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Neurosteroids and Female Reproduction: Estrogen Increases 3β-HSD mRNA and Activity in Rat Hypothalamus

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A central event in mammalian reproduction is the LH surge that induces ovulation and corpus luteum formation. Typically, the LH surge is initiated in ovariectomized rats by sequential treatment with estrogen and progesterone (PROG). The traditional explanation for this paradigm is that estrogen induces PROG receptors (PR) that are activated by exogenous PROG. Recent evidence suggests that whereas exogenous estrogen is necessary, exogenous PROG is not. In ovariectomized-adrenalectomized rats, estrogen treatment increases hypothalamic PROG levels before an LH surge. This estrogen-induced LH surge was blocked by an inhibitor of 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD), the proximal enzyme for PROG synthesis. These data indicate that estrogen induces *de novo* synthesis of PROG from cholesterol in the hypothalamus, which initiates the LH surge. The mechanism(s) by which estrogen up-regulates neuro-PROG is unknown. We investigated whether estrogen increases 1) mRNA levels for several proteins involved in PROG synthesis and/or 2) activity of 3β-HSD in the hypothalamus. In ovariectomized-adrenalectomized rats, estrogen treatment increased 3β-HSD mRNA in the hypothalamus, as measured by relative quantitative RT-PCR. The mRNAs for other proteins involved in steroid synthesis (sterol carrier protein 2, steroidogenic acute regulatory protein, and P450 side chain cleavage) were detectable in hypothalamus but not affected by estrogen. In a biochemical assay, estrogen treatment also increased 3β-HSD activity. These data support the hypothesis that PROG is a neurosteroid, produced locally in the hypothalamus from cholesterol, which functions in the estrogen positive-feedback mechanism driving the LH surge. (*Endocrinology* 146: 4386–4390, 2005)

ONE OF THE important questions confronting neuroendocrinology is the relationship between steroids produced by peripheral steroidogenic organs (*e.g.* adrenals and gonads) and steroids produced by the central nervous system, neurosteroids (1). We have demonstrated that systemic estrogen treatment of ovariectomized and adrenalectomized (OVX-ADX) rats increases hypothalamic progesterone (PROG) levels (2). The estrogen-induced increase of neuro-PROG precedes the LH surge, and the LH surge is blocked by an inhibitor of 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD), the enzyme that converts pregnenolone to PROG (2). These data suggest that *de novo* synthesis of PROG from cholesterol in the hypothalamus is a critical part of the estrogen positive-feedback mechanism that stimulates the LH surge.

All cell types in the nervous system, particularly macroglia, have steroidogenic potential (3, 4). Astrocytes have high levels of P450 side chain cleavage (P450scc) (5, 6) and predominantly synthesize and secrete PROG (7, 8). Astrocytes also express estrogen receptor-α (ERα) and ERβ (9, 10), suggesting that estrogen could stimulate PROG synthesis in these cells. Indeed, in enriched cultures of neonatal cortical astrocytes, PROG levels in media increase in response to estrogen treatment (8). Furthermore, astrocytes cultured from the hypothalamus respond to estrogen with increased PROG synthesis, but only after puberty (11). Although there is no evidence to date, *in vivo*, it remains possible that estrogen might stimulate neuro-PROG synthesis in neurons.

Proteins involved in steroidogenesis are expressed in a region-specific manner in the brain. For example, both sterol carrier protein 2 (SCP-2) and steroidogenic acute regulatory protein (StAR) are expressed in the hypothalamus and other regions (12–14). SCP-2 is involved in intracellular transport of cholesterol to mitochondria (12). StAR, considered to be the rate-limiting step in steroidogenesis, transports cholesterol from the outer to the inner mitochondrial membrane (13). On the inner mitochondrial membrane, P450scc converts cholesterol to pregnenolone, and then 3β-HSD metabolizes pregnenolone to PROG in the endoplasmic reticulum. Whether estrogen regulates these four proteins to increase neuro-PROG levels remains unclear (2).

The present study examines whether estrogen regulates the expression of cholesterol transport proteins and steroidogenic enzymes in the hypothalamus. We used OVX-ADX

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Abbreviations: Ct, Threshold cycle; EB, estradiol benzoate; ERα, estrogen receptor-α; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase; OVX-ADX, ovariectomized and adrenalectomized; P450scc, P450 side chain cleavage; PLSD, protected least significant difference; PR, progesterone receptor; PROG, progesterone; SCP-2, sterol carrier protein 2; StAR, steroidogenic acute regulatory protein.

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rats treated with estrogen and collected tissue at various times after treatment to establish a time course of estrogen stimulation of mRNA expression of steroidogenic proteins. Relative quantitative RT-PCR was used to measure levels of SCP-2, StAR, P450scc, and 3β-HSD mRNAs. In a parallel experiment, we used a biochemical assay to measure the effects of estrogen treatment on 3β-HSD activity.

Materials and Methods

Animals

Experimental procedures were approved by the Chancellor's Animal Research Committee at University of California, Los Angeles. Animals in this study were adult Long-Evans female rats weighing 200–225 g (Charles River Laboratories, Raleigh, NC). Animals were ovariectomized and adrenalectomized by the supplier. Food and water were provided *ad libitum*. Drinking water included 0.9% sodium chloride and corticosterone (50 mg/liter). Corticosterone is synthesized from PROG, but this is considered an irreversible reaction under physiological conditions. Thus, neuro-PROG is unlikely to be derived from exogenous corticosterone. In addition, previous studies indicate that circulating levels of PROG are basal in OVX-ADX rats given corticosterone in the drinking water (2). The animals were maintained on a 12-h light, 12-h dark cycle, with lights on at 0600 h. Steroid treatments were started between 28 and 35 d after ovariectomy and adrenalectomy, as in previous studies (2). Separate sets of rats were used for mRNA expression and enzyme activity studies.

Measurement of mRNA

All rats were housed two per cage and acclimated to handling by being handled at least once per day for 1 wk before the experiment. On d 1, rats were treated sc with 50 μg 17β-estradiol benzoate (EB) (Sigma Chemical Co., St. Louis, MO) in 0.1 ml safflower oil or with oil vehicle at noon. Rats treated with oil vehicle were anesthetized and decapitated immediately (0-h group, n = 6). Rats treated with EB were anesthetized and decapitated 12 h (n = 5), 24 h (n = 5), or 44 h (n = 5) later. Brains were quickly removed and briefly chilled in ice-cold RNase-free PBS. The hypothalamus-preoptic area was quickly dissected on ice. The rostral limit of the hypothalamus-preoptic area dissection contained the anteroventral periventricular nucleus (AVPV), and the caudal limit was at the rostral pole of the mammillary bodies. The dorsal limit was at the dorsal apex of the third ventricle, and the lateral limits were at the lateral edges of the medial preoptic area. Tissue blocks were frozen immediately on dry ice and stored at −80 C.

The mRNA levels of SCP-2, StAR, P450scc, 3β-HSD (type I), and PROG receptors (PR) were measured by relative quantitative RT-PCR. Because PR mRNA levels are increased after estrogen treatment (15), PR mRNA served as a positive control for EB treatment. Two sets of PCR primers for PR were used; one set recognized both PR-A and PR-B isoforms, and a second set recognized only PR-B (16). β-Actin was used as an endogenous housekeeping gene for normalization. Total RNA was isolated from tissue using Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) and included DNase treatment. RNA integrity was confirmed by 1.2% agarose gel electrophoresis. Single-stranded cDNA was synthesized from total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA).

The cDNA was subjected to PCR. SYBR Green or TaqMan chemistries were used with primers described in Table 1. Primers and probes were designed using Primer3 software (The Whitehead Institute, Boston, MA). Primers for PR were published previously (16). For measurement of StAR mRNA only, a TaqMan probe was used (HEX-TGC TAC CAT GTA GGC AGA CAG GAG TT-BHQ1). Primer and probe specificities were confirmed by a BLAST software-assisted search of a nonredundant nucleotide sequence database (National Library of Medicine, Bethesda, MD). Template dilutions using hypothalamic cDNA determined the efficiency of amplification for all mRNA examined. Melting curve analyses, agarose gel electrophoresis, and/or sequencing of PCR products confirmed PCR product specificity. The experiments were conducted on an Mx3000p Real-Time PCR System (Stratagene). Samples were run in triplicate, and positive controls (ovarian cDNA) and negative controls (no cDNA) were included in every PCR run.

Relative gene expression data were calculated using the comparative threshold cycle (Ct) method (Applied Biosystems User Bulletin 2, 2001). Briefly, the Ct was determined for each gene of interest. Within each subject, ΔCt was calculated as the difference in Ct between target mRNA and β-actin mRNA. ΔΔCt was calculated as the difference between ΔCt for each sample and the average ΔCt for the 0-h group (baseline). The change in target mRNA, relative to baseline, was calculated as $2^{-\Delta\Delta Ct}$. Changes in mRNA expression were expressed as a percentage of the 0-h group.

Measurement of 3β-HSD activity

A separate set of rats was used to measure 3β-HSD activity. On d 1, rats were treated sc with 50 μg EB (in 0.1 ml safflower oil) or oil vehicle at noon and were returned to their home cages. Rats were killed 2 or 44 h after treatment. Rats were rapidly anesthetized with isoflurane and decapitated within 3 min of removal from their cages. This was done to avoid rapid effects of stress on 3β-HSD activity (17). The brain was immediately removed and briefly chilled in ice-cold PBS. A hypothalamic-preoptic area block was dissected as described above. In addition, an amygdaloid block was collected because this area has high 3β-HSD activity (18). The amygdaloid block extended rostrocaudally from the medial amygdala, anterodorsal to the medial amygdala, and posterodorsal where the lateral ventricle emerges. The medial border paralleled the optic tract and the lateral border was a parallel cut on the lateral limit of the lateral amygdala. Perpendicular cuts to the previously described cuts were made just dorsal to the central amygdala and ventral to the basal medial amygdala. Tissue was frozen on dry ice and stored at −80 C.

3β-HSD activity was measured using an *in vitro* assay that quantifies the conversion of [^{3}H]pregnenolone to [^{3}H]PROG using thin layer chromatography (TLC) (19, 20). The assay methods were based on previous

TABLE 1. Primer sequences for relative quantitative RT-PCR

	Primers	GenBank accession no.
β-Actin	FW: AGG GAA ATC GTG CGT GAC AT RV: TCC AGG GAG GAA GAG GAT GC	NM_031144
PR-A/B	FW: CTT TGT TTC CTC TGC AAA AAT TG RV: GTA TAC ACG TAA GGC TTT CAG AAG G	NA
PR-B	FW: CAG ACC AAC CTG CAA CCA GAA RV: AGT CCT CAC CAA AAC CCT GGG	U06637/L16921
SCP-2	FW: AGG GGA TGG ATT CAA GGC RV: CAC GAA CTC TTC CCC TTC C	NM_138508
StAR	FW: TCA GAG TAG CAG CTC CCT TGT TTG RV: CTC CAA ATC CTG AAA CGG GAA TGC	NM_031558
P450scc	FW: CAG CGG TTC ATC GAC GC RV: TCT GGA GGC ATG TTG AGC A	J05156
3β-HSD (type I)	FW: CCC ATA CAG CAA AAG GAT GG RV: GCC GCA AGT ATC ATG ACA GA	M38178

FW, Forward; RV, reverse; NA, not applicable.

studies, in which results using TLC were verified by gas chromatography-mass spectrometry (20). Briefly, tissue was homogenized in 300 μl ice-cold sucrose-phosphate buffer with a glass-Teflon homogenizer. Homogenates were centrifuged for 30 min at 1000 × *g* at 4 C. Supernatants (180 μl) were incubated with [^{3}H]pregnenolone (800 nM) (NEN Life Science Products, Boston, MA). [^{3}H]Pregnenolone was repurified before use. Incubations included the cofactor nicotinamide adenine dinucleotide (20 μl; 1 mM final concentration). Radioinert PROG cold trap (800 nM) (Steraloids, Newport, RI) was included in the incubation to reduce the metabolism of formed [^{3}H]PROG. Control tubes contained everything but tissue. Incubations were carried out for 60 min at 37 C with shaking. This incubation duration was based on previous studies in rat sciatic nerve (20, 21), songbird hypothalamus and telencephalon (17), and our preliminary studies. Reactions were terminated by snap freezing. To correct for procedural losses, tubes containing a known amount of [^{3}H]PROG were processed in parallel.

Steroids were extracted using diethyl ether (three times). Radioinert pregnenolone, PROG, and 5α-dihydroprogesterone were added as markers, and steroids were separated using TLC. Silica plates were run in chloroform:ethyl acetate (3:1) for 28 min (two times). Steroids were visualized with primulin and UV light. The bands were scraped off the glass, and steroids were eluted from the silica with methanol (three times). Radioactivity was measured using a liquid scintillation counter (Packard 2200CA Tricarb scintillation counter). The counts per minute were corrected for background and procedural losses and then converted to femtomoles of PROG. Protein content was quantified using the Bradford method. Activity was reported as fmol PROG/mg protein.

Statistics

Relative changes in gene expression were quantified by the comparative Ct method (see above). ΔCt values were analyzed by ANOVA. *Post hoc* analyses were conducted with Fisher's protected least significant difference (PLSD) tests. For 3β-HSD activity assays, results from separate biochemical assays were analyzed with *t* tests. Values of $P \leq 0.05$ were considered significant.

Results

Estrogen-induced mRNA expression in hypothalamus

β-Actin mRNA levels were not affected by estrogen treatment (ANOVA, F = 0.26, $P = 0.85$), and the Ct values were similar across groups (17.53 ± 0.25, 17.53 ± 0.13, 17.43 ± 0.26, and 17.31 ± 0.07 for 0-, 12-, 24-, and 44-h groups, respectively). Therefore, β-actin mRNA levels were used as the baseline, and all expression data were normalized to β-actin mRNA levels for each subject.

As a positive control for EB action, PR expression was measured using PCR primers that recognized either both PR-A and PR-B (PR-A/B) isoforms or the PR-B isoform only. EB significantly increased PR-A/B mRNA expression (Fig. 1; ANOVA, F = 6.80, $P = 0.003$). Compared with the 0-h group, PR-A/B mRNA expression was significantly higher in the 12-, 24-, and 44-h groups (Fisher's PLSD, $P < 0.05$ in all cases). PR-A/B mRNA levels did not differ significantly among the 12-, 24-, and 44-h groups (Fisher's PLSD, $P > 0.05$ in all cases). Using PCR primers specific for the PR-B isoform, we also observed an EB-induced increase in PR-B mRNA levels (Fig. 1; ANOVA, F = 25.95, $P < 0.0001$). PR-B mRNA expression was significantly higher in the 12-, 24-, and 44-h groups relative to the 0-h group (Fisher's PLSD, $P < 0.05$ in all cases). PR-B mRNA levels were not different among the 12-, 24-, and 44-h groups (Fisher's PLSD, $P > 0.05$ in all cases).

Next, we examined mRNAs coding for proteins involved in PROG synthesis (Fig. 2). EB treatment did not significantly affect the expression of mRNA for the two cholesterol carrier proteins, SCP-2 (F = 1.11, $P = 0.37$) and StAR (F = 0.84, $P = 0.49$). Similarly, EB treatment did not change P450scc mRNA levels during the experimental period (F = 1.06, $P = 0.39$). In contrast, EB treatment significantly increased 3β-HSD mRNA levels in the hypothalamus (Fig. 2D; F = 6.71, $P = 0.003$). 3β-HSD mRNA expression was significantly elevated over baseline at both the 24- and 44-h time points after EB treatment (Fisher's PLSD, $P < 0.05$ in both cases). In addition, the mRNA expression levels of the 24- and 44-h groups were significantly greater than the 12-h group (Fisher's PLSD, $P < 0.05$ in both cases). The greatest increase in 3β-HSD mRNA levels was 143% over baseline at 24 h. The levels were 70% over baseline at 44 h.

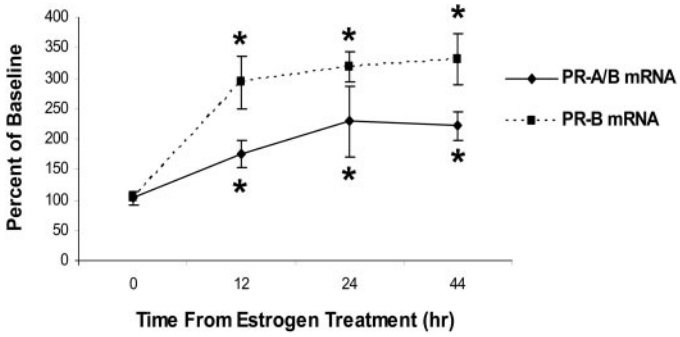

FIG. 1. Effects of estrogen treatment on PR mRNA in the hypothalamus of OVX-ADX rats. Subjects were treated with 50 μg EB for 0, 12, 24, or 44 h (n = 6, 5, 5, and 5, respectively). Primers were designed to recognize both PR-A and PR-B isoforms (PR-A/B) or PR-B only. The mRNA levels were measured by relative quantitative RT-PCR, normalized to β-actin mRNA, and expressed as a percentage of baseline (0-h group). EB treatment significantly increased mRNA levels of PR-A/B and PR-B within 12 h. Levels were not significantly different among 12-, 24-, and 44-h time points. *, Significantly greater than 0-h group ($P < 0.05$, Fisher's PLSD).

Estrogen increased 3β-HSD activity in hypothalamus

To verify that an increase in 3β-HSD mRNA levels was reflected in enzyme activity, the conversion of [^{3}H]pregnenolone to [^{3}H]PROG was measured. In the hypothalamus, EB significantly increased 3β-HSD activity by 59% at 44 h after treatment (Fig. 3; *t* test, $t = 2.35$, $P = 0.04$). In contrast, in the amygdala, EB had no effect on 3β-HSD activity at 44 h (Fig. 3; $t = 0.60$, $P = 0.56$), indicating a region-specific effect of estrogen. To determine whether enzyme activity was increased more quickly than the 3β-HSD mRNA levels, the synthesis of PROG was measured 2 h after EB treatment. At 2 h, EB did not significantly affect 3β-HSD activity in the hypothalamus ($t = 1.49$, $P = 0.17$) or amygdala ($t = 0.87$, $P = 0.41$).

Discussion

The major findings of the present experiments are that in the hypothalamus, estrogen treatment increases 1) the conversion of pregnenolone to PROG and 2) the mRNA expression of 3β-HSD, the enzyme catalyzing this conversion. These data indicate that peripheral estrogen can regulate local synthesis of PROG in the hypothalamus. Previous results indicated that estrogen treatment of OVX-ADX rats increased PROG levels in the hypothalamus (2), but whether estrogen affected cholesterol transport proteins or steroido-

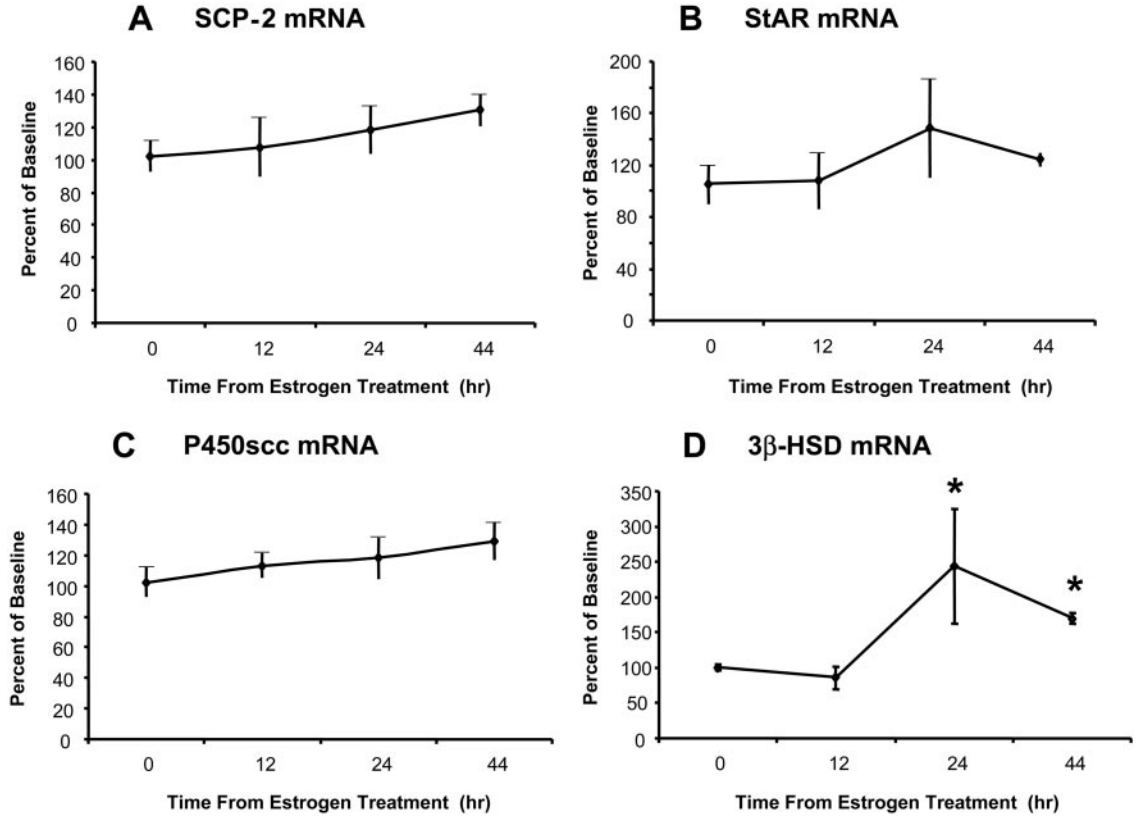

FIG. 2. Effects of estrogen treatment on mRNA levels of SCP-2 (A), StAR (B), P450scc (C), and 3β-HSD (D) in the hypothalamus of OVX-ADX rats. Subjects were treated with 50 μg EB for 0, 12, 24, or 44 h (n = 6, 5, 5, and 5, respectively). The mRNA levels were measured by relative quantitative RT-PCR and expressed as a percentage of baseline (0-h group). EB treatment significantly increased 3β-HSD mRNA levels ($F = 6.075$, $P = 0.003$). *, Significantly greater than 0- and 12-h groups.

genic enzymes was unknown. The present studies indicate that one mechanism through which estrogen increases neuro-PROG levels is by up-regulation of 3β-HSD mRNA levels within the brain. This is an important point, because relatively little is known about the regulation of neurosteroids *in vivo*.

In addition to 3β-HSD mRNA, we detected the mRNAs for SCP-2, StAR, and P450scc in the adult female hypothalamus.

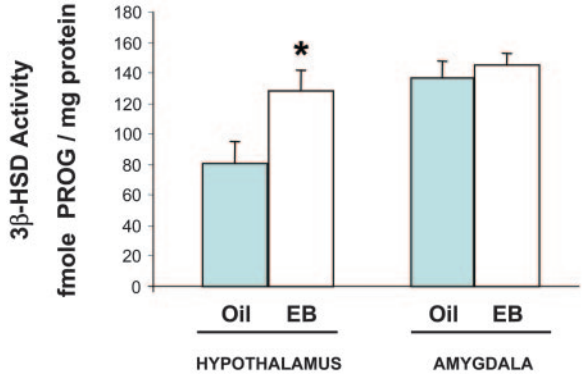

FIG. 3. Effects of estrogen treatment on 3β-HSD activity in the hypothalamus and amygdala of OVX-ADX rats. Subjects were treated with 50 μg EB (n = 6) or oil vehicle (n = 5) for 44 h. 3β-HSD activity was determined by measuring the *in vitro* conversion of [^{3}H]pregnenolone to [^{3}H]PROG. *, Significantly greater than oil-treated controls.

Furthermore, OVX-ADX rats lack an obvious peripheral source of pregnenolone, the substrate for 3β-HSD, but OVX-ADX animals have high PROG levels in the hypothalamus after EB treatment. Taken together, the data support the hypothesis that the hypothalamus is able to synthesize PROG *de novo* from cholesterol. Thus, PROG would be a true neurosteroid in this case.

Proteins involved in PROG synthesis, such as SCP-2, StAR, P450scc, and 3β-HSD, are present in the brains of a wide variety of vertebrates (17, 22–24). However, little is known about how these proteins are regulated *in vivo* to modify neurosteroid levels. Recent work has emphasized the importance of StAR as a rate-limiting step in steroid production (13); however, the present data point to an additional role for 3β-HSD in regulating neuro-PROG concentrations in the hypothalamus. In contrast, in ovarian and placental cells, estrogen facilitation of PROG synthesis may not involve increased P450scc or 3β-HSD mRNA (25, 26).

Our previous studies suggest that estrogen induction of neuro-PROG is a critical part of the positive feedback mechanism through which estrogen induces the LH surge (2). According to this hypothesis, estrogen increases the expression of both PR and 3β-HSD in the hypothalamus. Therefore, neuro-PROG can activate the newly expressed PR. Activated PRs permit the release of GnRH (27), stimulating the LH surge to regulate ovulation and luteinization of the ruptured follicle. In OVX-ADX rats, estrogen treatment specifically

increases hypothalamic PROG levels after 45 h (earlier time points have not been examined) (2). This increase in hypothalamic PROG occurs before the LH surge, which is 51–53 h after estrogen treatment in our colony (2). In the present studies, estrogen treatment increased hypothalamic PR mRNA within 12 h, 3β-HSD mRNA expression after 24 and 44 h, and 3β-HSD activity after 44 h. These results are consistent with the data on hypothalamic PROG levels and the ability of a 3β-HSD inhibitor to block the LH surge (2). In addition, recent data suggest a role for neuro-PROG in the expression of female reproductive behavior, particularly proceptive behavior (28). Thus, neuro-PROG may be important for the coordination of ovulation and sexual behavior in female rats.

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