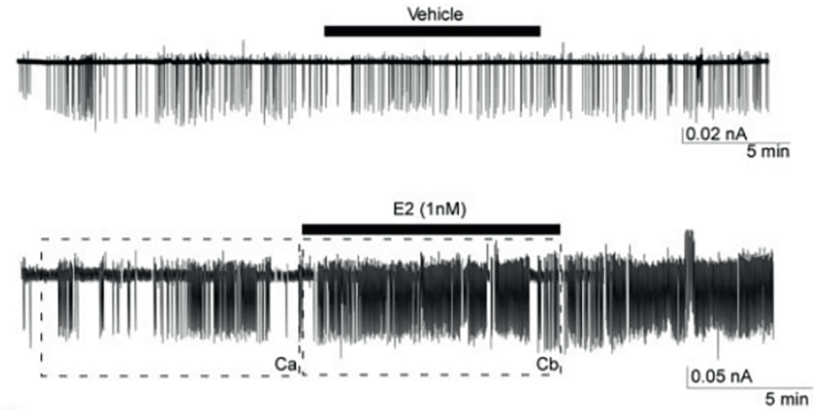

Figure 1.
Example showing the effects of vehicle (top) or estradiol (E_2, bottom) on single unit activity of GnRH neurons. Note that significant increases in firing activity are seen shortly after the start of exposure to 1 nM estradiol, but not vehicle. Figure adapted from Abe et al. [18].

when applied to cultured monkey GnRH neurons. In fact, when estradiol was directly applied to GnRH neurons, single unit firing activity with patch-clamp recording, and synchronization of intracellular calcium oscillations among GnRH neurons were dramatically increased [17, 18]. More specifically, exposure of GnRH neurons to estradiol (1 nM) for 10 minutes resulted in robust increases in firing frequency, as shown by **Figure 1** [18]. The latency of increased firing rate was 60 and 120 seconds after the estradiol application in the media, and the response lasted for as long as patch clamp seal integrity was maintained, up to 25 minutes, 15 minutes beyond removal of estradiol from the media [18]. Using Ca^{2+} imaging, we also found that application of 17β-estradiol (1 nM) to the culture media resulted in increased frequency of intracellular Ca^{2+} oscillations within minutes, and enhanced synchronization of Ca^{2+} pulses among GnRH neurons [19, 20], as illustrated by **Figure 2**. These observations clearly indicate estradiol induces a rapid, direct, excitatory action. Do these cells release the decapeptide GnRH in response to estradiol? To answer this question, we used a perifusion system in which media was continuously infused, while perifusates were collected at a 10-minute interval for several hours. As shown in **Figure 3**, we found that treatment with estradiol (1 nM) resulted in a significant increase in GnRH decapeptide release within 10 minutes and elevated GnRH release remained throughout the entire 20-minute estradiol treatment, and began returning to baseline levels approximately 30 minutes after estradiol removal. In contrast, vehicle treatment induced no effect [21]. These *in vitro* experiments demonstrate that estradiol rapidly stimulates

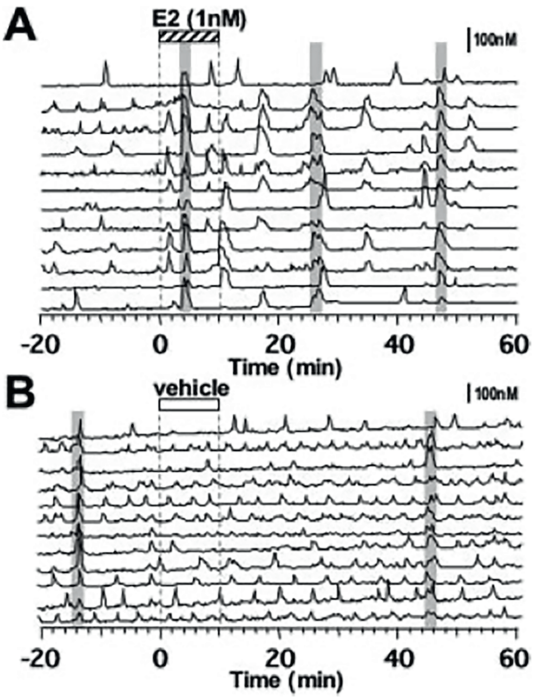

Figure 2.
Effects of estradiol (E_2, A) and vehicle (B) on Ca^{2+} oscillations and synchronization among cultured GnRH neurons. Each line represents the Ca^{2+} signal from a sigle GnRH neuron. Note that after the intial 20 min control recording, either E_2 or vehicle was infused for 10 min, and recording was continued an additional 60 min. Vertical gray bars indicate the synchronized Ca^{2+} increases among recorded GnRH neurons. Figure adapted from Abe et al. [19].

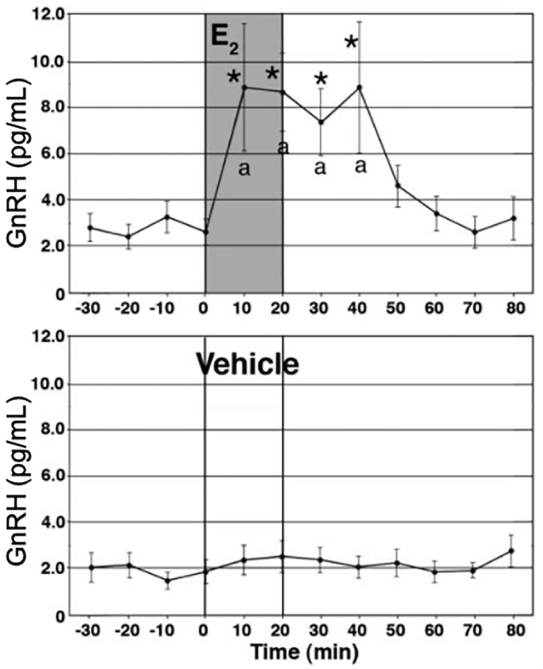

Figure 3.
In vitro *treatment of GnRH neurons with estradiol (E_2) for 20 min (top), but not vehicle (bottom), resulted in significant increases in GnRH release. The gray bar indicates E_2 treatment.* *$P < 0.05$ *versus before* E_2. *a,* $P < 0.05$ *versus vehicle at the corresponding time. Figure adapted from Noel et al. [21].*

firing activity, frequency of the intra-cellar Ca^{2+} oscillations, synchronization of Ca^{2+} oscillations among GnRH neurons, and decapeptide GnRH release.

3. Mechanisms of estrogen action in GnRH neurons *in vitro*

What is the mechanism initiating the rapid and direct estrogen action on GnRH neurons? We began to examine this question by treating cells with ICI182,780 (ICI), an ERα and ERβ receptor antagonist. Using patch clamp recording, we found that administration of ICI completely blocked estradiol-induced increases in firing activity [18], while failed to block estradiol-induced increases in intracellular Ca^{2+} oscillations and synchronization of Ca^{2+} oscillation among GnRH neurons [19]. Importantly, ICI failed to significantly attenuate estradiol-induced increases in GnRH release [21]. We next investigated possible estrogen action through ERα and ERβ by treating these cells with ERα and ERβ siRNA prior to E_2 treatment [20]. The results with qPCR indicated knockdown of respective receptor mRNA by >90% confirming the effectiveness of the treatment [20]. Results of intracellular Ca^{2+}imaging, however, indicate that, similar to ICI, siRNA knockdown failed to alter E_2-induced changes in intracellular Ca^{2+} activity [20]. Taken together, these findings indicate that rapid estrogen action resulting in alteration of intracellular Ca^{2+} oscillations and peptide release in GnRH neurons are independent of the mechanism mediated through ERα and ERβ. Interestingly, changes in cellular firing pattern, assessed by patch clamp recording, were sensitive to ICI, suggesting the presence of a mechanism for rapid excitatory estrogen action mediated through ERα and/or ERβ, which does not lead to rapid release of GnRH peptide.

Since rapid excitatory estrogen action resulting in GnRH release is not mediated through ERα or ERβ, how did estradiol induce its action on GnRH neurons? Toward approaching this question, we next investigated whether E_2 initiates its actions at the cell membrane, cytoplasm, and/or nucleus. We treated cells with estrogen dendrimer conjugate (EDC), which is unable to pass through the nuclear membrane. Administration of EDC, but not dendrimer conjugate itself as a control, induced similar effects as estradiol on Ca^{2+} imaging including significant increases in Ca^{2+} oscillations, and increased synchronous oscillations among cells [19]. Treatment of GnRH neurons with EDC also resulted in significant elevations in GnRH release [21]. The similar effects of EDC with those of E_2 suggest that the mechanism initiating E_2 action lies within the cytoplasm and/or cell membrane. We next applied estradiol-17 hemisuccinate BSA (E_2-BSA), which cannot cross the cell membrane. Upon treatment, we found that E_2-BSA induced significant elevations in GnRH release, although the increase in response to E_2-BSA was not as large, nor as long lasting as E_2 alone [21]. Collectively, the results from this series of experiments are interpreted to mean that (1) a significant portion of estrogen initiating its action, similar to the recent work of Faure et al. [22] occurs at the cell membrane levels of GnRH neurons, but a minor estrogen action may occur at cytoplasm, and (2) estradiol action at the cell membrane is responsible for stimulation of GnRH peptide release, because cell membrane impermeable estradiol resulted in stimulation of GnRH release.

Accordingly, we have hypothesized that the G protein-coupled receptor 30 (GPR30), a seven transmembrane G-protein receptor, mediates rapid E_2 action in GnRH neurons, as it has been reported that GPR30 mediates membrane action of estrogens in cancer cells [23]. Using RT-PCR we found that GPR30 mRNA is expressed in GnRH neurons in culture as well as in the hypothalamus of rhesus monkeys [21]. In addition, with immunostaining, we observed GPR30 positive expression in a subset of GnRH neurons in the rhesus monkey hypothalamus [21]. To investigate whether GPR30 is involved in rapid estrogen actions, we employed an siRNA knockdown approach similar to that we previously describe for ERα and ERβ. We found that while estradiol stimulated Ca^{2+}oscillations and synchronization among GnRH neurons in cells transfected with control siRNA, siRNA knockdown of GPR30 significantly attenuated, but did not eliminate, these changes [21]. We further examined whether GPR30 mediates rapid estrogen action by applying G1, a specific GPR30 agonist [24]. The results show that treatment of GnRH neurons with G1, but not vehicle, resulted in a significant increase in frequency of Ca^{2+} oscillations, similar to those seen with estradiol treatment [21], although G1 did not significantly affect synchronization of Ca^{2+} oscillations among GnRH neurons [21]. Moreover, G1 treatment facilitated GnRH release (unpublished observation). Taken together, these data are consistent with the hypothesis that E_2, through GPR30, imparts rapid stimulatory action on GnRH neurons.

siRNA knockdown of GPR30 partially, not completely, blocked effects of estradiol, suggesting the possible involvement of additional receptors in rapid estrogen action. We have examined the possible involvement of the receptor encoding diphenylacrylamide (STX). STX is an estrogenic compound and it has been shown to elicit effects similar to those with estrogens on energy metabolism, body temperature regulation, and reproduction, among others [25–27]. Moreover, the receptor for STX (STX-R) is located on the cell membrane, and differs from GPR30, ERα, and ERβ [25–28]. Importantly, STX dependent signaling, including response to the GABA agonist, baclofen, on patch clamp, is not affected by respective mouse knockouts of ERα, ERβ, and GPR30 receptors in POMC neurons in the arcuate nucleus of the hypothalamus [25–28]. Additionally, it has been demonstrated that STX-R is sensitive to

ICI [26]. Accordingly, we hypothesized that a portion of the E_2 effects we observed in cultured GnRH neurons are mediated through STX-R. We tested this possibility with a series of *in vitro* experiments. We found that STX treatment of GnRH neurons *in vitro* at 10 nM and 100 nM resulted in dose dependent GnRH release (**Figure 4**), and increased frequency and synchronizations of Ca^{2+} oscillations among GnRH neurons [29]. These effects were unaffected by siRNA knockdown of GPR30 but were blocked by treatment with ICI, consistent with other studies in which ICI blocked the effects of STX [25–28]. As a known downstream pathway of STX action is phospholipase

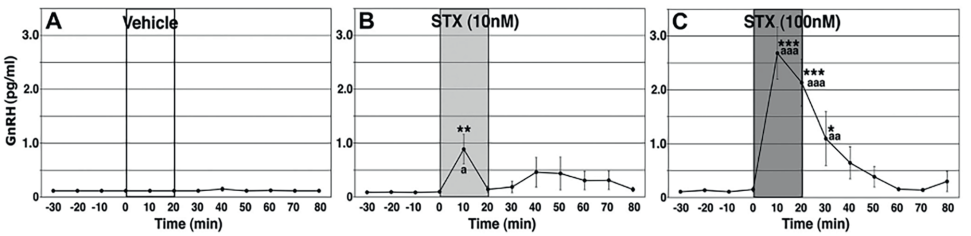

Figure 4.
Exposure of GnRH neurons to STX stimulated GnRH peptide release in vitro *in a dose dependent manner. After a 30-min control period, cells were exposed to vehicle (A), 10 nM STX (B), or 100 nM STX (C) and samples were collected for an additional 80 min.* $^{*}P < 0.05$ *vs. before STX.* $^{**}P < 0.01$ *vs. before STX.* $^{***}P < 0.001$ *vs. before STX.* $^{a}P < 0.05$ *vs. vehicle.* $^{aa}P < 0.01$ *vs. vehicle.* $^{aaa}P < 0.0001$ *vs. vehicle. Figure adapted from Kenealy et al. [29].*

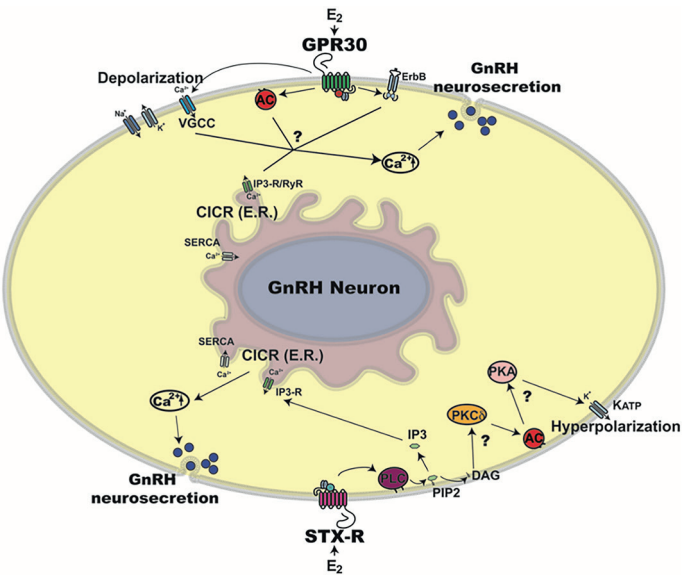

Figure 5.
This schematic illustration summarizes the mechanism of direct estradiol (E_2) action on the GnRH neuron. Estradiol (E_2) interacts with the membrane receptors GPR30 and STX-R, which ultimately lead to changes in intracellular Ca^{2+}oscillations. Question marks in the schema represent speculative mechanisms that require experimental validation. Abbreviations: GPR30, G protein coupled receptor 30; AC, adenylyl cyclase; CICR, calcium induced calcium release; DAG, diacylglycerol; ER, endoplasmic reticulum, ErbB, epidermal growth factor receptor; IP3, inositol triphosphate; IP3-R, inositol triphosphate receptor; KATP, ATP sensitive potassium channel; 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. GPR30, G protein coupled estrogen receptor. Adapted from Kenealy et al. [30].

C (PLC), we examined whether PLC is involved in rapid estrogen action in GnRH neurons. We found that inhibition PLC with U73122 blocked STX effects. Because STX and GPR30 are both G protein-coupled receptors, we reasoned that application of the G protein coupled receptor blocker, pertussis toxin (PTX), would attenuate E_2 effects. Indeed, treatment with PTX blocked E_2 induced increases in Ca^{2+} oscillation frequency and percent of stimulated cells [21].

Collectively, this series of *in vitro* experiments established that E_2 rapidly stimulates intracellular Ca^{2+} activity, and GnRH peptide release in primate GnRH neurons, and these effects are mediated, in part, by membrane G-coupled receptors GPR30 and STX-R, [30] as summarized schematically in **Figure 5**. The results from *in vitro* experiments have a number of implications. First, rapid effects, occurring within minutes, after E_2 treatment indicate a non-genomic mechanism, as they do not require transcription and translation. Second, enhanced synchronization of Ca^{2+} pulses among GnRH neurons following E_2 treatments appears to be an underlying mechanism responsible for synchronization among GnRH neurons *in vivo*. Hence, it is possible that estradiol may modulate coordination of pulsatile GnRH release *in vivo*. Third, and most importantly, the presence of multiple estrogen receptor mechanism on GnRH neurons, rather than a single receptor mechanism, would facilitate a complex regulation of estrogen action.

4. Estradiol infusion into the stalk-median eminence stimulates release of GnRH and LH *in vivo*

In order to examine whether direct stimulatory effects of estradiol on GnRH release occur *in vivo*, we used a microdialysis method. This approach requires initial surgical placement of a cranial pedestal on the monkey skull [31]. With aid of an x-ray ventriculogram obtained during the cranial pedestal implantation surgery, a microdialysis probe is inserted into the stalk-median eminence (S-ME) through the cranial pedestal. **Figure 6** summarizes the setup for microdialysis experiments. This approach has several advantages for cellular study in non-human primates. First, we are able to deliver experimental agents to, and collect samples from, the S-ME, a small specific area in the bottom of the hypothalamus. This approach greatly reduces off-target effects of systemically administered agents while enabling us to directly modify GnRH secretory activity at neuroterminals, and to measure release of GnRH, rather than measuring LH as a surrogate GnRH marker. Second, microdialysis experiments can be performed in unanesthetized conscious monkeys, as they are well-trained in all aspects of the experimental set up. Third, samples can be obtained from the S-ME continuously at a time interval, such as 10–20 minutes, depending on the sensitivity of measurements, for up to a 12-hour period under our animal protocol.

Using the microdialysis method in ovariectomized (OVX) adult rhesus monkeys, we observed that EB infusion (100 nM) directly to the S-ME for 20 minutes stimulated GnRH release within 20 minutes [32]. Moreover, continuous S-ME infusion of EB for 4 and 7 hours, respectively, results in sustained increases in GnRH release and circulating LH concentration for several hours (**Figure 7A**) [33]. S-ME infusion of vehicle resulted in no changes (**Figure 7B**) [33]. In a separate experiment, subcutaneous injection of EB (50 μg/kg) lowered circulating LH for several hours (**Figure 7C**), [33] consistent with previous work from this lab [34], and the widely known negative feedback action of E_2 on GnRH and LH release. The results showing that infusion of EB directly to the S-ME, where GnRH neuroprocesses are

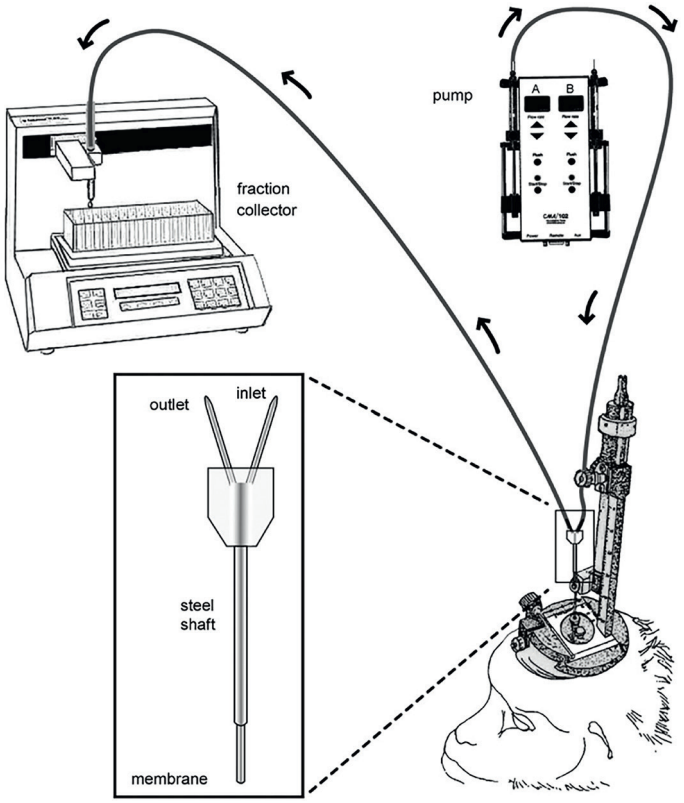

Figure 6.
Schematic illustration of the microdialysis method in the rhesus monkey. A custom-made microdialysis probe (insert) for the rhesus monkey was inserted into the stalk-median eminence of the hypothalamus through a permanently implanted cranial pedestal. The inlet of the probe is attached by tubing to a syringe pump that continuously infuses perfusion fluid, with or without experimental challenges. A probe outlet directs perfusate to a fraction collector for serial sampling and subsequent analysis. Arrows indicate the direction of fluid flow through the microdialysis probe and tubing. Figure adapted from Frost et al. [31].

concentrated, stimulates release of GnRH in adult, OVX, rhesus monkeys. These results are opposite from the effects of subcutaneously injected EB. Nevertheless, as local infusion of a small dose of estradiol mimics the role of neuroestradiol in the hypothalamus, we can speculate that neuroestradiol in the S-ME plays a role in regulation of GnRH release *in vivo*.

The rapid actions of estradiol on GnRH neurons *in vitro* lend support to the role of neuroestrogens as neurotransmitters [5]. The rapid stimulatory effects of estradiol on GnRH neurons observed *in vitro* experiments differs from canonical negative and positive feedback action of estradiol on GnRH release *in vivo*. Hence, similar rapid excitatory effects on GnRH release can be induced by electrical stimulation of the basal hypothalamus, we hypothesized that electrical stimulation also release estradiol. Indeed, application of electrical stimulation of the medial basal hypothalamus led to release of GnRH and estradiol in the S-ME *in vivo* (**Figure 8**), [32] while sham stimulation failed to induce any discernable increases in release of GnRH or estradiol [32].

Based on these results we reasoned that infusion of estradiol into the S-ME would elicit neuroestradiol release *in vivo*. In the experiment, we infused estradiol benzoate

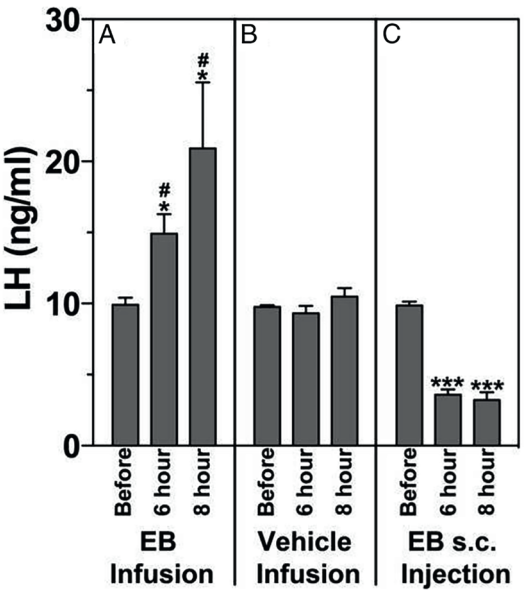

Figure 7.
*(A) Continuous EB infusion into the stalk-median eminence (S-ME) for 7-hour period stimulated prolonged release of circulating LH. (A) Serum samples were obtained before, 6, or 8 hours after the initiation of the 7-hour EB infusion. (B) Vehicle infusion into the S-ME did not change LH levels at the correspondent time. (C) For comparison, the effects of subcutaneous (s.c.) EB injection on LH release is shown. *P < 0.05 compared to before infusion. # P < 0.05 compared to corresponding time with vehicle treatment. ***P < 0.001 compared to before s.c. EB injection. Figure reproduced from Kenealy et al. [33].*

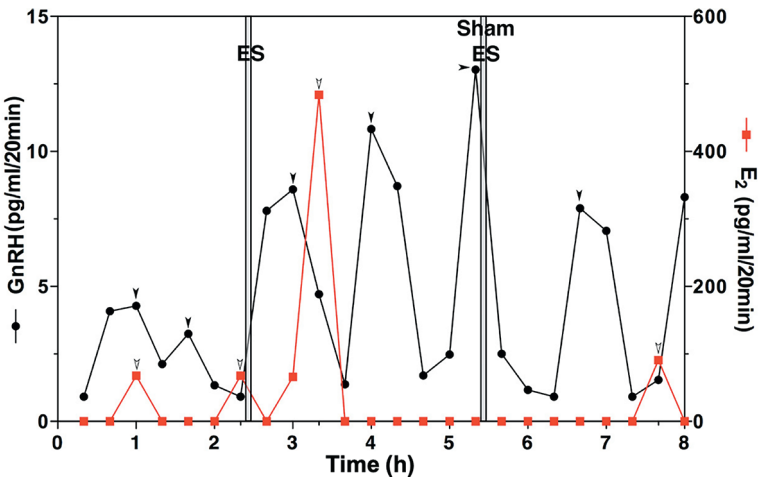

Figure 8.
Electrical stimulation (ES) of the basal hypothalamus, but not sham ES, elicits release of estradiol (E_2) into the stalk median eminence in conscious, adult, ovariectomized rhesus monkeys using microdialysis. Vertical bars indicate time period of ES or sham ES stimulation. Arrows mark peaks of estradiol and GnRH. Figure adapted from Kenealy et al. [32].

(EB) into the S-ME using microdialysis, and assessed changes in estradiol concentrations in dialysates. For measurements of estradiol concentrations, we employed liquid chromatography followed by mass spectrometry (LC/MS), instead of conventional immunologically based methods (i.e., EIA or RIA), because LC/MS differentiates

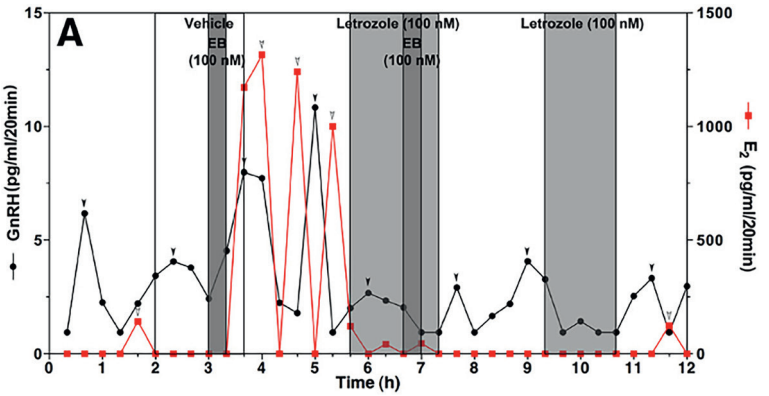

Figure 9.
Administration of estrogen benzoate (EB) by microdialysis to the S-ME of conscious, adult, OVX, rhesus macaques resulted in increases in GnRH release and E_2 release into the S-ME. Co-administration of the aromatase inhibitor letrozole with EB blocked these effects. Administration of letrozole alone had little effect. Dark gray bars represent times of EB infusion. Lighter gray bars represent times of letrozole infusion. White (unshaded) represent times of letrozole vehicle infusion. Figure adapted from Kenealy et al. [32].

between E_2 and EB molecules. The results indicate that EB infusion to the S-ME stimulated rapid release of estradiol as well as GnRH. Interestingly, the release of estradiol was pulsatile with peaks reaching over 1000 pg./mL. Moreover, infusion of EB in the S-ME in the presence of the aromatase inhibitor letrozole failed to increase in release of estradiol and GnRH (**Figure 9**). Collectively, these findings indicate importance of neuroestrogens locally synthesized in the basal hypothalamus in regulation of GnRH release.

5. Involvement of neuroestrogens in regulation of the LH surge

Based on the finding of neuroestrogens' role in GnRH pulsatility, we next investigated whether neuroestrogens play any role in the preovulatory GnRH/LH surge. As the GnRH/LH surge, similar to that seen during the late preovulatory phase, can be induced by a high dose of EB injection in ovariectomized females [32, 33], a group of ovariectomized females were implanted with capsules containing estradiol s.c., and two weeks later, a high dose of EB was injected in the presence or absence of the aromatase inhibitor letrozole. Results indicated that while in the absence of letrozole EB induced LH surges, in the presence of letrozole the EB induced-LH surge was significantly attenuated (**Figure 10**) [35]. Nevertheless, we needed to conduct another experiment, in which the role of hypothalamic neuroestrogens is directly examined, as aromatase enzyme is distributed widely throughout the body and letrozole was administered systemically. Accordingly, we applied letrozole to the S-ME directly and continuously for 6 hours, corresponding to hours 42–48 post-EB injection, the time when the expected EB-induced LH surge occurs. The results indicated that letrozole infusion in the S-ME greatly attenuated the LH surge (**Figure 11**) [35]. Together, these findings clearly indicate the significant role of neuroestrogen during the LH/GnRH surge. Collectively, we propose a modified concept on the positive feedback action of estradiol on GnRH/LH surge generation. In **Figure 12** we have included the role of hypothalamic kisspeptin/neurokinin B/dynorphin neurons, known as *KNDy neurons*, as they are the

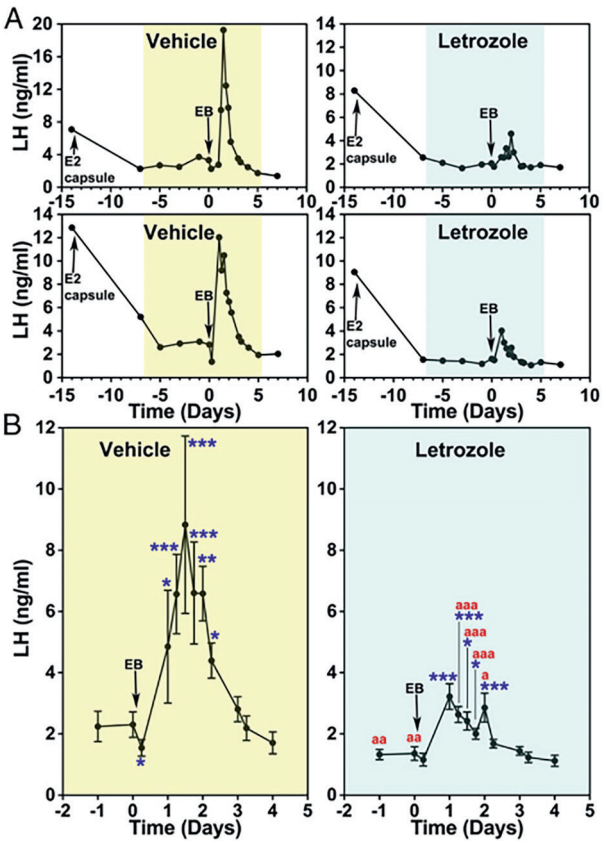

Figure 10.
*Part A shows examples of circulating LH concentration from individual adult, OVX, rhesus macaques in which they received an s.c. E_2 secreting capsule two weeks prior to a single s.c. injection of EB (30 μg) to induce an LH surge. Daily vehicle or letrozole s.c. injections began 7 days prior to, and continued 5 days following the EB injection. Part B shows average LH surge curves averaged across treated animals. Observe the markedly attenuated curves in the letrozole treated animals. *P < 0.05 vs. before EB treatment. **P < 0.01 vs. before EB treatment. ***P < 0.001 vs. before EB treatment. [a]P < 0.05 vs. corresponding time in vehicle treated group. [aa]P < 0.01 vs. corresponding time in vehicle treated group. [aaa]P < 0.001 vs. corresponding time in vehicle treated group. Figure adapted from Kenealy et al. [35].*

major target of circulating estradiol [36–38]. In sheep, rats, and mice, kisspeptin (K), neurokinin B (N), and dynorphin (Dy) colocalize in the same neurons and they are the direct upstream regulator of GnRH neurons. While kisspeptin and neurokinin B initiate a GnRH pulse, dynorphin terminates the pulse. Although the colocalization rate of kisspeptin, neurokinin B, and dynorphin in a single neuron is rare in primates, including humans, [39] the role of KNDy neurons in primates are very similar to those reported in sheep and rodents. For instance, our lab has demonstrated that administration of kisspeptin and senktide (a neurokinin B receptor agonist) to the S-ME via microdialysis, respectively induce rapid and pronounced release of GnRH in rhesus monkeys [40, 41]. KNDy neurons play important roles in sex steroid hormone feedback control. For example, ovariectomy has been shown to increase expression of kisspeptin and neurokinin B, and reintroduction of female gonadal steroid hormones a reduction, consistent with a negative feedback mechanism [42–45]. KNDy neurons also express female sex steroid hormone receptors including ERα. As we have detailed throughout this

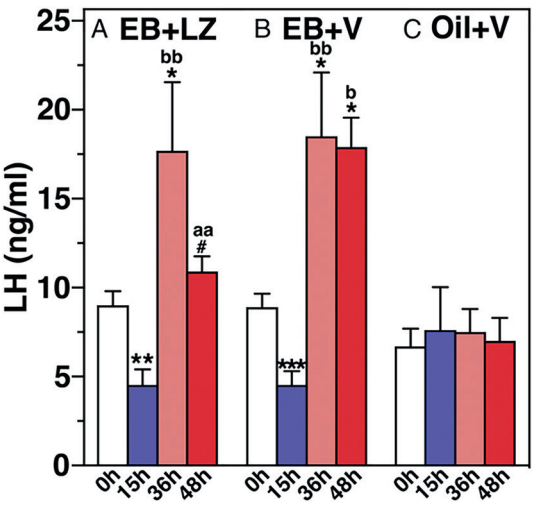

Figure 11.
We tested the effects of letrozole (LZ) administration via microdialysis to the hypothalamic S-ME on hours 42-48 h post administration of an s.c. EB injection, a time of anticipated LH surge. LH was measured from blood samples collected at the time of EB injection, 15 h, 36 h, and 48 h post EB injection. Shown are results for animals that received an (A) EB injection and letrozole infusion, (B) an EB injection with letrozole vehicle infusion, and (C) an EB vehicle (oil) injection with infusion of letrozole vehicle. $^{*}P < 0.01$ *vs. 0 h.* $^{**}P < 0.01$ *vs. 0 h.* $^{***}P < 0.001$ *vs. 0 h.* $^{b}P < 0.05$ *vs. corresponding time in Oil+V group.* $^{bb}P < 0.01$ *vs. corresponding time in Oil+V group.* $^{aa}P < 0.01$ *vs. corresponding time in EB + V group.* $^{\#}P < 0.05$ *vs. 36 h time in EB + LZ group. Figure adapted from Kenealy et al. [35].*

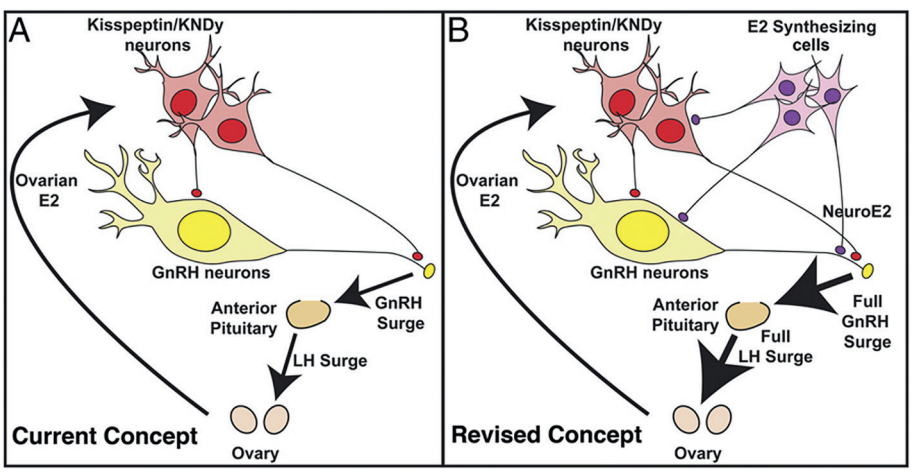

Figure 12.
(A) A schematic illustrating the historically traditional view of E_2 positive feedback in LH surge generation. In this view, ovarian E_2 reaches kisspeptin/KNDy neurons, that express ERα, are stimulated by E_2 and in turn, stimulate GnRH a surge leading to gonadotropin generation of an LH surge. Data from our laboratory suggests a revised concept (B). Ovarian E_2 stimulates kisspeptin/KNDy neurons and E_2 synthesis in the hypothalamus, which together stimulate release of a GnRH surge. It appears that stimulation of GnRH neurons by newly synthesized neuroestrogen is required to generate a full GnRH surge, and consequently, a full LH surge. Figure adapted from Kenealy et al. [35].

text, an underappreciated HPG axis regulatory mechanism is that circulating ovarian estradiol stimulates synthesis of hypothalamic neuroestrogens, which in turn, contributes to feedback loop regulation of the HPG axis.

6. Conclusion

We found, in adult, OVX, rhesus monkeys, that neuroestrogen, synthesized in the hypothalamus in an aromatase-dependent manner stimulates GnRH release when continuously administered for 20 minutes, 4 hours, and 7 hours, and appears required for generation of a full LH surge in our model of pre-ovulatory LH surge. We found that administration of EB to the hypothalamic S-ME induces synthesis of neuroestrogen locally. *In vitro*, we found treatment of GnRH neurons with E_2 to rapidly stimulate Ca^{2+} oscillations and intercellular synchronization and GnRH release. These effects are mediated, at least in part, through membrane G-couple protein receptors STX-R and GPR30. Collectively, these experiments, performed in non-human primates, and many virtually impossible to replicate in humans, position hypothalamic synthesis of neuroestrogen as a key regulator of GnRH release, and hence, the ovulatory cycle.

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Conflict of interest

The authors have no conflicts to disclose.

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