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

Brian P. Kenealy, Amita Kapoor, Kathryn A. Guerriero, Kim L. Keen, James P. Garcia, Joseph R. Kurian, Toni E. Ziegler, and Ei Terasawa

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 (E2). 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 E2 is removed. We found that (1) brief infusion of E2 benzoate (EB) to the S-ME rapidly stimulated release of GnRH and E2 in the S-ME of ovariectomized monkeys, (2) electrical stimulation of the MBH resulted in GnRH release as well as E2 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 E2. These findings reveal the importance of neuroestradiol as a neurotransmitter in regulation of GnRH release. How circulating ovarian E2 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 (E2) 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 E2 induces rapid, direct, excitatory actions, including stimulation of GnRH release within 10 min (Abraham 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 E2 exposure distinctively differs from the classical negative and positive feedback actions of circulating ovarian E2 on GnRH release.

Recently, the concept that E2 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 E2 inducing sex behavior in quails and rats; (2) the presence of enzymes necessary for de novo synthesis of E2 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 E2 synthesis and release in the rodent and songbird brain; and (5) acute synaptic E2 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 E2 observed in GnRH neurons in vitro is indicative of E2 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-medial 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 E2 in microdialysate samples with liquid chromatography-mass spectrometry (LC/MS). Subsequently, we examined whether brief infusion of EB stimulates release of GnRH and E2. LC/MS is an excellent tool for measuring neuroestradiol 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 E2 synthesis and release in the rodent and songbird brain; and (5) acute synaptic E2 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 E2 observed in GnRH neurons in vitro is indicative of E2 as a neurotransmitter or neuromodulator of GnRH release in vivo.

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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
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 E2 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 300 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 E2 levels were measured by LC/MS or LC/MS/MS. In Experiment 3, we examined the effects of EB on release of GnRH and E2 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 (Scherer; 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 E2, as determined by LC/MS analysis. In Experiment 4, we examined whether the aromatase inhibitor letrozole blocks EB-induced release of GnRH and/or E2. 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 treated with isoflurane anesthesia as previously described (Gearing and Terasawa, 1988; Frost et al., 2008). On the day of a microdialysis experiment, a microdive stylet 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 (∼0.5 × 0.5 mm²) 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). Ventrilcugraphs were used for positioning of 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, 96.0 mm in length, 0.6 mm in diameter), with a polyacrylamide and sulfone 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 CaCl₂, 0.85 mM MgCl₂; 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 E2 analysis with LC/MS. RIA buffer (0.3% BSA, 0.1 M PO4, 0.15 M NaCl, 0.1% NaN₃, pH 7.8) was added to dialysate for GnRH assay. No buffer was added to dialysates for E2, 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 E2 was assessed *in vitro* by (1) inducing artificial CSF through microdialysis probes placed in a reservoir containing E2 at 100 nM while dialysates were continuously collected at 10 min intervals (anteriorialysis) or (2) inducing EB 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 E2 in *in vitro* was examined, as E2 concentrations 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; Noell et al., 2009).

**GnRH RIA.** RIA for GnRH measurement was conducted using the R42 antiserver provided by Dr. Terry Net (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 E2 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)-E2 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 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 d5-E2 (506.2), against d5-E2 (mass-to-charge ratio...
(m/z), 511.2). For E2 analysis, a qualifier ion was monitored (m/z, 706.5). Standard calibration curves for E2 concentrations were 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0 pg/tube. (Sigma-Aldrich). Assay sensitivity of this E2 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 E2 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 2.6μ 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 by 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-E2 for E2. Standard calibration curves for E2 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 E2 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, E2 concentrations measured by LC/MS and LC/MS/MS method are combined, as these two procedures yielded consistent results. In all experiments, E2 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 min 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 E2 levels, while one-way ANOVA was conducted comparing total number of experiments conducted versus mean GnRH and E2 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 E2 were confirmed as pulses by the Pulsar program (Merriam and Wachter, 1982). Assay variations for GnRH and E2 were described by equations of Y = 3.38 X + 3.14/100 and Y = 0.093X + 0.38/100, 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 neuroestrodial, 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. 1A, C), whereas vehicle infusion did not induce any change (Fig. 1B, 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 E2 stimulus in vivo and that neuroestrodial in the S-ME may play a role in GnRH release.

ES of the medial basal hypothalamus-ME elicits release of GnRH and E2

If E2 is released in the S-ME, excitation of the medial basal hypothalamus (MBH)-ME by application of ES should induce E2 release in OVX monkeys. In fact, NMDA infusion stimulates E2 release in the rat hippocampus (Hojo et al., 2004), whereas glutamate infusion suppresses E2 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 E2 release. To accommodate measurement of GnRH and E2 in the same samples, we collected dialysate at 20 min intervals and divided each sample in half for GnRH measurement with RIA and E2 measurement with LC/MS. The peak recovery of E2 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 E2 release with a 40–60 min delay (Fig. 2A).

Statistical analysis further indicated that while ES significantly stimulated GnRH release (Fig. 2, compare B, C), ES tended to initially suppress E2 release, followed by a significant increase in E2 release (Fig. 2, compare D, E). The suppression of E2 levels 0–40 min after ES was not significantly different from before ES. E2 peak levels after the ES challenge were also significantly higher than before the challenge (Table 2). The latency to the first E2 peak after the initiation of ES was significantly different from before ES and also significantly higher than during 20–60 min (###p < 0.001; D vs C) and also significantly higher (##p < 0.01; D vs E) than sham ES at the corresponding time period. Similarly, the E2 increase at 40–60 min after ES was significantly higher (**p < 0.01) than before ES (B) and also significantly higher (##p < 0.01; D vs E) than sham ES at the corresponding time period. Interestingly, the E2 values at 0–20 and 20–40 min after ES were significantly lower than during 40–60 min (###p < 0.001 and ##p < 0.01, 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 E2

Because brief infusion of EB stimulates GnRH release and ES stimulated both release of GnRH and E2, we speculated that EB-induced GnRH release might be accompanied by E2 release. Previous reports indicate that E2 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 E2 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. 3A), 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. 3C) and also higher than vehicle during the corresponding time period (Fig. 3D). Vehicle infusion did not elicit any changes (Fig. 3B, D). Significantly higher GnRH peak levels after the EB challenge were also seen when compared with the 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 E2 release with a peak amplitude of >1 ng/ml (Fig. 3A). This is in contrast to the results from vehicle infusion (Fig. 3B). In fact, repeated E2 pulses seen after EB infusion were “induced E2” release, as during the 150–180 min of control sampling before EB challenges, in OVX animals E2 pulses were either undetectable (17 of 31 experiments) or a couple of E2 pulses with a mean peak amplitude of...
281 ± 129 pg/ml (Table 2) were seen (14 of 31 experiments). Importantly, circulating E2 is not the source of E2 in the hypothalamus, as E2 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 E2 release was significantly higher than those during the control period (Fig. 3E) as well as the corresponding period in vehicle control (Fig. 5F). Significantly higher E2 peak levels after the EB challenge were also seen when compared with EB peak levels before the challenge or vehicle control (Table 2). The peak latency of EB-induced E2 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 E2 release were strikingly similar to those of GnRH release, and none of the E2 pulse parameters differed from GnRH pulses. Importantly, with the exceptions of peak level and peak latency, the E2 pulse parameters between EB-induced and ES-induced E2 changes were similar to each other (Table 2).

An aromatase inhibitor suppresses GnRH release and blocks EB-induced GnRH and E2 release

After finding that EB induces E2 release from the S-ME, we next tested whether endogenous E2 release plays any role in regulating GnRH release by infusion of the aromatase inhibitor letrozole into the S-ME of OVX female rhesus monkeys. We first examined whether letrozole infusion modifies the EB-induced GnRH and E2 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. 4A). While vehicle treatment did not affect the EB-induced release of GnRH and E2 (Fig. 4B,E), letrozole treatment eliminated the EB-induced release of GnRH and E2 (Fig. 4C,F). These results indicate that letrozole inhibits the EB-induced release of both GnRH and E2.

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. 4A). Letrozole infusion not only suppressed GnRH pulses during the drug infusion (Fig. 4A), but also significantly suppressed mean release of GnRH (Fig. 4D). 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 E2 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., 2009). We did not, however, know which locations were ideal for E2 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 E2 concentrations. Our results were similar to those previously reported (Gearing and Terasawa, 1988). That is, higher GnRH levels were seen 0–1 mm posterior to 0–1 mm anterior (Fig. 5D) and 0–1 mm ventral than 0–1 mm dorsal (Fig. 5F) from the infundibular recess. There was no difference in lateral positions between 0–0.5 and 0.5–1 mm from the midline (Fig. 5B). In contrast, significantly higher E2 levels were seen 0.5–1 mm than 0–0.5 mm lateral from the midline (Fig. 5C), whereas there was no difference in anterior–posterior (Fig. 5E) or dorsal–ventral (Fig. 5G) position. Thus, there appears to be a location within our sampling area ideal for both GnRH and E2 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 E2 and GnRH levels.

Examination of possible EB conversion to E2, in vitro

Results from in vitro experiments indicate that there was no EB conversion to E2 in female monkey plasma, nor breakdown to E2 in artificial CSF. Moreover, in monkey CSF (500 µl) there was no measurable E2 in two of four monkey CSF samples, whereas a small single E2 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 E2 was small (<100 pg/ml), and the mole-to-mole conversion from EB to E2 was 0.28 and 0.52% (Table 4). Finally, using a perfusion system as previously described (Noel et al., 2009), we tested whether 20 min EB (100

Table 2. Effects of ES and EB infusion of the MBH-ME region on release of GnRH and E2 in OVX female rhesus monkeys

<table>
<thead>
<tr>
<th>Measured neurohormones</th>
<th>GnRH</th>
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<td>Sham ES (N = 9)</td>
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<td>ES</td>
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<tr>
<td></td>
<td>Peak after (pg/ml)</td>
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<td></td>
<td>Peak latency (min)</td>
<td>24.4 ± 2.9**</td>
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<td></td>
<td>Peak duration (min)</td>
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<td></td>
<td>IPI (min)</td>
<td>66.7 ± 4.7</td>
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<td>Treatments</td>
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<tr>
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<td>Peak after (pg/ml)</td>
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<td>IPI (min)</td>
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Data are mean ± SEM, N: Total number of experiments conducted. *p < 0.05; **p < 0.01; ***p < 0.001 versus vehicle or sham; *p < 0.05; **p < 0.10; ***p < 0.001 versus mean before treatment; *p < 0.001 versus E2.
Figure 3. Direct infusion of EB into the S-ME induces release of GnRH (black line with filled circle) and E₂ (red line with filled square) in vivo. A–F, Representative cases (A, B) and group data (means ± SEM) from EB-infused (C, D) or vehicle-infused (E, 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 E₂ release (A). While the amplitude of GnRH responses appears to be EB dose-dependent, the amplitude of E₂ did not vary. In contrast, vehicle infusion did not cause any significant effects (B). In this case, a single spontaneous E₂ 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 E₂ 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 E₂ 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.

Discussion

In this study, we examined the role of neuroestradiol in regulation of GnRH release in OVX female monkeys, in which circulating E₂ 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 E₂ release in the S-ME at concentrations as high as or higher than during the preovulatory phase; and (3) local

nm) perfusion through hypothalamic tissue (MBH and dorsal hypothalamus) yielded E₂. In two of the three cases, no E₂ was detected while in the third case a small amount of E₂ 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 E₂ peaks (Fig. 3A) occurring in vivo with a latency of 26.3 ± 3.2 min were due to EB metabolism to E₂.

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 E₂ 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 E₂, whereas EB infusion in the presence of letrozole failed to stimulate release of GnRH or E₂ (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 E₂ 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 (****p < 0.001, *p < 0.05 for letrozole plus EB, ****p < 0.001 for letrozole alone). Likewise, E₂ levels during both letrozole treatments (F, G) were lower than those during EB control (E, ***p < 0.001 for letrozole plus EB, ****p < 0.001 for letrozole alone). E₂ levels during EB with vehicle treatment (E) were also significantly higher than before infusions (***p < 0.01).
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 E2, rapidly elicits release of GnRH and E2 and that E2 release in the S-ME has a role in modulating GnRH release.

The EB-induced E2 release (peak levels >1000 pg/ml) observed in this study is of hypothalamic origin. First, spontaneous E2 release (80–400 pg/ml) in the hypothalamus was much higher than circulating E2 levels (~8 pg/ml). Second, although there was a delay, E2 levels at 0.5–1 mm posterior were significantly higher than at 0–0.5 mm lateral from the hypothalamic tissues. Thus, the EB-induced E2 release is not due to conversion of infused EB (hydrolysis or endogenous esterase action), a single E2 peak should appear. Fourth, the EB-induced E2 release is absent under the influence of the aromatase inhibitor letrozole. Fifth, a series of in vitro experiments examining whether conversion of EB to E2 occurs within the sampling period indicated that there is no consistent E2 peak after EB perfusion through monkey CSF and hypothalamic tissues. Thus, the EB-induced E2 peaks are not due to EB conversion. Collectively, the E2 measured in the present study represents neuroestradiol release in the hypothalamus.

Both neurons and glia synthesize E2 in vitro (Zwain and Yen, 1999). However, E2 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 E2 in response to brain injury (Saldanha et al., 2009), gliosis due to cannula insertion is an unlikely source of E2 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 E2 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., 2003; 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 E2 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 E2 in the brain, although the degree of adrenal contribution is unclear. In either case, E2 measured from the S-ME in this study is rapidly synthesized in the brain.

The present study shows that spontaneous E2 release was seen in only ~50% of the cases examined without any particular pattern in association with GnRH pulses, whereas E2 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 E2 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) E2 levels within the S-ME were site-specific and the probe was not always inserted in a position with the highest E2 levels, because we aimed for locations with higher GnRH levels.

In the present study we found that EB-induced E2 release is pulsatile and the peak durations and IPIs of the EB-induced GnRH and E2 release were strikingly similar. Yet, the causal relationship between the EB-induced or ES-induced GnRH pulses and E2 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 E2 from the ovary, pulsatile release of E2 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 E2 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 E2 action is mediated by GPR30 (Noel et al., 2009). If in vivo rapid E2 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 E2 release. The specific receptors mediating the rapid action of E2 (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 E2 elicits a rapid, direct excitatory action on GnRH neurons. This indicates that the rapid excitatory action of E2 on GnRH release in vivo reported here is completely different from the classical negative and positive feedback effects of E2 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 previously focused on the long-term (in hours) influence of E2, not the rapid (in minutes) action of neuroestradiol, in the hypothalamus. Additionally, direct measurement of E2 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 E2 directly into the MBH or systemic administration of E2 indicate that the GnRH neuronal system may differentiate responses to E2 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 E2 from the ovaries, as outlined below.

Infusion of letrozole suppressed spontaneous as well as the EB-induced GnRH release, suggesting that E2 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 E2-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, E2 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 E2 changes from the general circulation, and are the dose and/or length of E2 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 E2 actions? Among these questions, the relationship between neuroestradiol and ovarian E2 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.

References


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