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Estradiol Stimulates Progesterone Synthesis in Hypothalamic Astrocyte Cultures

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The brain synthesizes steroids *de novo*, especially progesterone. Recently estradiol has been shown to stimulate progesterone synthesis in the hypothalamus and enriched astrocyte cultures derived from neonatal cortex. Estradiol-induced hypothalamic progesterone has been implicated in the control of the LH surge. The present studies were undertaken to determine whether hypothalamic astrocytes derived from female neonatal or female postpubertal rats increased production of progesterone in response to an estradiol challenge. Estradiol induced progesterone synthesis in postpubertal astrocytes but not neonatal astrocytes. This estradiol action was blocked by the estrogen receptor antagonist ICI 182,780. Previously we had demonstrated that estradiol stimulates a rapid increase in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) spikes in neonatal cortical astrocytes acting through a membrane estrogen receptor. We now report that estradiol also rapidly increased $[Ca^{2+}]_i$ spikes in hypothalamic astrocytes. The membrane-impermeable estradiol-BSA construct also induced $[Ca^{2+}]_i$ spikes. Both estradiol-BSA and estradiol were blocked by ICI 182,780. Depleting intracellular Ca^{2+} stores prevented the estradiol-induced increased $[Ca^{2+}]_i$ spikes, whereas removing extracellular Ca^{2+} did not prevent estradiol-induced $[Ca^{2+}]_i$ spikes. Together these results indicate that estradiol acts through a membrane-associated receptor to release intracellular stores of Ca^{2+}. Thapsigargin, used to mimicked the intracellular release of Ca^{2+} by estradiol, increased progesterone synthesis, suggesting that estradiol-induced progesterone synthesis involves increases in $[Ca^{2+}]_i$. Estradiol treatment did not change levels of steroid acute regulatory protein, P450 side chain cleavage, 3β-hydroxysteroid dehydrogenase, and sterol carrier protein-2 mRNAs as measured by quantitative RT-PCR, suggesting that *in vitro*, estradiol regulation of progesterone synthesis in astrocytes does not depend on transcription of new steroidogenic proteins. The present results are consistent with our hypothesis that estrogen-positive feedback regulating the LH surge involves stimulating local progesterone synthesis by hypothalamic astrocytes. (*Endocrinology* 148: 782–789, 2007)

RECENTLY WE PROPOSED that the *de novo* synthesis of progesterone in the hypothalamus (neuroprogesterone) is part of the mechanism that initiates the LH surge in rats. Estradiol produced by the ovary and peaking on the afternoon of proestrus acts on the hypothalamus to increase the *de novo* synthesis of progesterone to produce locally elevated levels of progesterone before the LH surge (1). Our hypothesis is that estrogen-positive feedback is mediated by the local synthesis of neuroprogesterone, which acts on estradiol-induced progesterone receptors in the hypothalamus to initiate the neural cascade leading to the surge release of LH from the anterior pituitary (1–3).

Steroidogenesis in the nervous system has been well established (for reviews, see Refs. 4–7). Specific cell types in the central nervous system (CNS) have been demonstrated to have particular biosynthetic pathways (8, 9). Neurons reportedly have the enzymes for primarily synthesizing estrogen, whereas oligodendrocytes preferentially produce pregnenolone (11; but see Ref. 10). According to this model, astrocytes may be the most active steroidogenic cells in the brain, expressing P450 side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), and cytochrome P450 aromatase. Based on these results, astrocytes appear to preferentially synthesize pregnenolone and progesterone (9). Previously we demonstrated that estradiol increased progesterone synthesis in neonatal cortical astrocytes, indicating that peripheral estrogen regulated the synthesis of neurosteroids (2). In a parallel series of studies, neonatal cortical astrocytes were demonstrated to express estrogen receptor (ER)-α and ERβ mRNAs (12). Some of the ER proteins were also present in the membrane fraction and mediated membrane actions of estradiol (12). For example, estradiol, in a dose-dependent and stereo-specific manner, rapidly increased free intracellular calcium ($[Ca^{2+}]_i$) that was inhibited by ICI 182,780, an ER antagonist. Membrane-impermeable 17β-diol-6-one-6-carboxymethyloxime-BSA (E-6-BSA) also rapidly induced $[Ca^{2+}]_i$ transients. In a Ca^{2+}-free medium, estradiol continued to induce $[Ca^{2+}]_i$ spikes, suggesting that intracellular stores of Ca^{2+} were involved. This was borne out when blockade of either phospholipase C (PLC) or inositol trisphosphate (IP_3) receptor or depletion of intracellular Ca^{2+} stores prevented the estrogen-induced rise in $[Ca^{2+}]_i$. Thus, in astrocytes, estradiol acts via membrane-associated ER to rapidly increase $[Ca^{2+}]_i$ through activation of the PLC-IP_3 pathway.

The present studies were undertaken to determine

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Abbreviations: $[Ca^{2+}]_i$, Free intracellular calcium; CNS, central nervous system; Ct, cycle threshold; DMSO, dimethylsulfoxide; E-6-BSA, 17β-diol-6-one-6-carboxymethyloxime-BSA; ER, estrogen receptor; GPCR, G protein-coupled receptor; HBSS, Hanks' balanced salt solution; 3β-HSD, 3β-hydroxysteroid dehydrogenase; IP_3, inositol trisphosphate; mGluR1, metabotropic glutamate receptor type 1; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; P450scc, P450 side-chain cleavage; SCP-2, sterol carrier protein-2; StAR, steroidogenic acute regulatory protein.

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whether hypothalamic astrocytes synthesize progesterone in response to estradiol stimulation and whether this estradiol-induced progesterone synthesis involves an increase of $[Ca^{2+}]_i$ as has been described for steroidogenesis in peripheral tissues. Hypothalamic astrocytes from neonatal and postpubertal female rats were cultured, stimulated with estradiol, and resulting changes in progesterone synthesis and $[Ca^{2+}]_i$ levels determined. Expression of proteins associated with steroidogenesis, steroidogenic enzymes, and steroid carrier proteins was assessed using quantitative RT-PCR. These results have been presented in preliminary form (13).

Materials and Methods

Primary cell cultures

Primary hypothalamic astrocyte cultures were established from Long-Evans female neonatal (d 0–2) or postpubertal rats (d 48–49; Charles River, Wilmington, MA). Hypothalamic tissues were enzymatically digested with trypsin and mechanically disassociated with a fire-polished glass Pasteur pipette. Cultures were grown for 7–10 d in DMEM/F12 (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in a T75 flask at 37 C. Once grown to confluence, astrocyte cultures were purified from oligodendrocytes and microglia using a technique based on the method of McCarthy and de Vellis (55) and previously used in our laboratory (2). Flasks were shaken at 200 rpm at 37 C on an orbital shaker for at least 24 h to eliminate oligodendrocytes. After each shaking, the astrocyte-enriched cultures were replaced with fresh medium. Ten thousand astrocytes were replated into each well of a 24-well culture plate and allowed another 48 h *in vitro* before beginning experiments. To confirm the purity of the cultures, the presence of glial fibrillary acidic protein (Chemicon, Temecula, CA) in cultured cells was assayed by immunocytochemistry. Cultures were counterstained with Hoechst-3342 (10 mg/ml; Invitrogen Corp., Carlsbad, CA) to visualize all nuclei. Cultures routinely contained greater than 95% glial fibrillary acidic protein-positive cells.

Before drug treatment, all experimental astrocyte cultures were steroid starved in DMEM/F12 (supplemented with 5% charcoal-stripped fetal bovine serum) for 24 h. After steroid-starvation, the cultures were treated with cyclodextrin-encapsulated 17β-estradiol (10^{-9} to 10^{-6} M; Sigma, St. Louis, MO), ICI 182,780 [1 μM in dimethylsulfoxide (DMSO); Tocris, Ellisville, MO], 17β-estradiol + ICI 182,780, or estradiol-free DMEM/F12 culture media for 48 h. Subsequently the conditioned media were collected, snap frozen, and stored at −80 C until assayed for progesterone levels. In another experiment studying the role of intracellular Ca^{2+} in neuroprogesterone synthesis, steroid-starved astrocyte cultures were pretreated with thapsigargin (1 μM in DMSO; Tocris) for 1 h. The culture media were collected as above, and new media with estradiol or control (steroid-free DMEM/F12 culture media) were added and incubated for 48 h and then collected.

Steroid RIA

Progesterone levels were quantified by RIA using a method modified from Abraham *et al.* (14; and as described in Ref. 15). Briefly, 0.8 ml of the sample with known amounts of tritiated steroids was extracted with 7 ml diethyl ether, and the extract was dried. The dried extract was dissolved in isooctane and applied to a Celite column (Sigma) for chromatographic separation of steroids. Fractions collected from the columns were dried and reconstituted in assay buffer. The 4-ml fraction contained both the radiolabeled and the immunoreactive progesterone. For routine analysis, progesterone was extracted from the astrocyte conditioned media (0.8 ml) with diethyl ether before the RIA. The mixture was thoroughly vortexed and placed in a dry ice/methanol bath until the aqueous phase was frozen. The ether phase containing progesterone was decanted into a glass test tube and ether evaporated under a nitrogen gas stream. Samples were reconstituted with 0.1 M PBS with 0.1% gelatin. Progesterone standards were prepared in 0.1 M PBS with 0.1% gelatin. The intraassay and interassay coefficients of variation were 5.7 and 7.2%, respectively. The presence of progesterone was verified by gas chromatography/mass spectroscopy (5975 GC/MS system; Agilent Technologies, Palo Alto, CA; with EI/CI package, equipped with an HP1 16.2 m × 0.2 mm × 0.11 μm column). Statistical significance was tested using a one-way ANOVA. Significant differences among means were determined using the Student-Newman-Keuls *post hoc* test. Differences were considered significant at the $P \leq 0.05$ level.

Intracellular Ca^{2+} measurements

Astrocyte-enriched cultures were grown on poly-L-lysine (0.1 mg/ml; Sigma) coated 15-mm glass coverslips until confluent. Cells were steroid starved for 24 h before calcium imaging. Astrocytes were incubated for 45 min with a calcium indicator, fura-2AM (2 μM; Molecular Probes, Eugene, OR), which was dissolved in DMSO and diluted in Hanks' balanced salt solution (HBSS; Mediatech) at 37 C before imaging. Coverslips were mounted in a RC-26 recording chamber on P-4 platform (Warner Instruments, Hamden, CT) and placed on stage of a Axiovert 100 TV inverted microscope (Zeiss, Thornwood, New York, NY). Cells were washed out twice from fura-2 and perfused with HBSS buffered with HEPES (20 mM) using a peristaltic pump (Rainin Instrument, Woburn, MA). Changes in the $[Ca^{2+}]_i$ were imaged on the Attofluor ratio vision digital fluorescence microscopy system (Atto Instruments, Rockville, MD) as previously described (12). Fluorescent intensity at 505 nm with excitation of 334 and 380 nm was followed by videomicroscopy and analyzed using AttoFluor software. For calcium imaging, drugs were used in the following concentrations: 10^{-7} M thapsigargin (Calbiochem, San Diego, CA); 10^{-6} M ICI 182,786; 10^{-9} to 10^{-6} M cyclodextrin-encapsulated 17β-estradiol; 10^{-6} M cyclodextrin-encapsulated 17α-estradiol; 1 μM 1, 3, 5(10)-estratrien-3, E-6-BSA (Steraloids, Newport, RI). Stock solutions were prepared in DMSO, except estradiol, which was prepared fresh in HBSS. Final working concentrations were diluted in HBSS. To remove any free estradiol from the E-6-BSA solution, an aliquot was dissolved in 50 mM Tris-HCl (pH 8.5) and filtered, as described (12). The retained fraction was resuspended in Tris-HCl and used immediately.

Quantitative RT-PCR analysis

mRNA levels were measured in postpubertal female rat astrocyte cultures by quantitative RT-PCR to determine whether estradiol increased the expression of steroidogenic enzymes, P450scc, and 3β-HSD, or carrier proteins, sterol carrier protein-2 (SCP-2) and steroidogenic acute regulatory protein (StAR). Total RNA was isolated from several cultures of Long-Evans postpubertal rat astrocytes using TRIZOL reagent (Invitrogen) as per the manufacturer's recommended protocol. The integrity of total RNA was confirmed by the presence of sharp bands corresponding to 18S and 28S rRNA when separated by electrophoresis on a 2% agarose gel. cDNA was synthesized from the total RNA in 20-μl reactions using anchored oligo $(dT)_{20}$ primers and Superscript III RNase H^- reverse transcriptase (Invitrogen). The reverse transcriptase was performed at 40 C for 15 min, 45 C for 15 min, 50 C for 15 min, and 55 C for 15 min. Real-time chemistry using SYBR Green was performed with primers specific to genes of interest provided in Table 1. Primers and probes were designed using Primer3 software (The Whitehead Institute, Boston, MA) and their specificity confirmed by a BLAST software-assisted search of a nonredundant nucleotide sequence database (National Library of Medicine, Bethesda, MD).

Experiments were conducted with an Mx3000P real-time PCR System (Stratagene, La Jolla, CA) using QuantiTect SYBR Green PCR master mix (QIAGEN, Valencia, CA). PCR conditions used for amplification were as follows: initial denaturation at 95 C for 15 min, 40 cycles of denaturation at 94 C for 15 sec, annealing at 54 C for 30 sec, and elongation at 72 C for 30 sec. Data were collected in real time at the end of each cycle. Negative controls, without reverse transcriptase, and water controls were included in each reaction. In addition to real-time and melting curve analysis of the reactions, amplified products were separated electrophoretically on 2% agarose gels with ethidium bromide, and visualized under UV light to confirm proper amplicon size and the absence of nonspecific products. All PCR products produced a single specific product.

Relative expression data were calculated according to the two Δ-threshold relative quantification method (3, 16). Normalization of cycle threshold (Ct) values was performed by subtracting the β-actin Ct

TABLE 1. Primers specific to genes of interest

Gene	Accession no.	Primers for SYBR Green
P450scc	J05156	FW:CAGCGGTTCATCGACGC RV:TCTGGAGGCATGTTGAGCA
3β-HSD	M38178	FW:CCTACCCAGGCAGACCATCC RV:ATGATGCTCTTCCTCATGGCC
StAR	NM_031558	FW:TCAGAGTAGCAGCTCCCTTGTTTG RV:CTCCAAATCCTGAAACGGGAATGC
SCP-2	NM_138508	FW:AGGGGATGGATTCAAGGC RV:CACGAACTCTTCCCCTTCC
β-Actin	NM_031144	FW:ATGAGCGGTTCCGATGCC RV:CAGCACTGTGTTGGCATAGAGG

FW, Forward; RV, reverse.

from the Ct values acquired for each sample from the gene of interest. To calculate the relative difference in the number of cycles between the samples, the average Ct value of the postpubertal hypothalamic samples was selected as a baseline. This number was subtracted from each of the Ct values that were previously normalized to β-actin. The relative difference between sample groups was calculated based on the difference in Ct values using $2^{-\Delta(\Delta Ct)}$. Because the selected region of the cDNA of interest doubles during each cycle, the relative amount of initial material was compared by raising 2 to the power of the value of the postnormalization- and postbaseline-adjusted cycle difference.

Results

Progesterone synthesis in neonatal vs. postpubertal hypothalamic astrocytes

Astrocyte primary cultures from both neonatal and postpubertal female rats were treated with estradiol (10^{-9} to 10^{-6} M) for 48 h to induce progesterone synthesis. Basal levels of progesterone were measured in the neonatal and postpubertal hypothalamic astrocyte cultures (Fig. 1A). Estradiol did not increase progesterone levels in neonatal hypothalamic astrocyte cultures, compared with the steroid-free DMEM/F12 group (df = 4,19; F = 2.8; P = 0.065). In postpubertal hypothalamic astrocyte cultures (10^{-9} M and 10^{-8} M), estradiol increased levels of progesterone above basal levels, but this increase was significant only at 10^{-6} M estradiol (Fig. 1A; df = 4,39; F = 8.6; $P < 0.05$; Student-Newman-Keuls, $P < 0.05$).

To test whether estradiol-induced progesterone synthesis was mediated through a classical ER, postpubertal astrocyte-enriched cultures were treated with an ER inhibitor, ICI 182,780, in the presence or absence of estradiol. ICI 182,780 blocked estradiol-induced progesterone synthesis (Fig. 1B; df = 3,12; F = 13.1; $P < 0.001$).

Estradiol-induced increase of $[Ca^{2+}]_i$ in hypothalamic astrocytes

To determine whether estradiol could induce a $[Ca^{2+}]_i$ spike in hypothalamic astrocytes, both neonatal and postpubertal astrocytes were imaged using fura-2AM. As previously observed in cortical astrocytes, estradiol induced a rapid $[Ca^{2+}]_i$ spike in both neonatal and postpubertal astrocytes, *in vitro*. A typical example of the effect of 17β-estradiol (20 nM) on postpubertal hypothalamic astrocytes is shown in Fig. 2A. The ED_{50} of the response was 15.9 nM. Estradiol stimulation produced a marked increase in $[Ca^{2+}]_i$ in 74% of cells tested (n = 36, from seven different cultures). The mean maximum amplitude of the $[Ca^{2+}]_i$ response to estradiol was 193.1 ± 14.2 nM in postpubertal hypothalamic astrocyte cultures. In neonatal cultures, the mean maximum amplitude was 235.0 ± 30.1 nM (n = 23). In postpubertal astrocytes, E-6-BSA (1 μM) mimicked the estrogen-induced $[Ca^{2+}]_i$ spike with a mean amplitude of 174 ± 18.9 (n = 11), suggesting a membrane mediated estradiol effect on $[Ca^{2+}]_i$ flux (Fig. 2, B and D). In Ca^{2+}-free medium [chelated with 5 mM 1,2-bis(o-

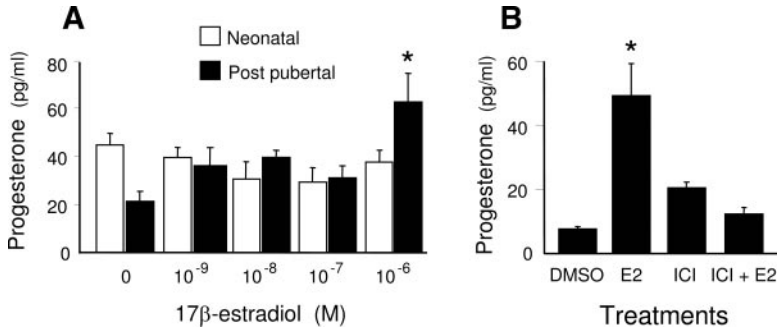

FIG. 1. Response of neonatal and postpubertal astrocytes *in vitro* to estradiol stimulation. A, Cells were steroid starved for 24 h and then incubated for 48 h with indicated concentrations of 17β-estradiol or 17β-estradiol-free media (0). Levels of progesterone in the supernatants were measured by RIA. Neonatal astrocytes did not increase progesterone levels in the media in response to any estradiol dose tested. Postpubertal astrocytes increased progesterone levels after treatment with estradiol. This increase was statistically significant at 10^{-6} M 17β-estradiol. Values are reported as mean ± SEM (n = 8). *, $P < 0.05$ (Student-Newman-Keuls), compared with 0 17β-estradiol. B, 17β-Estradiol (E2; 10^{-6} M) induction of progesterone levels in media from primary postpubertal hypothalamic astrocyte cultures from female rats. Steroid-starved astrocytes were incubated for 48 h with E2-free media containing DMSO, E2, ER antagonist ICI 182,780 (ICI, dissolved in DMSO), and ICI + E2. Levels of progesterone in the supernatants were measured by RIA. Data are mean ± SEM (n = 4). *, Values significantly greater than all other treatment groups ($P < 0.05$, Student-Newman-Keuls).

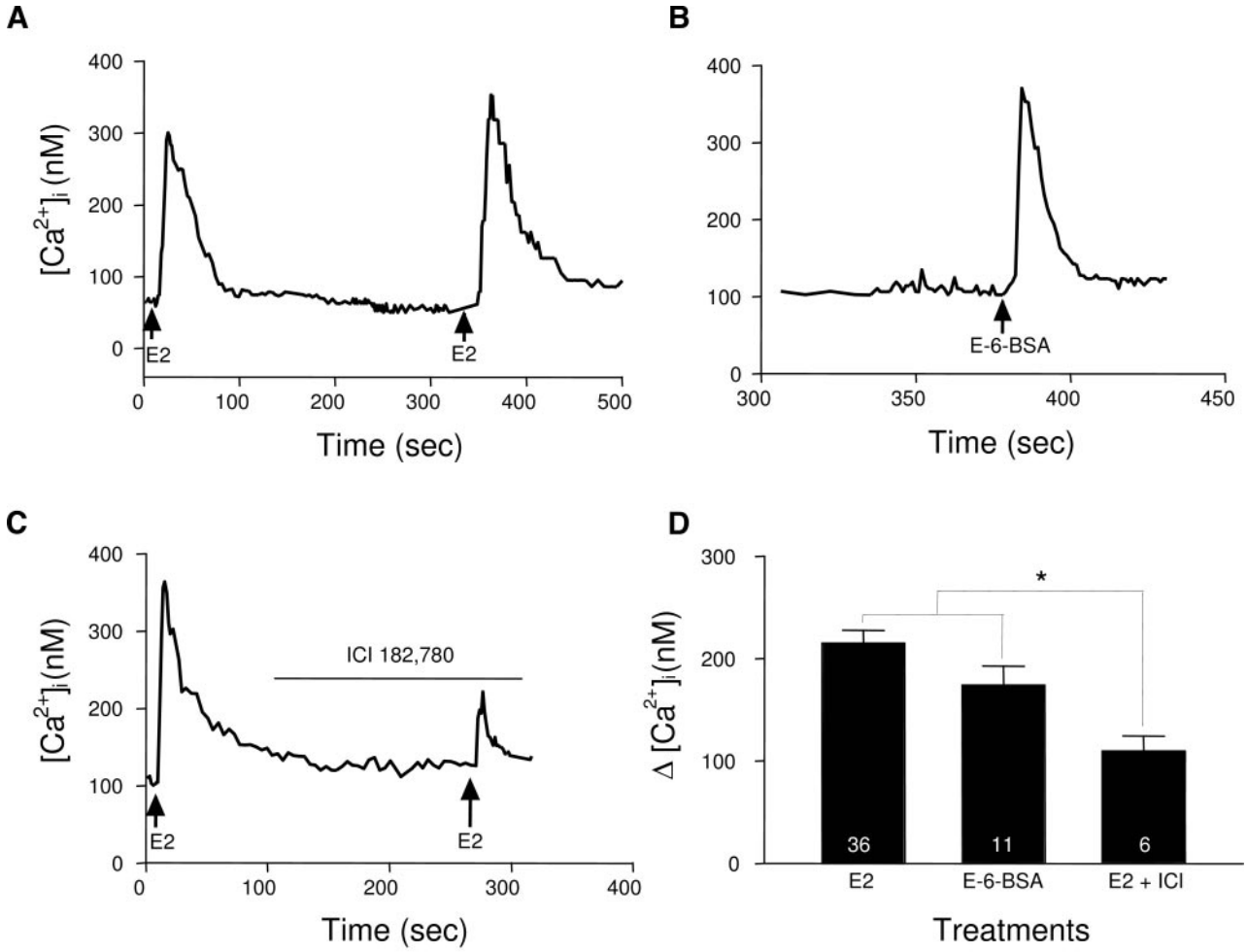

FIG. 2. 17β-Estradiol (E2; 10^{-8} M) induced $[Ca^{2+}]_i$ response in astrocyte-enriched cultures from postpubertal female rats measured by Ca^{2+} imaging fluorescent digital microscopy. A, Changes in $[Ca^{2+}]_i$ (nM) after E2 bath stimulation. E2 rapidly induced a $[Ca^{2+}]_i$ spike that could be reactivated after a 5-min washout period after the initial E2 treatment. B, Real-time changes in $[Ca^{2+}]_i$ after bath application of E-6-BSA (10^{-6} M). E-6-BSA treatment mimicked the E2-induced $[Ca^{2+}]_i$ spike, indicating that estrogen interacted with ERs associated with the plasma membrane. C, Real-time changes in $[Ca^{2+}]_i$ of astrocytes treated with ICI 182,780 (10^{-6} M) and E2 (10^{-8} M). Pretreatment with ICI 182,780 inhibited the E2-induced $[Ca^{2+}]_i$ spike. D, Mean changes in $[Ca^{2+}]_i$ of astrocytes treated with E2, E-6-BSA, or E2 + ICI 182,780. The E2- and E-6-BSA-induced $[Ca^{2+}]_i$ were equivalent, and the E2-induced $[Ca^{2+}]_i$ was significantly reduced with pretreatment with ICI 182,780. The number of observations is indicated in each column. *, Significantly greater than E2 + ICI ($P < 0.05$).

aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid], estradiol produced similar responses to those observed in Ca^{2+}-containing medium, indicating that the $[Ca^{2+}]_i$ spikes were produced through the release of intracellular stores of Ca^{2+} as previously reported with cortical astrocytes (12).

To determine whether the ER mediating these rapid actions of estradiol was similar to the classic intracellular ERs, we tested 17α-estradiol, which did not stimulate $[Ca^{2+}]_i$ spikes (data not shown). Furthermore, the estradiol effect was blocked by 1 μM ICI 182,780 (Fig. 2, C and D). Together, these effects suggest that estradiol action is through a membrane associated receptor with the pharmacology of a classic ER.

Thapsigargin-induced progesterone synthesis in astrocyte cultures

Treatment of postpubertal astrocytes with thapsigargin, which rapidly releases IP_3-sensitive intracellular Ca^{2+} stores, such as those in the smooth endoplasmic reticulum, induced $[Ca^{2+}]_i$ spikes that resembled the spikes induced by estradiol (Fig. 3A). A 1-h application of thapsigargin significantly increased levels of progesterone, compared with control (Fig. 3B; $df = 5,23$; $F = 6.9$; $P < 0.001$). In cultures incubated with estradiol and thapsigargin for 1 h, no further increase was observed (Fig. 3B; Student-Newman-Keuls, $P < 0.05$). In cultures in which the media were changed after an hour of thapsigargin incubation and then incubated for another 48 h in fresh media with or without estradiol, progesterone levels in the supernatant did not increase significantly (Fig. 3B; Student-Newman-Keuls, $P > 0.05$).

Expression of mRNAs coding for proteins associated with progesterone steroidogenesis

Steroidogenesis requires the conversion of cholesterol to pregnenolone through the action of the C27 cholesterol side chain cleavage cytochrome P450 enzyme (P450scc) that is located on the inner mitochondrial membrane. The first step

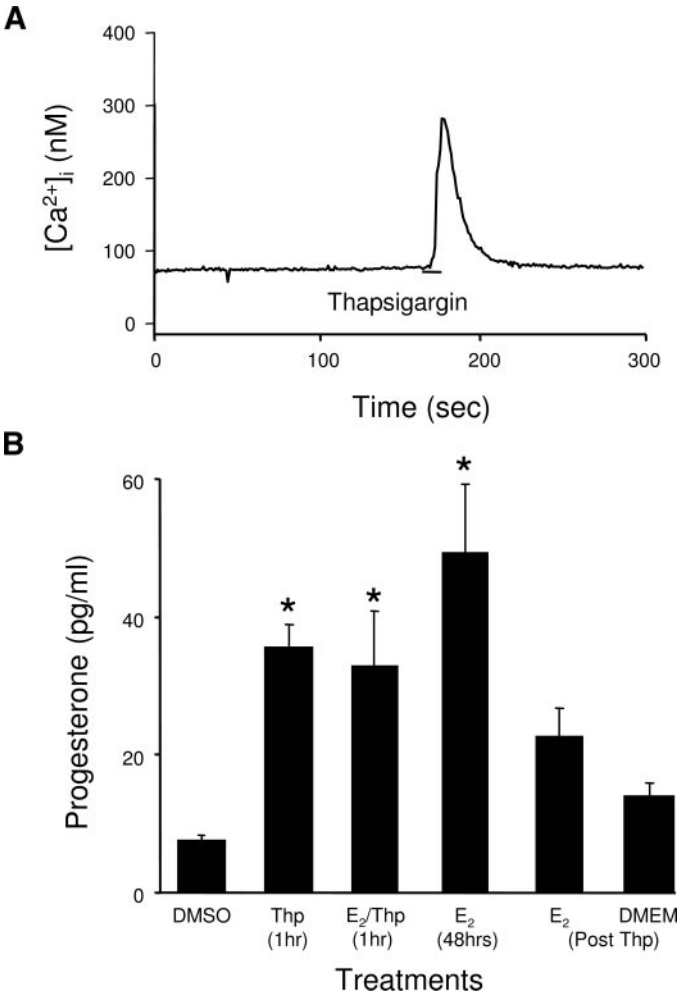

FIG. 3. The effects of thapsigargin on induction of $[Ca^{2+}]_i$ spikes and progesterone synthesis in postpubertal hypothalamic astrocytes from female rats. A, Real-time changes in $[Ca^{2+}]_i$ levels in astrocytes after treatment with thapsigargin (10^{-7} M). Treatment with thapsigargin, a potent inhibitor of Ca^{2+}-ATPase, depleted intracellular Ca^{2+} stores. Stimulation with thapsigargin induced an increase of $[Ca^{2+}]_i$ that was similar to that of estradiol. B, Effect of thapsigargin on progesterone synthesis in astrocyte cultures obtained from postpubertal female rats. Astrocytes were treated with thapsigargin (Thp) or Thp (10^{-7} M) supplemented with 10^{-6} M estradiol (E2/Thp) for 1 h. Media were collected and replaced with either estradiol-free DMEM/F12 (DMEM Post Thp) or 10^{-6} M estradiol (E2 48 h Post Thp). The progesterone concentration in the supernatant significantly increased after 48 h treatment with E2 or an hour of Thp or Thp + E2. When the media were replaced with DMEM/F12 (DMEM) after Thp treatment, there was no increase of progesterone concentration above baseline. After an hour of Thp treatment, 48 h of E2 did not statistically increase the concentration of progesterone in the supernatant. Data are mean ± SEM (n = 4). *, Values significantly different ($P < 0.05$), compared with the control media, DMEM + DMSO.

in steroidogenesis is the delivery of cholesterol to the mitochondrion mediated by SCP-2 (17), in which it is then shuttled to the inner membrane by StAR (18). Pregnenolone is converted to progesterone by 3β-HSD in the smooth endoplasmic reticulum (19–22). In the ovary, StAR-mediated transport is the rate-limiting step of steroidogenesis (reviewed in Ref. 19). Because estradiol induced progesterone synthesis in postpubertal astrocytes, only these cells were used to measure estradiol effects on mRNA levels of genes coding for proteins involved in steroidogenesis in these cells. Interestingly, estradiol treatment did not increase P450scc, 3β-HSD, StAR, or SCP-2 mRNA levels, compared with control cultures sampled at 24 and 48 h as measured by RT-PCR (one-way ANOVA effect of treatment $P = 0.579$, $df = 1,8$, $F = 0.334$; $P = 0.899$, $df = 1,8$, $F = 0.0175$; $P = 0.500$, $df = 1,8$, $F = 0.500$; $P = 0.177$, $df = 1,8$, $F = 2.190$, respectively).

Discussion

The major findings of the present experiments are that estradiol induced progesterone synthesis in cultured postpubertal hypothalamic astrocytes and that this stimulation was mediated through an increase in $[Ca^{2+}]_i$. Interestingly, a basal level of progesterone was present in media from cultured neonatal astrocytes, but only postpubertal astrocytes responded to estradiol with increased progesterone levels. These data suggest that estradiol-induced progesterone synthesis in astrocytes is developmentally regulated. The response of postpubertal astrocytes *in vitro* was similar to adult hypothalamus that had been treated with estradiol *in vivo* (1). In both experiments, estradiol increased progesterone synthesis. The present observations are also consistent with hypothalamic nonresponsiveness to estradiol-positive feedback before puberty (23, 24). Why neonatal hypothalamic astrocytes do not respond to estradiol stimulation is presently not clear. Quantitative RT-PCR identified mRNAs for progesterone synthesis-associated proteins in cultured neonatal astrocytes and measurement of progesterone in neonatal astrocyte conditioned media indicate that these cells are capable of synthesizing progesterone. Although neonatal astrocytes did not respond to estradiol stimulation by increasing progesterone synthesis, estradiol did induce $[Ca^{2+}]_i$ spikes. Both neonatal and postpubertal astrocytes responded to estradiol, but there were slight differences. For example, in neonatal astrocytes, the effective concentration at 50% (EC_{50}) of estradiol was 12.7 nM and the rise time 21.5 ± 3.6 sec, whereas in postpubertal cortical astrocytes, the EC_{50} = 15.9 nM and rise time = 36.4 ± 5.4 sec. The most striking difference between the neonatal and postpubertal astrocytes in terms of $[Ca^{2+}]_i$ was that estradiol-induced $[Ca^{2+}]_i$ oscillations were observed only in astrocytes from postpubertal rats, and these were the astrocytes in which estradiol-induced progesterone synthesis was detected.

In peripheral steroidogenic tissues, $[Ca^{2+}]_i$ is vital to the intracellular signaling pathways that activate steroidogenesis (25–29). We had previously demonstrated that estradiol rapidly induced $[Ca^{2+}]_i$ spikes in cortical astrocytes (12). Although the neonatal cortical and postpubertal hypothalamic astrocytes were similar in estradiol-induced $[Ca^{2+}]_i$ flux and their ability to synthesize progesterone, the relationship of increased $[Ca^{2+}]_i$ had not been connected to increased progesterone synthesis until now. In the present study, we demonstrate that estradiol increases steroidogenesis that is dependent of estradiol-induced $[Ca^{2+}]_i$.

The estradiol action on $[Ca^{2+}]_i$ flux was rapid (<2 min), blocked by ICI 182,780 and stereospecific (17α-estradiol was ineffective). As previously reported in cortical astrocytes (12), the estradiol effect was due to release of intracellular

Ca^{2+} stores, as evidenced by similar responses to estradiol in standard and Ca^{2+}-free media. However, estradiol stimulated $[Ca^{2+}]_i$ spikes were blocked when internal stores of Ca^{2+} were depleted by thapsigargin, as reported for other nonexcitable cells (30). Previous results had suggested a mechanism through which estradiol may induce progesterone synthesis in astrocytes involving the release of IP_3-sensitve Ca^{2+} stores (12). In the present study, estradiol stimulated $[Ca^{2+}]_i$ flux and increased progesterone synthesis in postpubertal astrocytes. To determine whether increasing $[Ca^{2+}]_i$ would stimulate progesterone synthesis, the sesquiterpene lactone, thapsigargin, was used to rapidly increase release of $[Ca^{2+}]_i$ by releasing Ca^{2+} from intracellular stores bypassing the IP_3-IP_3 receptor mechanism (Ref. 31 and references therein). Thapsigargin caused a rapid and transient increase of $[Ca^{2+}]_i$ (Fig. 3A). A 1-h treatment of postpubertal astrocytes with thapsigargin alone increased progesterone levels in the supernatant (Fig. 3B), which was similar to 1-h treatment with thapsigargin and estradiol. These results suggest that estradiol and thapsigargin activate the same intracellular pathway to stimulate progesterone synthesis. The idea that estradiol increased progesterone synthesis by elevating $[Ca^{2+}]_i$ was supported by the demonstration that levels of progesterone induced by estradiol treatment for 48 h were not significantly different from those induced by the 1-h treatment with thapsigargin. It is important that after thap-

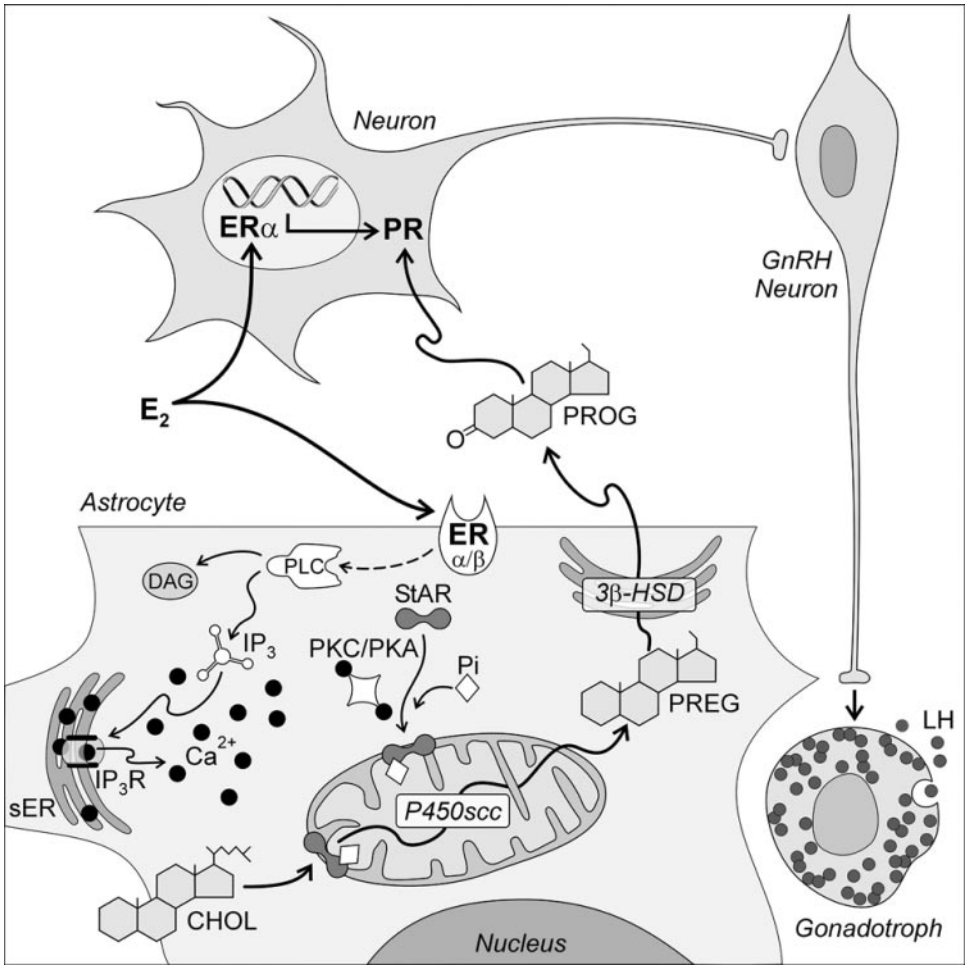

FIG. 4. A model of estradiol action on hypothalamic cells involved in the regulation of the LH surge based on present results and previous findings (3, 51, 52). Circulating estradiol acts on neurons and astrocytes. The astrocyte has been magnified to illustrate the potential intracellular pathway of membrane estradiol signaling that regulates progesterone (PROG) synthesis. Estradiol acts on membrane ERs, which have been reported in astrocyte membranes. Activated ERs increase $[Ca^{2+}]_i$ through a PLC/ IP_3 receptor (IP_3R) mechanism. The source of the elevated $[Ca^{2+}]_i$ is the smooth endoplasmic reticulum (sER). In the present study, estradiol-induced release of Ca^{2+} was mimicked with thapsigargin. Calcium and PKA and possibly PKC phosphorylate StAR before import into the mitochondrion (*e.g.* Ref. 53). The StAR-mediated transport of cholesterol (CHOL) through the mitochondrial intermembrane space is the rate-limiting step of steroidogenesis. In the inner mitochondrial membrane, CHOL is converted to pregnenolone (PREG) by cytochrome P450scc. PREG is converted to PROG in the sER via the action of 3β-HSD isomerase and diffuses out of the astrocyte to encounter estradiol-induced PROG receptors (PRs) in neurons. PR-expressing neurons transmit environmental and circadian signals to GnRH neurons, stimulating release at the median eminence. GnRH in turn initiates the surge release of LH from anterior pituitary gonadotrophs. Several aspects of the model remain to be determined. StAR is present in astrocytes, but the mechanism of its regulation has not been elucidated. In peripheral steroidogenic tissues, both PKA and PKC mediate StAR activation, and it is assumed to be the same in astrocytes. In addition,the localization of PR neurons that regulate GnRH neurons is thought to be along the rostral third ventricle and in the anteroventricular periventricular nucleus and rostral periventricular nucleus (54). DAG, Diacylglycerol; Pi, inorganic phosphate.

sigargin treatment and replacement of DMEM, estradiol treatment for 48 h did not significantly increase progesterone concentration in the supernatant. This is most likely due to the inability of the intracellular Ca^{2+} pools to be restored after depletion because thapsigargin poisons nonmitochondrial Ca^{2+}-ATPase pumps of IP_3-sensitive intracellular Ca^{2+} pools (32).

Whereas the present results strongly implicate a membrane ER such as was demonstrated in cortical astrocyte membranes that signals through the PLC-IP_3 pathway (12), the proximal signaling mechanism of this receptor remains to be elucidated. One possibility is that the membrane ER is itself a G protein-coupled receptor (GPCR) that stimulates PLC. This is a possibility; our results suggest that the membrane receptor is a classic ER, but neither ERα nor ERβ has the typical seven-membrane pass structure of known GPCRs. An alternative proposal is that membrane ERs require another protein, metabotropic glutamate receptor type 1 (mGluR1), to activate intracellular signaling pathways (33). The mGluR1 is a GPCR protein-coupled receptor that activates the PLC-IP3 pathway in neurons and astrocytes (33, 34). Thus, the ER/mGluR1 complex may initiate signaling through the PLC pathway stimulating IP_3 production that in turn activates IP_3 receptors, causing the release of Ca^{2+} (12). This increase of $[Ca^{2+}]_i$ could activate a protein kinase, either protein kinase A (PKA) or protein kinase C (PKC), leading to protein phosphorylation and activation of StAR, a mechanism identified for regulation of steroidogenesis (19, 35).

The idea that steroidogenesis is rapidly activated without involving gene expression is supported by quantitative RT-PCR data. Estradiol did not alter mRNA levels of proteins related to steroidogenesis. Thus, although astrocytes in culture synthesize progesterone and respond to estradiol, the mechanism may be different from that found *in vivo* in which 24 h after estradiol treatment, the expression of 3β-HSD mRNA was increased in the hypothalamus (3). It is possible that in an enriched astrocyte culture that does not have neurons, important neuron-glia interactions are missing (36, 37). These interactions may be necessary for estradiol to increase transcription of 3β-HSD as observed *in vitro* (3). The enriched astrocyte culture model, however, revealed an estradiol nongenomic mechanism intrinsic to astrocytes and points to questions about neural-glial interactions that require further study.

The present results suggest that in addition to an estradiol-induced increase in 3β-HSD expression (3), estradiol also regulates progesterone synthesis through a nongenomic mechanism. For most of the estrous cycle, estradiol provides negative feedback suppressing the GnRH network, but on the afternoon of proestrus, estradiol provides positive feedback inducing the LH surge (38). Our previous studies showed that estradiol-induced increase in locally synthesized hypothalamic progesterone, neuroprogesterone, is a critical part of the positive feedback mechanism (1). We hypothesize that neuroprogesterone binds to estradiol-induced progesterone receptors, activating the GnRH network and causing the surge release of LH that is critical for ovulation (Fig. 4). The present study provides important information about the role of astrocytes in this process and demonstrates a nongenomic estradiol action on $[Ca^{2+}]_i$ through which progesterone synthesis is regulated.

Astrocytes have been traditionally relegated to supporting roles in the CNS. The main function of these cells was thought to be structural support and maintenance of the extracellular environment [*e.g.* blood brain barrier and potassium concentration (22)]. Recently it has become clear that astrocytes have important roles in brain function. Astrocytes express voltage-sensitive channels (39–41), produce Ca^{2+} waves (42) and oscillations (43), and control glutamate uptake (44). Astrocytes have been shown to modulate neural activity (45, 46), neurogenesis (47), plasticity (48), and synaptogenesis (49). In terms of neuroendocrinology, the idea that astrocytes synthesize sex steroid hormones dates back to the pioneering work of Baulieu and colleagues (50). More recently it has been postulated that among the various cells of the CNS, astrocytes preferentially synthesize and release progesterone (9), and we had demonstrated that estradiol stimulated the *de novo* synthesis of progesterone in neonatal cortical astrocytes (2) and hypothalamic astrocytes (this study).

In summary, the present results expand our understanding of peripheral estradiol regulation of neuroprogesterone synthesis. We demonstrated that estradiol can stimulate the *de novo* synthesis of progesterone in hypothalamic astrocytes and that this response is developmentally regulated: inducible in postpubertal but not neonatal cultures. Furthermore, estradiol appears to regulate progesterone synthesis via nongenomic actions through a classic ER associated with the plasma membrane that stimulates the release of $[Ca^{2+}]_i$, and through genomic regulation of 3β-HSD mRNA expression (3). Both of these mechanisms appear to be important for estradiol to induce progesterone synthesis in the hypothalamus, as part of the brain mechanism responsible for initiating the estrogen-positive feedback of the LH surge.

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