Systems/Circuits

Neuroestradiol in the Hypothalamus Contributes to the Regulation of Gonadotropin Releasing Hormone Release

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Release of gonadotropin releasing hormone (GnRH) from the medial basal hypothalamus (MBH)/median eminence region (S-ME) is essential for normal reproductive function. GnRH release is profoundly regulated by the negative and positive feedback effects of ovarian estradiol (E_2). Here we report that neuroestradiol, released in the S-ME, also directly influences GnRH release in ovariectomized female monkeys, in which the ovarian source of E_2 is removed. We found that (1) brief infusion of E_2 benzoate (EB) to the S-ME rapidly stimulated release of GnRH and E_2 in the S-ME of ovariectomized monkeys, (2) electrical stimulation of the MBH resulted in GnRH release as well as E_2 release, and (3) direct infusion of an aromatase inhibitor to the S-ME suppressed spontaneous GnRH release as well as the EB-induced release of GnRH and E_2. These findings reveal the importance of neuroestradiol as a neurotransmitter in regulation of GnRH release. How circulating ovarian E_2 interacts with hypothalamic neuroestrogens in the control of GnRH release remains to be investigated.

Introduction

Gonadotropin releasing hormone (GnRH) in the hypothalamus is released into the pituitary portal circulation and controls pituitary-gonadal function. Gonadal steroids, in turn, modify GnRH neuronal function via negative and positive feedback action. The concept of negative and positive feedback effects of estradiol (E_2) on GnRH release and gonadotropin release in females, which are known to occur with latencies of ~2 h and over 12–24 h, respectively, has been well established (Ramirez et al., 1964; Ferin et al., 1974; Levine et al., 1985; Mizuno and Terasawa, 2005). In contrast, recent *in vitro* studies from our laboratory and those of others show that brief exposure of cultured GnRH neurons to E_2 induces rapid, direct, excitatory actions, including stimulation of GnRH release within 10 min (Abrahám et al., 2003; Temple et al., 2004; Abe and Terasawa, 2005; Abe et al., 2008; Chu et al., 2009; Noel et al., 2009). This rapid excitatory response of GnRH neurons to a brief E_2 exposure distinctively differs from the classical negative and positive feedback actions of circulating ovarian E_2 on GnRH release.

Recently, the concept that E_2 can function as a neurotransmitter in the brain has gained acceptance (Balthazart and Ball, 2006; Saldanha et al., 2011). This is based on extensive literature detailing (1) rapid actions of E_2 inducing sex behavior in quails and rats; (2) the presence of enzymes necessary for *de novo* synthesis of E_2 in the brain; (3) the presence of aromatase, the enzyme responsible for conversion of androgens into estrogens, in perikarya, and in presynaptic terminals; (4) the rapid timing of E_2 synthesis and release in the rodent and songbird brain; and (5) acute synaptic E_2 actions in the rat hippocampus and songbird auditory system (MacLusky et al., 1986; Naftolin et al., 1996; Cross and Roselli, 1999; Hojo et al., 2004; Cornil et al., 2005; Peterson et al., 2005; Remage-Healey et al., 2008; Mukai et al., 2010; McEwen et al., 2012). While neuroestrogens are better characterized in these behavioral contexts, it is unknown whether neuroestrogens also play a role in regulation of GnRH release. We hypothesized that the rapid excitatory action of E_2 observed in GnRH neurons *in vitro* is indicative of E_2 as a neurotransmitter or neuromodulator of GnRH release *in vivo*.

To test this hypothesis, we used a microdialysis method to examine whether a brief direct infusion of estradiol benzoate (EB), mimicking neuroestradiol in the stalk-median eminence region (S-ME), induces rapid GnRH release in ovariectomized (OVX) female rhesus monkeys *in vivo*. We next examined whether excitation of the S-ME by electrical stimulation (ES) induces release of E_2 in microdialysate samples with liquid chromatography-mass spectrometry (LC/MS). Subsequently, we examined whether brief infusion of EB stimulates release of GnRH and E_2. LC/MS is an excellent tool for measuring neuroestradiol in these studies, as it distinguishes infused EB from E_2 release in the S-ME. Finally, we examined whether local infusion of the aromatase inhibitor letrozole modifies release of GnRH and E_2. The results indicate that neuroestradiol released in the hypothalamus plays a role in modifying GnRH release.

Materials and Methods

Animals. A total of 16 OVX female rhesus monkeys (*Macaca mulatta*; 48.7 ± 4.8 months of age) were used in the microdialysis experiments. All

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animals were born and raised at the Wisconsin National Primate Research Center and housed in pairs (cages 172 × 86 × 86 cm) in a room with a 12 h light/dark cycle and at a controlled temperature (22°C). Animals were fed a standard diet of Teklad Primate Chow (Harlan) twice per day and water was available *ad libitum*. Enrichment, including fresh fruit, was provided daily. The protocol was approved by the Animal Care and Use Committee, University of Wisconsin-Madison in accordance with the guidelines of the National Institutes of Health and U.S. Department of Agriculture.

Experimental design. In Experiment 1, we examined the effects of EB on GnRH release in six animals. After 2 h of control sampling, EB (10 nM) was infused into the S-ME for 20 min, while dialysates were collected at 10 min intervals. GnRH levels in dialysates were measured by RIA. In Experiment 2 we examined whether application of ES to the S-ME modifies release of E_2 and GnRH in nine animals. The methods and parameters for ES were similar to those previously described (Claypool et al., 1990). Briefly, the electrode was connected to the cathode and the cranial pedestal was connected to the anode of a stimulator (Model S-88, Grass Instruments). Pulse parameters were monitored using a cathode ray oscilloscope during ES. After control samples were obtained for a period of 2.5–3 h, ES with monophasic square waves with pulse duration of 0.5 ms and amplitude of 500 μA (baseline to peak) at a frequency of 50 Hz was applied to the S-ME for 5 min at 2–3 h intervals. GnRH levels in dialysates were measured by RIA and E_2 levels were measured by LC/MS or LC/MS/MS. In Experiment 3, we examined the effects of EB on release of GnRH and E_2 in 10 animals. After 2.5–3 h of control sampling, EB at 100 nM (or 10 nM in a few cases) was infused for 20 min through the dialysis probe, while dialysates were collected at 20 min intervals. EB for infusion was sterilized by first boiling EB in a vial (Schering; in oil) in a water bath for 30 min, and then serially diluting to the final concentration with sterile artificial CSF. Boiling and dilution with artificial CSF did not convert EB into E_2 as determined by LC/MS analysis. In Experiment 4, we examined whether the aromatase inhibitor letrozole blocks EB-induced release of GnRH and/or E_2. Letrozole was dissolved in DMSO, sterilized by boiling in a water bath (letrozole is stable at 100°C), and serially diluted to a concentration of 100 nM in artificial CSF. After ≥1 h of control sampling, letrozole or vehicle (control) was infused for a 60 min period before EB infusion for 20 min and letrozole or vehicle control infusion was continued for an additional 20 min after the EB infusion, while dialysates were continuously collected. Two to 3 h after the first challenge with letrozole or vehicle control, a second challenge with either vehicle control or letrozole was infused for 80 min. The order of challenges was randomized. This experiment was conducted in four animals. To conserve limited resources, all 16 OVX monkeys were assigned to several experiments described above.

Cranial pedestal implantation and guide cannula insertion. All animals were well adapted to the primate chair, experimental conditions, and researchers before experimentation. At least 1 month before experimentation, all animals were implanted with cranial pedestals under isoflurane anesthesia as previously described (Gearing and Terasawa, 1988; Frost et al., 2008). On the day of a microdialysis experiment, a microdrive unit was secured onto the pedestal, and a guide cannula with an inner stylet was inserted into the brain 5 mm above the ME as previously described (Frost et al., 2008). For ES, a monopolar electrode, consisting of an insulated stainless steel wire (0.13 mm in diameter), except for a small area ($\sim 0.5 \times 0.5\ mm^2$) of the tip, was attached to the outside of the guide cannula. The length of the guide cannula was increased to 95.5 mm to allow for a stimulation site as close as possible to the sample collection site. In this case, the inner stylet protruded <0.5 mm from the end of the guide cannula. A similar electrode was successfully used for ES of the S-ME using a push–pull perfusion method (Claypool et al., 1990). Ventriculographs were used for positioning the site of stimulation and dialysate sample collection in the S-ME as previously described (Gearing and Terasawa, 1988; Frost et al., 2008).

Microdialysis. Immediately after the guide cannula placement, the monkey was placed in a primate chair. A microdialysis probe (stainless steel shaft 96.0 mm in length, 0.6 mm in diameter), with a polyarylethersulfone membrane (20 kDa molecular mass cutoff, 5.0 mm in length, 0.5 mm in diameter) was inserted into the guide cannula so that the tip of the probe was located in the S-ME as previously described (Frost et al., 2008). CNS perfusion fluid (artificial CSF, 147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$ 0.85 mM $MgCl_2$; Harvard Apparatus) containing bacitracin (4 U/ml) was infused through the influx tubing at 2 μl/min using a 2.5 ml Hamilton syringe (Hamilton) and a CMA/102 microdialysis pump. Dialysates were collected into 12 × 75 borosilicate tubes at 10 or 20 min intervals with a fraction collector (model FC203B, Gilson) for ≤12 h. Dialysate samples collected at 20 min intervals were divided into two vials at 20 μl each, one for measurement of GnRH with RIA and the other for E_2 analysis with LC/MS. RIA buffer (0.3% BSA, 0.01 M PO_4, 0.15 M NaCl, 0.1% NaN_3, pH 7.8) was added to dialysate for GnRH assay. No buffer was added to dialysates for E_2 analysis, as GnRH buffer interferes with LC/MS readings. All samples were then immediately frozen on dry ice and stored at −80°C until assayed. During the experiments, animals were provided monkey chow, fresh fruit, treats, and water *ad libitum*, and allowed in close proximity to a partner monkey for visualization and vocal interaction. After experiments, the microdialysis probe and guide cannula were removed and monkeys were returned to their home cage for ≥3 weeks before another experiment.

In vitro *experiments.* In the first series of *in vitro* experiments, the recovery rate of E_2 was assessed *in vitro* by (1) infusing artificial CSF through microdialysis probes placed in a reservoir containing E_2 at 100 nM while dialysates were continuously collected at 10 min intervals (anterodialysis) or (2) infusing E_2 at 100 nM for 20 min through microdialysis probes into a reservoir containing artificial CSF, with the reservoir changed every 10 min (retrodialysis). In the second series of *in vitro* experiments, possible EB conversion to E_2 *in vitro* was examined, as E_2 increases induced by EB infusion could be due to hydrolysis and/or conversion of EB by an endogenous esterase. We first infused EB at 100 nM for 20 min (2 μl/min speed) through a microdialysis probe in (1) artificial CSF, (2) monkey plasma, or (3) monkey CSF obtained by spinal tap, while dialysates were continuously collected. Detailed methods for the *in vitro* experiment have been described previously (Frost et al., 2008; Noel et al., 2009).

GnRH RIA. RIA for GnRH measurement was conducted using the R42 antiserum provided by Dr. Terry Nett (Colorado State University, Fort Collins, CO) as previously described (Gearing and Terasawa, 1988). Assay sensitivity was 0.02 pg/tube. Intra-assay and interassay coefficients of variation were 8.1 and 11.3% respectively.

Steroid hormone extraction from dialysate samples. Dialysate samples for E_2 analysis were extracted using the following protocol. Samples and standards were diluted in 500 μl of ultrapurified bottled water (Fisher Scientific). For internal standard, 50 pg of deuterated 5 (d5)-E_2 was used. One milliliter of methyl tert butyl ether (Fisher Scientific) was then added, vortexed vigorously, and incubated at room temperature for 5 min. The top organic phase containing steroids was transferred into a new tube with a glass pipette, evaporated to dryness by air stream and heated water bath (60°C), and then resuspended in 100 μl of ethanol and 500 μl of water. A second liquid–liquid extraction was performed with dichloromethane (Fisher Scientific). The lower dichloromethane organic phase containing steroids was transferred into a clean test tube and evaporated to dryness. Samples were then resuspended in 25 μl of $NaHCO_3$ buffer and E_2 was derivitized with 50 μl of dansyl chloride (200 mg/ml in acetonitrile; Fisher Scientific), heated at 40°C for 4 min, and transferred into minivials for LC/MS or LC/MS/MS. Serum E_2 concentrations in 500 μl of samples were similarly extracted and measured with LC/MS.

LC/MS. A 30 μl volume of the extract was injected onto the column (Kinetex 150 × 2.1 mm, 2.6 μm, 100A; Phenomenex). A gradient of purified water with 0.1% formic acid (Solution A) and acetonitrile (Fisher Scientific) with 0.1% formic acid (Solution B) was used at a 200 μl/min flow rate. Gradient of Solution B was increased from 50 to 75% over 5 min, then to 90% for 6–10 min, reaching 100% by 13 min and maintained for an additional 10 min. Subsequently, Solution B was reduced to 50% over 2 min and held at 50% for 10 min to prepare for the next injection. The LC/MS system consisted of an Agilent 1100 series HPLC pump and mass spectrometer, single quad (Agilent). We used positive ionization mode with a voltage of +4000 and single ion monitoring (SIM) mode. Quantitative results were obtained in SIM mode with a response factor for E_2 (506.2), against d5-E_2 [mass-to-charge ratio

(m/z), 511.2]. For E_2 analysis, a qualifier ion was monitored (m/z, 706.5). Standard calibration curves for E_2 concentrations were 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0 pg/tube. (Sigma-Aldrich). Assay sensitivity of this E_2 analysis was 1.85 pg/tube. Extraction recovery, interassay, and intra-assay coefficients of variation determined by a pooled sample of 20 μl of artificial CSF spiked with 160 pg of E_2 were 97.5 ± 0.7, 15.7, and 4.5%, respectively.

LC/MS/MS. Samples were analyzed on a QTRAP 5500 quadruple linear ion trap mass spectrometer (AB Sciex) equipped with an atmospheric pressure chemical ionization source. The system included two Shimadzu LC20ADXR pumps and a Shimadzu SIL20ACXR autosampler. A sample of 30 μl was injected onto a Phenomenex Kinetex 2.6u C18 100A, 100 × 2.1 mm column (Phenomenex) for separation using a mobile phase: water with 1% formic acid (Solution A) and acetonitrile with 1% formic acid (Solution B), with a flow rate of 200 μl/min. Three percent Solution B was held for 3 min followed by 50% Solution B for the next 0.10 min, then maintained for 2.9 min, followed by an increase to 67% Solution B for 15 min and an increase to 100% Solution B over the next 3 min. This was held for 7 min before the system was returned to initial conditions of 3% Solution B over 0.1 min and held for the final 9.9 min of each run.

Mass spectrometer results were generated in positive-ion mode with the following optimized voltages: corona discharge current, 3 V; entrance potential, 10 V. The source temperature was 500°C. The gas settings were as follows: curtain gas, 30 psi; nebulizing gas, 20 psi; collisionally activated dissociation gas, medium. Quantitative results were recorded as multiple reaction monitoring area counts after determination for the response factor for each compound and internal standard. Internal standard was d5-E_2 for E_2. Standard calibration curves for E_2 concentrations were 300, 150, 75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17, and 0 pg/tube. The linearity was $r > 0.9990$ and the curve fit was linear with $1/x$ weighting. None of the compounds of interest were detected in blank or double blank samples. Interassay and intra-assay coefficients of variation determined by a pooled sample of 20 μl of artificial CSF spiked with 160 pg of E_2 were 17.1 and 6.4%, respectively.

Statistical analyses. In Experiments 1–3, means of all data points at 10 min (Experiment 1) or 20 min (Experiments 2 and 3) intervals were calculated. The control period before the treatment was 20 min in Experiment 1 and 60 min in Experiments 2 and 3. In Experiment 4, means of the 80 min period during the treatments and the 60 min period before and after the treatments were calculated in each animal. For statistical analysis, E_2 concentrations measured by LC/MS and LC/MS/MS method are combined, as these two procedures yielded consistent results. In all experiments, E_2 mean values contained undetectable data points as 0 pg/ml. Subsequently, two-way ANOVA with repeated measures followed by Bonferroni's *post hoc* test was applied for statistical comparison. In Experiment 3 we did not include the data from EB concentrations at 10 nM for statistical analysis because we only had two cases. Unpaired two-tailed Student's *t* tests were conducted comparing probe position and interval between two probe insertions versus mean GnRH and E_2 levels, while one-way ANOVA was conducted comparing total number of experiments conducted versus mean GnRH and E_2 levels. One-way ANOVA was also conducted for statistical analysis of Table 2. In Experiments 1–3, animals contributed once to each treatment group. In cases in which >1 of the same challenge was applied, we only included the first challenge. In Experiment 4, each animal contributed to two experiments for the analysis.

EB-induced or ES-induced release of GnRH and E_2 were confirmed as pulses by the Pulsar program (Merriam and Wachter, 1982). Assay variations for GnRH and E_2 were described by equations of $Y = 3.38X + 3.14/100$ and $Y = 0.093X + 0.381/100$, respectively. The cutoff criteria for pulse determination G1, G2, G3, G4, and G5 were 5.78, 2.60, 1.92, 1.46, and 1.13, respectively.

Results

Brief exposure to EB induces GnRH release

Using an *in vivo* microdialysis method in OVX female monkeys, we first examined whether brief EB infusion, mimicking neuroestradiol, to the S-ME induces GnRH release. EB was infused for 20 min through the microdialysis probe, while dialysates were continuously collected at 10 min intervals. The results indicate that EB (10 nM) infusion rapidly stimulated GnRH release (Fig. 1*A*,*C*), whereas vehicle infusion did not induce any change (Fig. 1*B*,*D*). Mean peak latency of the EB-induced GnRH release was 13.3 ± 3.3 min. These results reveal that, similar to our *in vitro* findings, the GnRH system responds rapidly to a transient E_2 stimulus *in vivo* and that neuroestradiol in the S-ME may play a role in GnRH release.

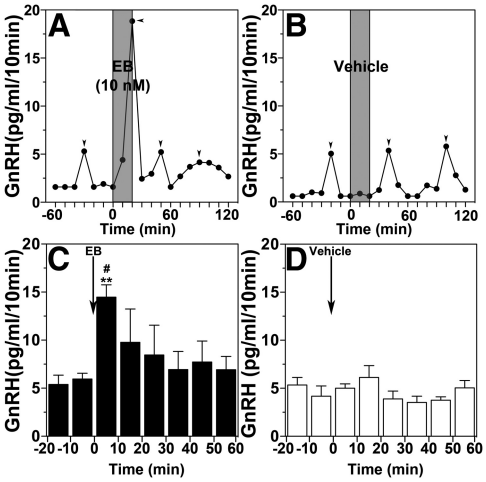

Figure 1. Brief direct infusion of EB into the S-ME induces GnRH release. ***A–D***, Representative cases (***A***, ***B***) and group data (means ± SEM) from EB-infused (***C***) or vehicle-infused (***D***) animals are shown. Time 0 designates the beginning of a 20 min EB or vehicle infusion (gray bars). Two-way ANOVA indicates that EB significantly stimulated GnRH release (***C***) over vehicle control (***D***, $p = 0.0018$). *Post hoc* analysis further indicates that GnRH levels during the first 10 min of EB infusion were significantly higher than those during the control period (***C***; $^{**}p < 0.01$) as well as the corresponding time period of vehicle infusion (***D***; $\#p < 0.05$). Arrowheads indicate pulses identified by Pulsar.

Table 1. Recovery rate of E_2 (100 nM) through microdialysis probes[a]

E_2 recovery through probe membrane (number of experiments)	Peak recovery rate (%)
Anterodialysis/E_2 uptake from reservoir ($n = 3$)	13.13 ± 2.39
Retrodialysis/E_2 infusion ($n = 3$)	10.81 ± 0.52

[a]The recovery rate of E_2 was assessed *in vitro* by (1) infusing artificial CSF through microdialysis probes placed in a reservoir containing E_2 at 100 nM while dialysates were continuously collected at 10 min intervals or (2) infusing E_2 at 100 nM for 20 min through microdialysis probes into a reservoir containing artificial CSF, with the reservoir changed every 10 min.

ES of the medial basal hypothalamus-ME elicits release of GnRH and E_2

If E_2 is released in the S-ME, excitation of the medial basal hypothalamus (MBH)-ME by application of ES should induce E_2 release in OVX monkeys. In fact, NMDA infusion stimulates E_2 release in the rat hippocampus (Hojo et al., 2004), whereas glutamate infusion suppresses E_2 release in the songbird cortex (Remage-Healey et al., 2008). Accordingly, we hypothesized that ES of the MBH-ME, which readily stimulates GnRH release in monkeys (Claypool et al., 1990), elicits increases in E_2 release. To accommodate measurement of GnRH and E_2 in the same samples, we collected dialysates at 20 min intervals and divided each sample in half for GnRH measurement with RIA and E_2 measurement with LC/MS. The peak recovery of E_2 through the microdialysis membrane was ~13% for anterodialysis and ~11% for retrodialysis (Table 1), similar to the recovery of GnRH through the microdialysis membrane (Frost et al., 2008).

ES was applied to the MBH-ME for 5 min at 150–180 min intervals, while dialysates were collected at 20 min intervals. The parameters of ES were based on our previous study (Claypool et al., 1990). For control, the effects of sham ES (the same procedure without passing a current) were examined. Application of ES to the MBH-ME for 5 min promptly stimulated GnRH release within 20–40 min and E_2 release with a 40–60 min delay (Fig. 2*A*).

Statistical analysis further indicated that while ES significantly stimulated GnRH release (Fig. 2, compare *B*, *C*), ES tended to initially suppress E_2 release, followed by a significant increase in E_2 release (Fig. 2, compare *D*, *E*). The suppression of E_2 levels 0–40 min after ES was not significantly different from before ES. E_2 peak levels after the ES challenge were also significantly higher than before the challenge (Table 2). The latency to the first E_2 peak after the initiation of ES was significantly different from the ES-induced GnRH peak latency (Table 2). The interpulse interval (IPI) and pulse duration of ES-induced E_2 release were not different from those of GnRH release (Table 2). Sham ES did not induce any significant effects (Fig. 2*A*,*C*,*E*).

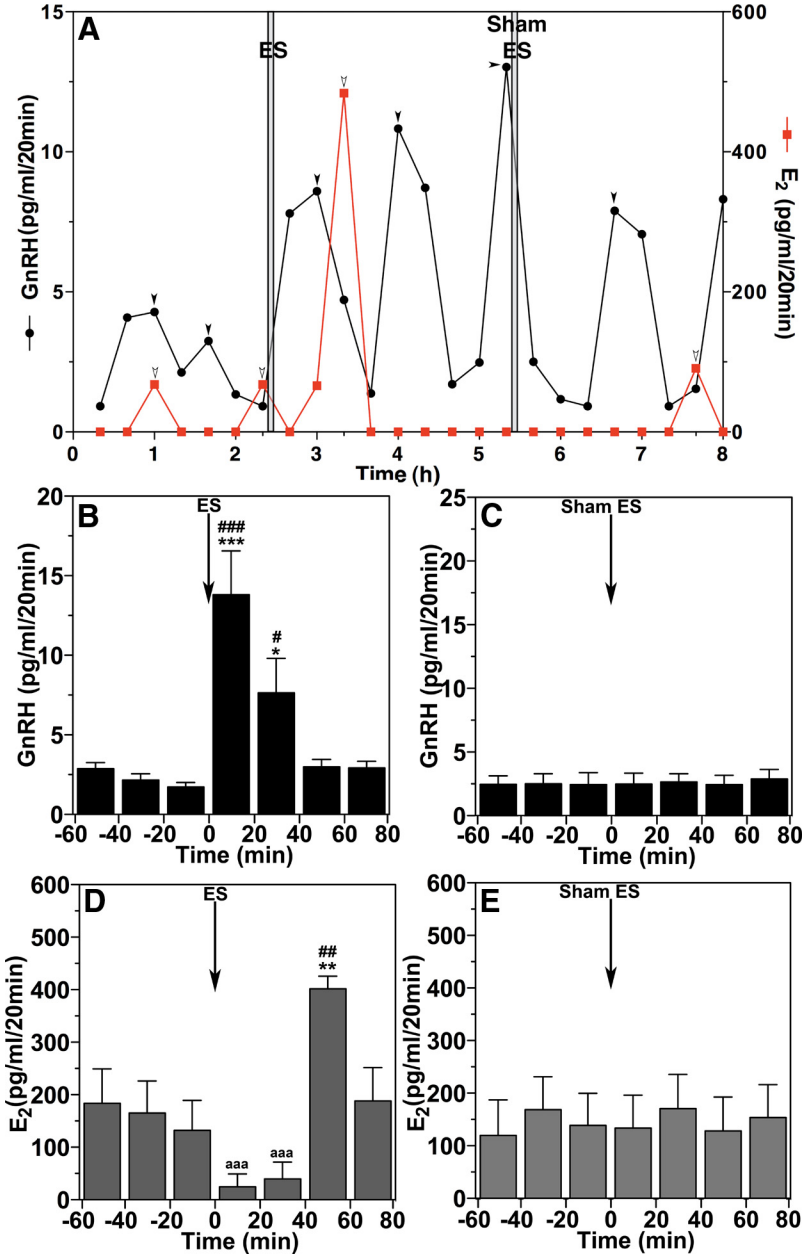

Figure 2. ES, but not sham ES, stimulates GnRH release (black line with filled circle), and E_2 release after an initial suppression (red line with filled square). ***A–E***, A representative case (***A***) and group data (means ± SEM) from ES (***B***, ***D***) or sham ES (***C***, ***E***) are shown. Time 0 designates the beginning of a 5 min ES or sham ES treatment (gray bars). Note that there were GnRH pulses and small E_2 fluctuations before ES application and that ES stimulated GnRH release, which was followed by E_2 release. Sham ES did not induce any significant change. Two-way ANOVA analysis further indicated that ES significantly stimulated release of GnRH (***B***; $p = 0.0006$) and E_2 (***D***; $p = 0.047$) over sham controls (***C***, ***E***). *Post hoc* test indicated that GnRH increases at 0–20 and 20–40 min after the initiation of ES were significantly higher (***$p < 0.001$ and *$p < 0.05$, respectively; ***B***) than before ES application and also significantly higher (###$p < 0.001$ and #$p < 0.05$; ***B*** vs ***C***) than those in sham ES at the corresponding time period. Similarly, the E_2 increase at 40–60 min after ES was significantly higher (**$p < 0.01$) than before ES (***D***) and also significantly higher (##$p < 0.01$; ***D*** vs ***E***) than sham ES at the corresponding time period. Interestingly, the E_2 values at 0–20 and 20–40 min after ES were significantly lower than during 40–60 min ($^{aaa}p < 0.001$ and $^{aaa}p < 0.001$, respectively), but neither value was significantly lower than before ES ($p > 0.05$ and $p > 0.05$, respectively). Arrowheads indicate pulses identified by Pulsar.

EB infusion induces release of E_2

Because brief infusion of EB stimulates GnRH release and ES stimulated both release of GnRH and E_2, we speculated that EB-induced GnRH release might be accompanied by E_2 release. Previous reports indicate that E_2 synthesis occurs within 30 min in the rat and songbird brains (Hojo et al., 2004; Remage-Healey et al., 2008). Accordingly, we assessed the effects of EB (or vehicle) on E_2 release as well as on GnRH release.

Twenty minute infusion of EB (100 nM) into the S-ME in OVX monkeys stimulated GnRH release (Fig. 3*A*), although in this case EB (10 nM) induced only a small GnRH increase, perhaps due to the 20 min sampling period. Group data indicated that mean GnRH release 0–20 and 20–40 min after 100 nM EB challenge was higher than before EB (Fig. 3*C*) and also higher than vehicle during the corresponding time period (Fig. 3*D*). Vehicle infusion did not elicit any changes (Fig. 3*B*,*D*). Significantly higher GnRH peak levels after the EB challenge were also seen when compared with before the challenge (Table 2). The peak latency after EB infusion was significantly shorter than after the initiation of vehicle infusion, but the peak duration and IPI after EB were not different from those after vehicle infusion (Table 2). The longer GnRH peak latency with EB (100 nM) and smaller responses induced by EB (10 nM) in this experiment compared with Experiment 1 was attributable to a longer sampling period (20 vs 10 min).

EB infusion in OVX monkeys elicited oscillatory E_2 release with a peak amplitude of >1 ng/ml (Fig. 3*A*). This is in contrast to the results from vehicle infusion (Fig. 3*B*). In fact, repeated E_2 pulses seen after EB infusion were "induced E_2" release, as during the 150–180 min of control sampling before EB challenges, in OVX animals E_2 pulses were either undetectable (17 of 31 experiments) or a couple of E_2 pulses with a mean peak amplitude of

Table 2. Effects of ES and EB infusion of the MBH-ME region on release of GnRH and E_2 in OVX female rhesus monkeys

Measured neurohormones	GnRH		E_2	
Treatments	ES ($N = 9$)	Sham ES ($N = 9$)	ES ($N = 9$)	Sham ES ($N = 9$)
ES				
Peak before (pg/ml)	3.1 ± 0.4	2.9 ± 1.0	242 ± 63 (5)*	236 ± 37 (4)*
Peak after (pg/ml)	14.0 ± 2.7^{aaa,bbb}	3.3 ± 0.9	437 ± 20^{a,b}	232 ± 72 (4)*
Peak latency (min)	24.4 ± 2.9^{a,ccc}	52.5 ± 10.0	64.4 ± 4.4	44.0 ± 8.4 (4)*
Peak duration (min)	42.2 ± 5.2	44.4 ± 7.3	37.8 ± 5.2	40.0 ± 8.2 (4)*
IPI (min)	66.7 ± 4.7	62.9 ± 8.1	64.0 ± 7.5 (5)*	60.0 ± 8.2 (4)*
Treatments	EB ($N = 10$)	Vehicle ($N = 10$)	EB ($N = 10$)	Vehicle ($N = 10$)
EB				
Peak before (pg/ml)	3.1 ± 0.4	3.7 ± 0.4	281 ± 129 (5)*	265 ± 80 (5)*
Peak after (pg/ml)	14.8 ± 4.3^{aa,bb}	3.3 ± 0.6	1285 ± 285^{a,b}	281 ± 53 (5)*
Peak latency (min)	26.0 ± 3.1^{aa}	58.0 ± 9.6	25.5 ± 2.8^{aa}	48.0 ± 4.9 (5)*
Peak duration (min)	38.0 ± 5.5	44.0 ± 5.8	32.0 ± 4.4	32.0 ± 3.3 (5)*
IPI (min)	62.0 ± 6.0	66.0 ± 5.0	70.0 ± 16.5 (8)*	65.0 ± 12.6 (5)*

Data are mean ± SEM, N: Total number of experiments conducted. ()*: Number of experiments used for calculation; in some experiments sufficient E_2 peaks were not present for calculation.
$^{a}p < 0.05$; $^{aa}p < 0.01$; $^{aaa}p < 0.001$ versus vehicle or sham; $^{b}p < 0.05$; $^{bb}p < 0.10$; $^{bbb}p < 0.001$ versus mean before treatment; $^{ccc}p < 0.001$ versus E_2.

281 ± 129 pg/ml (Table 2) were seen (14 of 31 experiments). Importantly, circulating E_2 is not the source of E_2 in the hypothalamus, as E_2 levels (8 ± 2 pg/ml, $n = 31$) in general circulation measured from the same OVX animals before experiments were far lower than levels in the hypothalamus.

Group data indicated that the EB-induced E_2 release was significantly higher than those during the control period (Fig. 3*E*) as well as the corresponding period in vehicle control (Fig. 3*F*). Significantly higher E_2 peak levels after the EB challenge were also seen when compared with E_2 peak levels before the challenge or vehicle control (Table 2). The peak latency of EB-induced E_2 release was significantly shorter than that after the initiation of vehicle infusion (Table 2), but the peak duration and IPI after EB were not different from those after vehicle infusion. Interestingly, the peak latency, peak duration, and IPI of E_2 release were strikingly similar to those of GnRH release, and none of the E_2 pulse parameters differed from GnRH pulses. Importantly, with the exceptions of peak level and peak latency, the E_2 pulse parameters between EB-induced and ES-induced E_2 changes were similar to each other (Table 2).

An aromatase inhibitor suppresses GnRH release and blocks EB-induced GnRH and E_2 release

After finding that EB induces E_2 release from the S-ME, we next tested whether endogenous E_2 release plays any role in regulating GnRH release by infusion of the aromatase inhibitor letrozole into the S-ME of OVX female monkeys. We first examined whether letrozole infusion modifies the EB-induced GnRH and E_2 release. As a control, vehicle, instead of letrozole, was similarly infused. After collection of control dialysates for 60 min, letrozole (100 nM) or vehicle was infused into the S-ME for 100 min, i.e., letrozole or vehicle infusion was initiated 60 min before a 20 min infusion of EB (100 nM) and was completed 20 min after the EB infusion (Fig. 4*A*). While vehicle treatment did not affect the EB-induced release of GnRH and E_2 (Fig. 4*B*,*E*), letrozole treatment eliminated the EB-induced release of GnRH and E_2 (Fig. 4*C*,*F*). These results indicate that letrozole inhibits the EB-induced release of both GnRH and E_2.

We also examined the effects of letrozole on spontaneous GnRH release. After collection of control dialysates for at least 60 min, letrozole (100 nM) was infused into the S-ME through the microdialysis probe for 80 min, while dialysates were continuously collected at 20 min intervals (Fig. 4*A*). Letrozole infusion not only suppressed GnRH pulses during the drug infusion (Fig. 4*A*), but also significantly suppressed mean release of GnRH (Fig. 4*D*). These results indicate that letrozole suppressed basal release of GnRH. Collectively, local neuroestradiol release in the S-ME in OVX monkeys significantly modifies GnRH release.

Site-specific variation of E_2 release in the S-ME

The distribution pattern of aromatase in the monkey MBH is not uniform (Naftolin et al., 1996). Our previous studies also indicate that GnRH concentrations assessed by push–pull perfusion or microdialysis methods are site-specific (Gearing and Terasawa, 1988; Frost et al., 2008). We did not, however, know which locations were ideal for E_2 release and therefore aimed for an area where GnRH levels are high. Thus, with probe tips positioned using x-ray ventriculographs, we investigated whether there was any variation in E_2 concentrations. Our results were similar to those previously reported (Gearing and Terasawa, 1988). That is, higher GnRH levels were seen 0–1 mm posterior than 0–1 mm anterior (Fig. 5*D*) and 0–1 mm ventral than 0–1 mm dorsal (Fig. 5*F*) from the infundibular recess. There was no difference in lateral positions between 0–0.5 and 0.5–1 mm from the midline (Fig. 5*B*). In contrast, significantly higher E_2 levels were seen 0.5–1 mm than 0–0.5 mm lateral from the midline (Fig. 5*C*), whereas there was no difference in anterior–posterior (Fig. 5*E*) or dorsal–ventral (Fig. 5*G*) position. Thus, there appears to be a location within our sampling area ideal for both GnRH and E_2 measurement that is slightly posterior, ventral, and lateral.

A retrospective analysis of our microdialysis experiments (Table 3) further suggested that neither the period between two microdialysis experiments nor the number of experiments (cannula insertions) were correlated with mean E_2 and GnRH levels.

Examination of possible EB conversion to E_2 *in vitro*

Results from *in vitro* experiments indicate that there was no EB conversion to E_2 in female monkey plasma, nor breakdown to E_2 in artificial CSF. Moreover, in monkey CSF (500 μl) there was no measurable E_2 in two of four monkey CSF samples, whereas a small single E_2 peak was seen in samples obtained 60–80 min after initiation of EB infusion in the remaining two monkey CSF samples, although the peak value of E_2 was small (<100 pg/ml), and the mole-to-mole conversion from EB to E_2 was 0.28 and 0.52% (Table 4). Finally, using a perifusion system as previously described (Noel et al., 2009), we tested whether 20 min EB (100

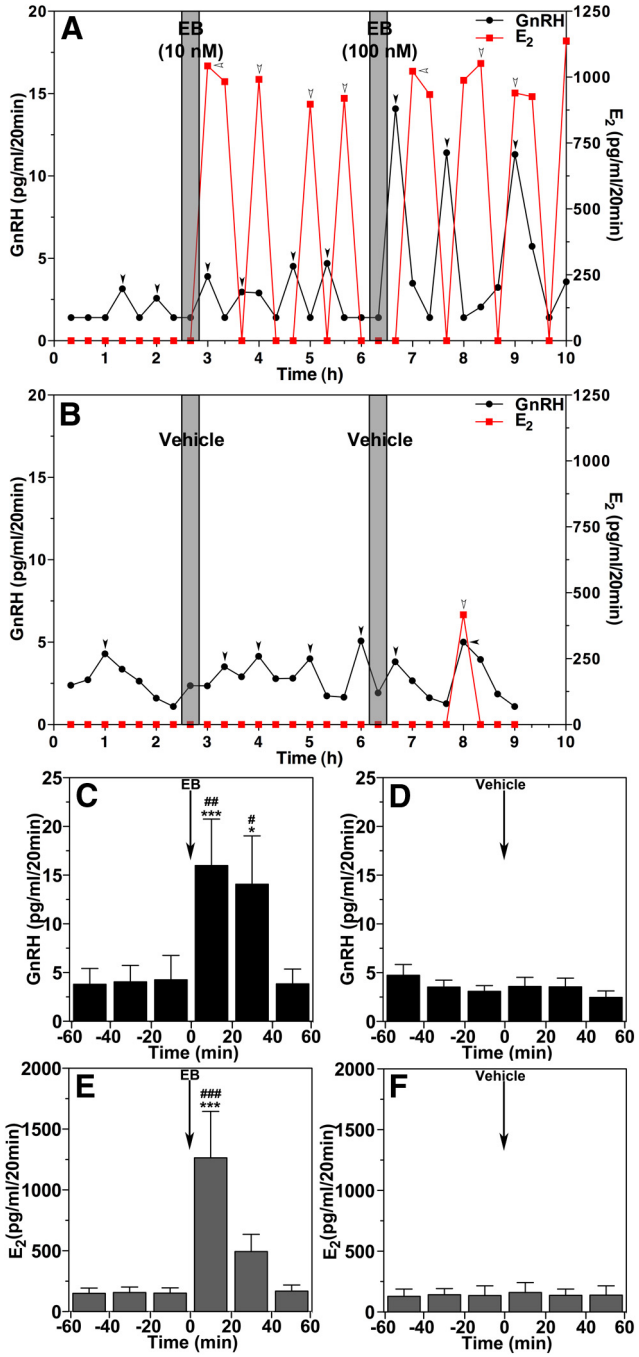

Figure 3. Direct infusion of EB into the S-ME induces release of GnRH (black line with filled circle) and E_2 (red line with filled square) *in vivo*. ***A–F***, Representative cases (***A***, ***B***) and group data (means ± SEM) from EB-infused (***C***, ***E***) or vehicle-infused (***D***, ***F***) animals are shown. Time 0 designates the beginning of a 20 min EB (10 or 100 nM) or vehicle infusion (gray bars). Note that EB induced increases in GnRH release and pulsatile E_2 release (***A***). While the amplitude of GnRH responses appears to be EB dose-dependent, the amplitude of E_2 did not vary. In contrast, vehicle infusion did not cause any significant effects (***B***). In this case, a single spontaneous E_2 peak was seen at 8 h. Two-way ANOVA analysis further indicated that EB (100 nM) significantly stimulated GnRH release (***C***; $p = 0.0098$) as well as E_2 release (***E***; $p = 0.0009$) over respective vehicle controls. *Post hoc* test further indicated that the GnRH increases at 0–20 and 20–40 min after EB infusion were significantly higher (***$p < 0.001$ and *$p < 0.05$, respectively) than before EB infusion (***C***) and the GnRH increase at 0–20 min after EB infusion was also significantly higher (##$p < 0.01$) than after vehicle infusion at the corresponding time period (***C*** vs ***D***). Similarly, the E_2 increase at 0–20 min after EB infusion was significantly higher (***$p < 0.001$) than before EB infusion (***E***) and also significantly higher (###$p < 0.001$) than after vehicle infusion at the corresponding period (***E*** vs ***F***). Arrowheads indicate pulses identified by Pulsar.

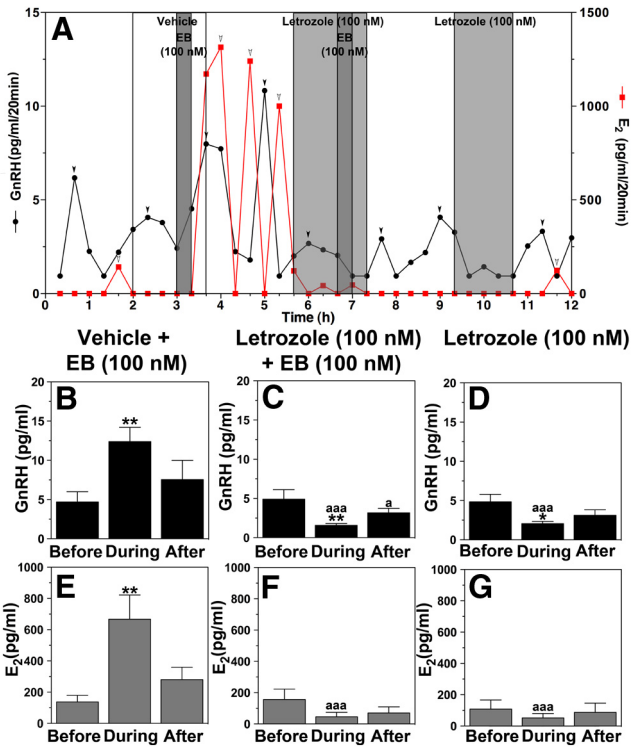

Figure 4. Infusion of letrozole (100 nM, light gray bars) into the S-ME inhibits spontaneous GnRH pulses as well as the EB-induced GnRH and E_2 increases. ***A–G***, An example (***A***) and the results of two-way ANOVA examining the effects of EB (***B***, ***E***), of letrozole on EB-induced release (***C***, ***F***), and of letrozole on spontaneous release (***D***, ***G***) are shown. In ***A***, an EB challenge (100 nM, dark gray bars) was first given for 20 min with vehicle. Subsequently letrozole (100 nM, light gray bars) was continuously infused for 100 min, during which EB (100 nM) infusion was added for 20 min (60–80 min after the initiation of letrozole infusion), and finally letrozole (100 nM) alone was infused for 80 min. Note that control EB infusion stimulated release of GnRH and E_2, whereas EB infusion in the presence of letrozole failed to stimulate release of GnRH or E_2 (***A***). Likewise, letrozole infusion alone blocked spontaneous GnRH pulses (***A***). ANOVA analysis indicated that letrozole in the presence of EB (***C***) or letrozole alone (***D***) significantly reduced mean GnRH release compared with EB control (***B***; $p < 0.0001$). Similarly, letrozole in the presence of EB (***F***) or letrozole alone (***G***) significantly reduced mean E_2 release compared with EB control (***E***; $p < 0.0001$). *Post hoc* test indicated that GnRH levels during control EB infusion (***B***) and during the letrozole infusion with (***C***) or without (***D***) EB were all significantly different from respective control (before) periods (***B–D***; **$p < 0.01$ for EB alone, **$p < 0.01$ for letrozole plus EB, *$p < 0.05$ for letrozole alone). GnRH levels during both letrozole treatments were significantly lower than those during EB with vehicle ($^{aaa}p < 0.001$, $^{a}p < 0.05$ for letrozole plus EB, $^{aaa}p < 0.001$ for letrozole alone). Likewise, E_2 levels during both letrozole treatments (***F***, ***G***) were lower than those during EB control (***E***; $^{aaa}p < 0.001$ for letrozole plus EB; $^{aaa}p < 0.001$ for letrozole alone). E_2 levels during EB with vehicle treatment (***E***) were also significantly higher than before infusions (**$p < 0.01$).

nM) perifusion through hypothalamic tissue (MBH and dorsal hypothalamus) yielded E_2. In two of the three cases, no E_2 was detected while in the third case a small amount of E_2 was detected 2.5 h after administration of EB. Again, the mole-to-mole conversion (0.02%) was small (Table 4). Thus, it is unlikely that the EB-induced E_2 peaks (Fig. 3*A*) occurring *in vivo* with a latency of 26.3 ± 3.2 min were due to EB metabolism to E_2.

Discussion

In this study, we examined the role of neuroestradiol in regulation of GnRH release in OVX female monkeys, in which circulating E_2 levels are minimal. We found that (1) local infusion of EB to the S-ME induces rapid GnRH release; (2) application of EB or ES to the S-ME evokes transient increases in E_2 release in the S-ME at concentrations as high as or higher than during the preovulatory phase; and (3) local

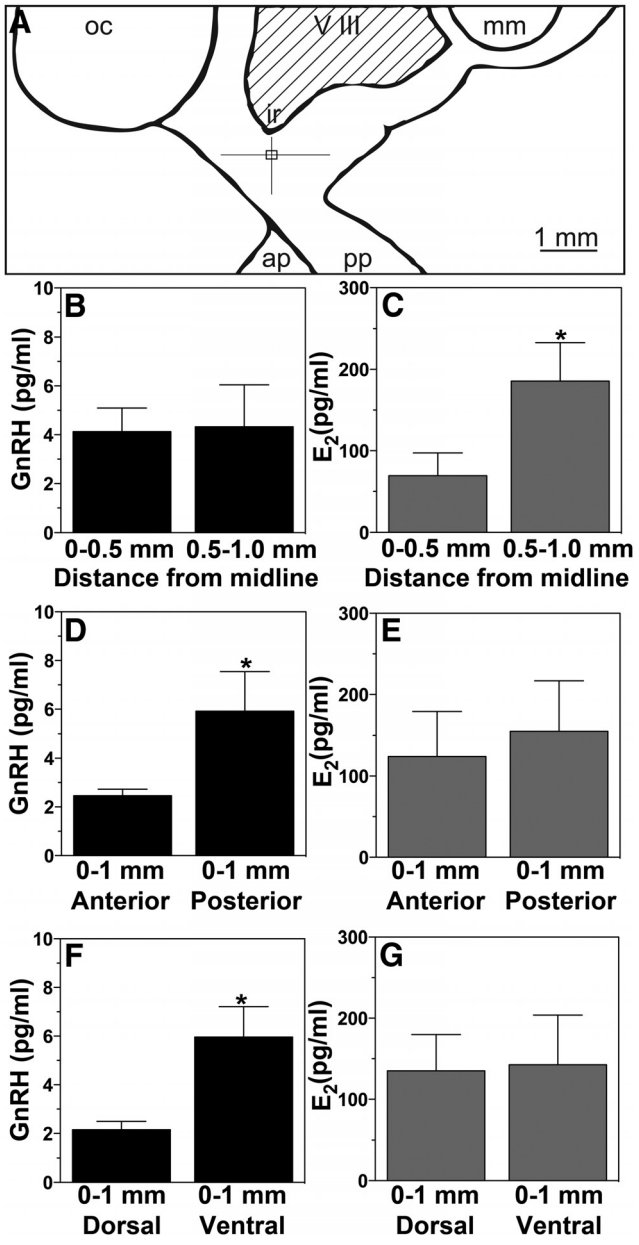

Figure 5. ***A–G***, Midsagittal diagram of the hypothalamus showing locations of the microdialysis probe tip (***A***) and comparison of probe tip position with GnRH (***B***, ***D***, ***F***) and E_2 levels (***C***, ***E***, ***G***). The distance from the midline (***B***, ***C***), the anterior–posterior position (***D***, ***E***), and the dorsal–ventral position (***F***, ***G***) in reference to the infundibular recess of the third ventricle are shown. The vertical and horizontal lines in ***A*** indicate the dorsal–ventral and anterior–posterior ranges of the sampling locations. The intersections of these lines indicate the mean cannula location, and the box delineates the SEM. The lateral position (from midline; mean ± SEM) was 0.5 ± 0.1 mm. GnRH levels 0–1 mm posterior were significantly >0–1 mm anterior from the infundibular recess ($p = 0.0471$ with unpaired Student's *t* test) and GnRH levels 0–1 mm ventral were significantly >0–1 mm dorsal from the infundibular recess ($p = 0.0241$ with unpaired Student's *t* test). E_2 levels at 0.5–1 mm were significantly higher than at 0–0.5 mm lateral from midline ($p = 0.0461$ with unpaired Student's *t* test). The number of experiments in this mapping was 31. ap, Anterior pituitary; ir, infundibular recess; mm, mammillary body; oc, optic chiasm; pp, posterior pituitary; V III, third ventricle.

infusion of the aromatase inhibitor letrozole suppresses spontaneous and EB-induced GnRH release. These observations are interpreted to mean that a local application of EB, mimicking endogenous E_2, rapidly elicits release of GnRH and E_2 and that E_2 release in the S-ME has a role in modulating GnRH release.

Table 3. The effects of the interval between experiments and the total number of experiments on E_2 and GnRH levels

Experimental conditions	Mean E_2 (pg/ml)	Mean GnRH (pg/ml)
Intervals between two probe insertions		
0–21 d from last experiment ($n = 17$)	127 ± 58^a	4.48 ± 1.46^b
22–50 d from last experiment ($n = 14$)	152 ± 59^a	3.26 ± 0.41^b
Total number of experiments		
1–5 experiments conducted ($n = 9$)	118 ± 58^c	3.75 ± 1.28^d
5–10 experiments conducted ($n = 8$)	179 ± 136^c	2.24 ± 0.36^d
10–15 experiments conducted ($n = 14$)	139 ± 59^c	4.54 ± 1.38^d

Retrospective analyses indicate that, reactive glia are not a likely a source of E_2 release in the S-ME, as neither time between two microdialysis experiments nor the number of experiments were correlated with mean E_2 and GnRH levels. The same letter indicates no difference between conditions by Student's *t* test (for the intervals between two probe insertions) or 1-way ANOVA (for the total number of experiments). [a]: Mean E_2 ($p = 0.7658$); [b]: Mean GnRH ($p = 0.4575$); [c]: Mean E_2 ($p = 0.8874$); [d]: Mean GnRH ($p = 0.5967$). Note that the total number of experiments can reach up to 15 times per animal, as the same animals were also co-assigned to two additional unrelated microdialysis sampling projects.

Table 4. EB (100 nM) conversion to E_2 *in vitro*

Conditions tested for EB to E_2 conversion	Total number of cases examined	Number of cases with E_2 peak present	Peak E_2 levels (pg/ml)	Conversion rate (%)
Artificial CSF	3	0	na	0
Monkey plasma	3	0	na	0
Monkey CSF	4	2	75, 90	0.28, 0.52
Hypothalamic tissues	3	1	2	0.02

Because E_2 increases induced by EB infusion could be due to hydrolysis and/or conversion of EB by an endogenous esterase, we conducted a series of validation experiments. We infused EB at 100 nM for 20 min (2 μl/min speed) through a microdialysis probe in 1) artificial CSF, 2) monkey plasma, or 3) monkey CSF obtained by spinal tap, while dialysates were continuously collected.

The EB-induced E_2 release (peak levels >1000 pg/ml) observed in this study is of hypothalamic origin. First, spontaneous E_2 release (80–400 pg/ml) in the hypothalamus was much higher than circulating E_2 levels (~8 pg/ml). Second, although there was a delay, ES elicited oscillatory increases in E_2 release in the S-ME. Third, EB infusion induces repeated E_2 peaks with uniform amplitude. If the E_2 peak is due to conversion of infused EB (hydrolysis or endogenous esterase action), a single E_2 peak should appear. Fourth, the EB-induced E_2 release is absent under the influence of the aromatase inhibitor letrozole. Fifth, a series of *in vitro* experiments examining whether conversion of EB to E_2 occurs within the sampling period indicated that there is no consistent E_2 peak after EB perifusion through monkey CSF and hypothalamic tissues. Thus, the EB-induced E_2 peaks are not due to EB conversion. Collectively, the E_2 measured in the present study represents neurocrine E_2 release in the hypothalamus.

Both neurons and glia synthesize E_2 *in vitro* (Zwain and Yen, 1999). However, E_2 measured in this study is of neuronal, not glial, origin because (1) immunohistochemical studies show that in the mammalian brain aromatase-expressing cells are primarily neurons, and not glia (Saldanha et al., 2009); and (2) although reactive glia produce E_2 in response to brain injury (Saldanha et al., 2009), gliosis due to cannula insertion is an unlikely source of E_2 in our study. A retrospective analysis suggests that neither the period between two microdialysis experiments nor the number of experiments (cannula insertions) was correlated with mean E_2 and GnRH levels.

The sites of dialysate collection were primarily in the S-ME, where GnRH neuroterminals are concentrated and few GnRH perikarya are present (Goldsmith et al., 1983). Thus, possible sites of neuroestradiol action are neuronal perikarya and neuroterminals, as the presence of aromatase in neuronal perikarya and neuroterminals has been described (Naftolin et al., 1996; Peter-

son et al., 2005; Mukai et al., 2010). However, it is also possible that neuroestradiol may influence GnRH release indirectly through many types of interneurons containing estrogen receptors, such as estrogen receptor α, including kisspeptin neurons in the arcuate nucleus (Franceschini et al., 2006). This is a conceivable pathway when EB is administered systemically. Nevertheless, unlike neurotransmitters, neither E_2 nor aromatase is found in vesicles (Naftolin et al., 1996; Peterson et al., 2005; Mukai et al., 2010). Neuroestradiol in the brain can be synthesized from cholesterol, as all enzymes necessary for estrogen synthesis are present in the brain. Alternatively, adrenal androgens can be converted to E_2 in the brain, although the degree of adrenal contribution is unclear. In either case, E_2 measured from the S-ME in this study is rapidly synthesized in the brain.

The present study shows that spontaneous E_2 release was seen in only ~50% of the cases examined without any particular pattern in association with GnRH pulses, whereas E_2 was released in a pulsatile manner in association with GnRH pulses when challenges, such as brief EB or ES, were applied. Moreover, infusion of letrozole consistently suppressed spontaneous GnRH pulses as well as the EB-induced GnRH increase, indicating neuroestradiol release contributes to GnRH release. A question arises: why was spontaneous E_2 release seen in only ~50% of the cases? There are two possible explanations: (1) a large portion of the dialysates collected at 20 min intervals may be below the LC/MS sensitivity; or (2) E_2 levels within the S-ME were site-specific and the probe was not always inserted in a position with the highest E_2 levels, because we aimed for locations with higher GnRH levels.

In the present study we found that EB-induced E_2 release is pulsatile and the peak durations and IPIs of the EB-induced GnRH and E_2 release were strikingly similar. Yet, the causal relationship between the EB-induced or ES-induced GnRH pulses and E_2 pulses is unclear. A future experiment with GnRH antagonist infusion would help to clarify whether GnRH release is necessary for neuroestradiol release. Nevertheless, in contrast to a prolonged elevation of E_2 from the ovary, pulsatile release of E_2 in the hypothalamus may be an important feature of neuroestradiol function in the S-ME. For example, GnRH is released into the portal circulation in a pulsatile manner and this pulsatility prevents desensitization of the G-protein-coupled GnRH receptors in the pituitary (Belchetz et al., 1978). Similarly, pulsatile release of E_2 may be necessary to prevent desensitization of the GnRH system in the MBH. In support of this speculation, our previous *in vitro* studies show that rapid E_2 action is mediated by GPR30 (Noel et al., 2009). If *in vivo* rapid E_2 action in the S-ME is also mediated through a G-protein-coupled receptor, e.g., GPR30, the vulnerability of the GnRH system to desensitization may be prevented by a pulsatile pattern of E_2 release. The specific receptors mediating the rapid action of E_2 (presumably action of neuroestradiol) in the S-ME are yet to be determined.

Our finding that EB infusion into the S-ME *in vivo* stimulated GnRH release with a short peak latency are consistent with previous *in vitro* observations that E_2 elicits a rapid, direct excitatory action on GnRH neurons. This indicates that the rapid excitatory action of E_2 on GnRH release *in vivo* reported here is completely different from the classical negative and positive feedback effects of E_2 on GnRH release, which require ~2 h and >24 h, respectively. Despite extensive publications regarding the role of neuroestradiol in courtship/reproductive behaviors, learning, and memory in rodents and birds, the importance of rapid neuroestradiol action in regulation of the hypothalamo-pituitary axis has been neglected. This is attributable to the fact that GnRH release is profoundly influenced by circulating E_2, and therefore studies previously focused on the long-term (in hours) influence of E_2, not the rapid (in minutes) action of neuroestradiol, in the hypothalamus. Additionally, direct measurement of E_2 from the hypothalamus is challenging and had not been conducted. The excitatory effect of EB by brief infusion discovered in the present study compared with the suppression of GnRH release induced by long infusion/crystalline implantation of E_2 directly into the MBH or systemic administration of E_2 indicate that the GnRH neuronal system may differentiate responses to E_2 based on the length of exposure or local concentration. We speculate that the GnRH neurosecretory system has a capacity to differentiate neuroestradiol released in the hypothalamus from elevated circulating E_2 from the ovaries, as outlined below.

Infusion of letrozole suppressed spontaneous as well as the EB-induced GnRH release, suggesting that E_2 release in the S-ME has a significant role in control of GnRH release. Perhaps, neuroestradiol is necessary for membrane excitability of GnRH neurons. Consequently, impaired membrane excitability in the absence of neuroestradiol during letrozole infusion may compromise the EB-induced GnRH release. Altogether, we hypothesize that neuroestradiol maintains homeostasis of GnRH neurons, such as membrane excitability. If GnRH neurons' membrane excitability is enhanced by neuroestradiol, pulsatility of GnRH release can be facilitated by signals originating from the putative pulse-generating system, i.e., kisspeptin/neurokinin B/dynorphin neurons (Lehman et al., 2010). Furthermore, activity of E_2-synthesizing neurons is also augmented by input from other neurons, as excitatory action of neuroestradiol is likely universal. Nevertheless, it appears that oscillatory release of GnRH and neuroestradiol are two independent systems, as indicated by the differential effects of EB (10 vs 100 nM) and ES, although the two systems readily influence each other. In contrast to these neuroestradiol mechanisms, E_2 from the ovary controls the GnRH neuronal system through a large number of interneurons expressing classical estrogen receptors.

Many questions remain to be answered: (1) What is the phenotype of neurons that express aromatase in the S-ME? (2) How does neuroestradiol interact with the major neurotransmitter systems, such as kisspeptin, GABA, and glutamate, to control GnRH function? (3) How does the GnRH system in the hypothalamus differentiate transient pulses of neuroestradiol from gradual E_2 changes from the general circulation, and are the dose and/or length of E_2 exposure distinguishing factors? (4) Does neuroestradiol release change during the menstrual cycle and, if so, how is it modified by ovarian steroid hormones? and (5) Which estrogen receptor subtypes are responsible for these rapid E_2 actions? Among these questions, the relationship between neuroestradiol and ovarian E_2 feedback in control of the GnRH release should be investigated soon. Nevertheless, the results of the present study provide a cornerstone for new concepts on neuroestradiol regulation of the reproductive system.

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