Cellular/Molecular

Direct Action of Estradiol on Gonadotropin-Releasing Hormone-1 Neuronal Activity via a Transcription-Dependent Mechanism

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Pulsatile secretion of gonadotropin-releasing hormone-1 (GnRH-1) is essential for reproduction. GnRH-1 induces gonadotropin release and is regulated by 17β-estradiol (E2). Although a subpopulation of GnRH-1 neurons expresses estrogen receptor (ER) β, it is unclear whether E2 acts directly on GnRH-1 neurons or indirectly through interneuronal connections. To test the hypothesis that E2 acts directly on GnRH-1 neurons to regulate neuronal activity, we used calcium imaging to monitor intracellular calcium oscillations in GnRH-1 neurons maintained in nasal explants. TTX was used to minimize synaptic input from other cells. Consistent with previous studies, TTX reduced the activity of individual GnRH-1 neurons to a basal level, while the population of cells maintained synchronized calcium oscillations. Exposure of GnRH-1 cells to TTX plus E2 increased the number of calcium peaks/cell, percentage of cells with ≥10 peaks, mean peak amplitude, and percentage of cells that contributed to each calcium pulse in explants maintained *in vitro* for 7 d (7 div) compared with TTX alone. These effects were induced within 30 min and were not mimicked by 17α-estradiol, E2 conjugated to BSA (which does not cross the plasma membrane), or seen at 21 div, when the percentage of GnRH-1 cells expressing ERβ transcripts declines. In addition, these effects were inhibited by the ER antagonist ICI 182,780 and prevented by inhibition of gene transcription. These data suggest that, via ERβ, E2 can rapidly act as a hormone-activated transcription complex and are the first to show that E2 directly increases GnRH-1 neuronal activity and synchronization.

Key words: **estrogen receptor β; LHRH; calcium imaging; pulsatility; estradiol; GnRH-1**

Introduction

17β-Estradiol (E2) regulates multiple neuronal functions, including neurogenesis (McEwen et al., 2001), reproductive behavior (Meisel and Sachs, 1994), and neuroendocrine output (Moore and Price, 1932; Levine et al., 1985). The classical mechanism of E2 action is through intracellular estrogen receptors (ERs), ERα and ERβ, acting as transcription factors (Kuiper et al., 1996). Additionally, rapid actions of E2 have been documented (Wong and Moss, 1991; Kelly and Levin, 2001). These rapid effects can either occur through ERα and ERβ (Razandi et al., 1999; Abraham et al., 2003) or through a novel, membrane-associated ER (Kelly and Levin, 2001; Toran-Allerand et al., 2002; Qiu et al., 2003).

Gonadotropin-releasing hormone-1 (GnRH-1) neurons regulate reproduction in all vertebrates. In females, E2-induced positive and negative regulation of GnRH-1 neurons occurs during the estrous and menstrual cycles (Levine et al., 1985; Karsch et al., 1987). The mechanism of E2 action on GnRH-1 neurons, however, remains unclear. ERβ plays a functional role in the neuroendocrine system of both peripubertal (Temple et al., 2003) and adult mice (Abraham et al., 2003). Recent reports indicate that a subpopulation of GnRH-1 neurons contains ERβ transcript (Skynner et al., 1999; Sharifi et al., 2002) and protein (Hrabovsky et al., 2001), consistent with a possible direct action of E2, via ERβ, on GnRH-1 neurons.

Successful reproduction relies on synchronized, pulsatile release of GnRH-1 peptide from multiple cells. Thus, sampling across the GnRH-1 neuronal population is critical to elucidate mechanisms underlying GnRH-1 pulsatility. Such sampling is difficult *in vivo* because GnRH-1 neurons are diffusely distributed from olfactory bulbs to caudal hypothalamus (Wray, 2002). Therefore, taking advantage of the extra-CNS origin of these cells, several groups have established an *in vitro* model of postmitotic, primary GnRH-1 cells in nasal explants. Individual GnRH-1 neurons in nasal explants exhibit electrical and synaptic properties similar to *in vivo* GnRH-1 neurons, including responses to GABAergic and glutamatergic stimulation (Kusano et al., 1995). In addition to depolarized-induced GnRH-1 release (Terasawa et al., 1999a; Moore and Wray, 2000), as *in vivo*, GnRH-1 neurons in nasal explants release GnRH-1 in a pulsatile manner (Terasawa et al., 1999a; Funabashi et al., 2000) with the interpulse interval being species dependent. Examination of the

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GnRH-1 neuronal population *in vitro* revealed increases in intracellular calcium, observed with calcium imaging, that correlate with GnRH-1 release (Terasawa et al., 1999a; Moore and Wray, 2000), that display species-specific synchronized calcium oscillations with periodicities similar to GnRH-1 release *in vivo* (Terasawa et al., 1999b; Moore et al., 2002), and that are maintained in the absence of synaptic input (Moore et al., 2002). Recent studies indicate that a subpopulation of GnRH-1 neurons *in vitro* coexpresses ERβ (Sharifi et al., 2002), with the percentage *in vitro* similar to that found *in vivo* (Skynner et al., 1999). Thus, GnRH-1 neurons in this *in vitro* system maintain many intrinsic characteristics of GnRH-1 neurons *in vivo* (Fueshko and Wray, 1994; Wray 2002).

In this study, calcium imaging was used to determine whether E2 acts directly on GnRH-1 neurons to modify neuronal activity in individual GnRH-1 neurons and the GnRH-1 neuronal population. Here, we show that E2 acts directly on GnRH-1 neurons as a DNA transcription factor to increase GnRH-1 neuronal activity and facilitate synchronized pulsatile activity within the GnRH-1 neuronal population.

Materials and Methods

Materials. For preparation of nasal explants, BSA, D-glucose, apotransferrin, putrescine, sodium selenite, bovine insulin, L-ascorbic acid, fluorodeoxyuridine, and thrombin were purchased from Sigma (St. Louis, MO). Gey's balanced salt solution, Ham's F-12 Nutrient Mixture, L-glutamine, and penicillin–streptomycin–nystatin antibiotic mixture were supplied by Invitrogen (Grand Island, NY). Chicken plasma was purchased from Cocalico Biologicals (Reamstown, PA). For treatment of nasal explants, E2, 17β-triol-6-one-6-carboxymethyloxine:BSA (BSA-E2), 17α-estradiol, 5,6-Dichlorobenzimidazole-1-β-D-ribofuranoside (DRB), and TTX were purchased from Sigma. ICI 182,780 was purchased from Tocris (Bristol, UK). For calcium imaging, Calcium Green-1 AM was purchased from Molecular Probes (Eugene, OR), and pluronic F-127 and DMSO were purchased from Sigma.

Nasal explant preparation. Nasal explants were prepared as described previously (Fueshko and Wray, 1994). Briefly, timed-pregnant NIH Swiss mice were obtained, and embryos were removed at embryonic day 11.5 (E11.5) in accordance with National Institutes of Health guidelines. Nasal regions were dissected under aseptic conditions and refrigerated in glucose-enriched Gey's balanced salt solution. Explants were adhered to coverslips via a plasma–thrombin clot and maintained in a defined serum-free medium (SFM) at 37°C in a humidified incubator with 5% CO_2. On the third day *in vitro*, nasal explants were treated with fluorodeoxyuridine (8×10^{-5} M) for 3 d to inhibit cell proliferation. On the sixth day *in vitro*, and every 2–3 d after, explants were provided with fresh SFM. The explants were used for experiments on culture days 6–8 or 20–22.

Calcium imaging. Calcium imaging was conducted using previously described methods (Moore et al., 2002). Briefly, nasal explants were exposed to the indicator dye Calcium Green-1 AM (2.7 mM in 80% DMSO/20% pluronic F-127, diluted 1:200 with SFM) for 20 min in a humidified chamber. Explants were then washed twice with fresh SFM (10 min each) and transferred to a heated perfusion chamber (37.5°C; Warner Instruments, Hamden, CT). Medium was perfused across the explant at a rate of 100 μl/min via a peristaltic pump (Spectra Hardware, Westmoreland City, PA). Intracellular calcium oscillations were visualized using a Nikon microscope equipped with a 20× fluorescence objective and an intensified CCD camera (Video Scope International, Sterling, VA). The camera shutter was controlled by a G4 Macintosh computer via imaging software (IP Labs; Scanalytics, Vienna, VA). Excitation wavelengths were 450–490 nm, and emission was monitored at 520–560 nm.

Analysis of calcium imaging data. Intracellular calcium was monitored every 20 sec for 100 min. At the end of the 100 min period, explants were exposed to an acute dose of KCl (20 mM) to cause an abrupt increase in intracellular calcium to confirm the viability of the cells. Using IP Labs, ODs were measured within each cell, and background values were subtracted; thus, the corrected OD values represent only intracellular events. The traces of intracellular calcium were then analyzed by PULSAR (Merriam and Wachter, 1982) (Fig. 1) to determine when calcium peaks occurred (OD readings 2 SDs above baseline). Time points when peaks were detected in each cell were compiled into a single file for the entire neuronal population within the imaged field. The MATLAB program divided these data into 1 min intervals and assigned either a 0 (no calcium peaks detected during that 1 min period) or a 1 (one or more significant calcium peaks detected). These data were then transformed by the WAVELET analysis program to determine when calcium pulses occurred (Fig. 1). A pulse was defined as a period of time when multiple GnRH-1 neurons displayed synchronous calcium oscillations. For each pulse, the duration from start to finish, the interpulse interval (the time from the

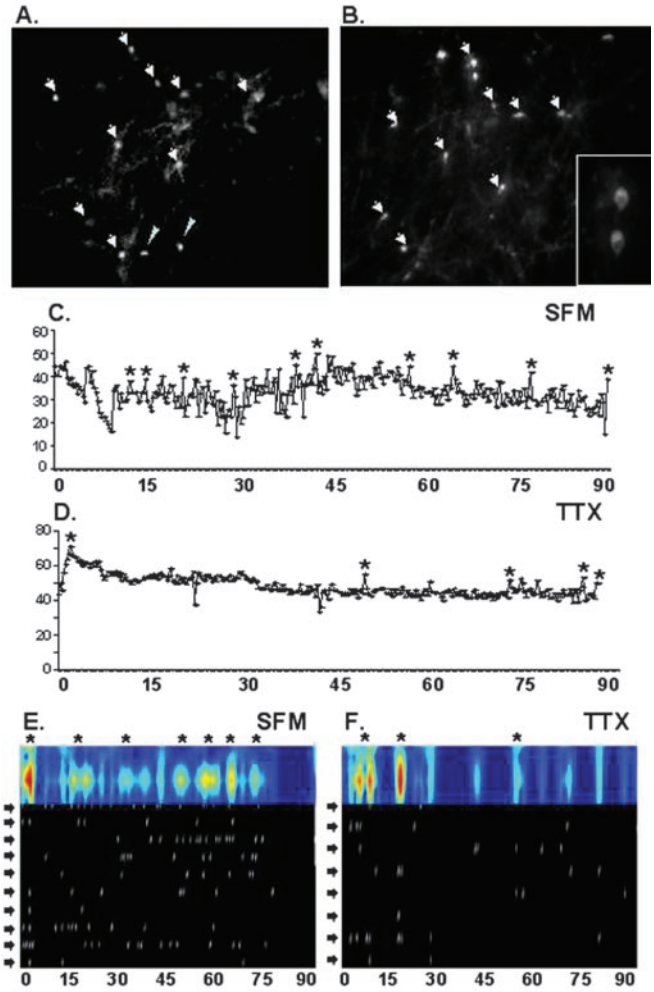

Figure 1. GnRH neurons exhibit synchronized calcium pulses. An example of cells visualized in a 7 div nasal explant labeled with Calcium Green (*A*), imaged for 100 min, followed by fixation and staining for GnRH-1 (*B*). The inset in *B* is a high magnification (40×) of two of the GnRH-1-immunoreactive cells. The arrowheads indicate Calcium Green-labeled cells that were also GnRH-1 positive ($n = 9$). The narrow arrowheads indicate cells that were labeled with calcium dye that were not GnRH-1 positive ($n = 2$). Note that after fixation (*B*), the explant shrinks slightly, and so the distances between cells in (*A*) and (*B*) will not be identical. Activity traces from individual GnRH-1 cells are shown in *C* (SFM) and *D* (TTX); the asterisk indicates a significant increase in intracellular calcium (peaks). Calcium peaks in GnRH-1 cells imaged in a single explant are shown in *E* (SFM; $n = 10$ cells) and *F* (TTX; $n = 8$ cells). In these panels, the *x*-axis is time in minutes. In the black and white section, each row is data from an individual cell (denoted by a black arrow), with the white lines representing the time at which a significant peak in intracellular calcium occurred. The colored trace above represents synchronized calcium oscillations across multiple cells (denoted with an asterisk). The more "intense" the pulsatile event, the closer to red the trace appears.

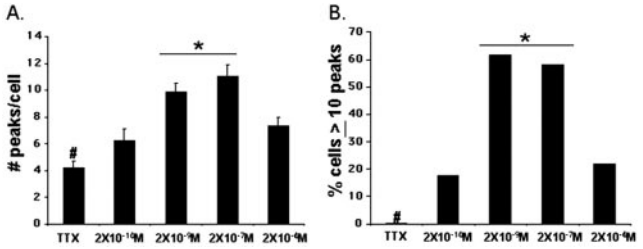

Figure 2. E2 has a direct effect on GnRH-1 neuronal activity. GnRH-1 neuronal responses to E2 were examined at four doses: 2×10^{-10}, 2×10^{-9}, 2×10^{-7}, or 2×10^{-6} M in the presence of TTX. E2 treatment increased the number of calcium peaks per cell (*A*; mean ± SEM) and percentage of cells with ≥10 peaks (*B*) in a dose-dependent manner. Each treatment group contained $n = 3$ explants, 6–8 div. *Significantly different from other E2 doses ($p < 0.05$); #significantly different from all other treatments.

start of one pulse to the start of the next pulse), and the number of cells contributing to the pulse were determined.

Immunocytochemistry. At the end of the data collection period, explants were fixed with 4% formalin in PBS for 1 hr. They were rinsed with PBS and incubated for 1 hr in 10% NGS with 0.1% sodium azide and 0.3% Triton X-100, followed by several rinses in PBS. Explants were then incubated overnight at 4°C in GnRH-1 antibody (SW1, 1:3000) (Wray et al., 1988). The next day, cultures were washed with PBS and incubated in goat anti-rabbit-conjugated Cy3 (1:800; Jackson ImmunoResearch, West Grove, PA). The staining was then compared with the calcium dye labeling to determine which cells were GnRH-1 cells (Fig. 1). Any cells that were not GnRH-1 positive were analyzed separately (<5%).

Transfections of MCF-7 cells. To assure that any effects of the BSA-E2 are not attributable to unconjugated E2, MCF-7 breast cancer cells were transfected with a construct containing an estrogen response element (ERE) linked to a luciferase reporter gene (Wang et al., 2003). Using the manufacturer's (Invitrogen, Carlsbad, CA) instructions, lipofectin was used to transfect this construct into MCF-7 cells. Briefly, cells were grown to 80–90% confluency in 6-well plates. For each well, 4 μg of DNA was brought up to 250 μl in OptiMEM medium. In a separate tube, 10 μl of lipofectin was also brought up to 250 μl in OptiMEM. After 10 min, the DNA and lipofectin mixtures were combined and allowed to incubate (20 min). This mixture was then added to 1.5 ml of OptiMEM (per well), and 2 ml of DNA–lipofectin construct was added to each well. After transfection (6–8 hr), the cells were treated with E2, BSA-E2, or no hormone for 18 hr. Cells were lysed, and their contents were harvested and assayed for luciferase activity. The only way for the luciferase to become activated is for E2 to bind to an intracellular ER, which then binds to the ERE. Therefore, the relative amount of luciferase activity accurately reflects free E2.

Experimental methods for 7 d in vitro *nasal explants.* Experimental groups consisted of two to four explants per treatment with an average of 14.8 ± 0.9 (range, 8–32) cells visualized/explant. The sodium channel blocker TTX was used to isolate GnRH-1 neurons from neuronal input to examine direct effects of E2 and E2 antagonist administration on calcium oscillations. All drugs were perfused continuously across the explant for the duration of the experiment and, where indicated, explants were treated after calcium dye loading before imaging. The following treatments were given in the presence of TTX (10^{-6} M in acetate buffer); E2 (2×10^{-6}, 2×10^{-7}, 2×10^{-9}, and 2×10^{-10} M in EtOH) (Fig. 2), 17α-estradiol (2×10^{-6} M in EtOH), E2 plus ICI 182,780 (2×10^{-7} M in EtOH), BSA-E2 (2×10^{-7} M), BSA-E2 plus ICI 182,780, E2 plus DRB (150 μM given at time 0, 10 min prior, or 30 min before the beginning of imaging), and DRB (30 min before incubation). To remove any free E2, BSA-E2 was dissolved in 50 mM Tris-HCl, pH 8.5, and filtered through a 3 kDa cutoff filter (Amicon, Beverly, MA) immediately before use (Stevis et al., 1999). The retentate was resuspended in SFM to its final concentration (2×10^{-7} M).

Several compounds were run on explants in the absence of TTX (Table 1). These included SFM plus vehicle (EtOH, acetate buffer, and 50 mM Tris-HCl), SFM plus E2 (2×10^{-7} M), SFM plus BSA-E2 (2×10^{-7} M), SFM plus BSA, and SFM plus DRB.

To determine the rapidity of E2 effects on calcium oscillations, a time course study was conducted. Four 7 d *in vitro* (div) explants were imaged during treatment with TTX for 30 min to establish a baseline. Estradiol was then added to the perfusate, and imaging was continued for an additional 80 min. The data were analyzed, and the percentage of cells contributing to each pulse was used for the following analysis. The data were separated into 10 min blocks, and each pulse was represented as the change in the percentage of cells contributing to the pulse relative to the percentage of cells contributing to pulses with TTX alone (41%). A scatterplot with a trendline was generated, and the time point at which the percentage of cells contributing to pulses reached the level of that for E2 plus TTX (66%) was determined.

Experimental methods for 21 div nasal explants. A previous study showed that expression of ERβ decreased significantly in GnRH-1 neurons from nasal explants older than 14 div (from ~50 to <25%) (Sharifi et al., 2002). Nasal explants at 21 div were therefore used as one mechanism to determine whether estrogenic effects were mediated through the ERβ. Experimental groups consisted of two to three explants per treatment with an average of 11.8 ± 1.1 (range, 8–21) cells visualized/explant. The treatments given at 21 div were similar to those given at 7 div (as described above).

Statistical analysis. Statistical comparisons of frequency data were calculated using χ^2 analysis. Comparisons of pulse duration, pulse amplitude, interpulse interval, peak amplitude, and number of calcium peaks per cell were made using ANOVA, followed by a Fisher's LSD *post hoc* test. All analyses were conducted using Statview statistical software (Abacus Concepts, Berkley, CA). χ^2 data were considered significantly different if $p < 0.01$. ANOVA data were considered significantly different if $p < 0.05$. All data are expressed as mean ± SEM.

Table 1. Summary of calcium imaging analysis of GnRH-1 neurons from non-TTX-treated explants at 7 and 21 div

	Number of cells with peaks (%)	Number of peaks/cell	Mean peak amplitude	Cells ≥10 peaks (%)	Percentage of cells contributing to each pulse
7 div					
SFM	43/44 (97)	9.3 ± 1.4	14.4 ± 0.3	14/44 (32)	43.5
E2	60/63 (95)	6.2 ± 0.8	15.2 ± 0.4	12/63 (19)	39.3
ICI	48/51 (94)	9.6 ± 1.7	14.9 ± 0.4	19/51 (37)	51.1
E2 plus ICI	34/36 (94)	9.5 ± 1.9	14.6 ± 0.5	21/36 (58)[a]	45.9
BSA	24/26 (92)	5.7 ± 1.0	16.6 ± 0.8	4/26 (15)	46.9
BSA-E2	42/42 (100)	8.2 ± 0.3	21.9 ± 0.6[a]	10/42 (24)	52.0
DRB	33/34 (97)	7.8 ± 1.2	17.8 ± 0.8	9/34 (25)	60.0[a]
21 div					
SFM	26/27 (96)	12.12 ± 2.6	15.3 ± 0.6	10/27 (38)	50.9
E2	14/14 (100)	6.2 ± 0.3[a]	11.7 ± 0.5	2/14 (14)	50.0

[a]Significantly different from SFM ($p < 0.05$ for ANOVA; $p < 0.01$ for χ^2).

Table 2. Summary of calcium imaging analysis of GnRH-1 neurons at 7 and 21 div in the presence of TTX

	Number of cells with peaks (%)	Number of peaks/cell	Mean peak amplitude	Cells ≥10 peaks (%)	Percentage of cells contributing to each pulse
7 div					
TTX	29/31 (94)	4.2 ± 0.5[a]	13.0 ± 1.1[a]	0/31 (0)[a]	40.6[a]
TTX plus 17α-estradiol	51/54 (94)	3.7 ± 0.4[a]	13.1 ± 0.4[a]	3/54 (6)[a]	41.3[a]
TTX plus E2	58/59 (98)	11.5 ± 0.8[b]	20.5 ± 0.6[b]	34/59 (58)[b]	65.8[b]
TTX plus E2 plus ICI	38/39 (97)	4.6 ± 0.6[a]	17.4 ± 0.9	4/39 (10)[a]	59.9[b]
TTX plus BSA-E2	66/66 (100)	6.3 ± 0.3[a]	21.8 ± 0.5[b]	7/66 (11)[a]	60.7[b]
TTX plus BSA-E2 plus ICI	20/21 (95)	3.7 ± 0.3[a]	16.7 ± 0.8[a]	0/21 (0)[a]	41.3[a]
21 div					
TTX	26/29 (90)	4.9 ± 0.7	10.9 ± 0.8	2/29 (7)	39.8
TTX plus E2	21/26 (81)	2.8 ± 0.6	9.7 ± 0.2	1/26 (4)	49.4

[a]Significantly different from TTX plus E2 within each age.

[b]Significantly different from TTX.

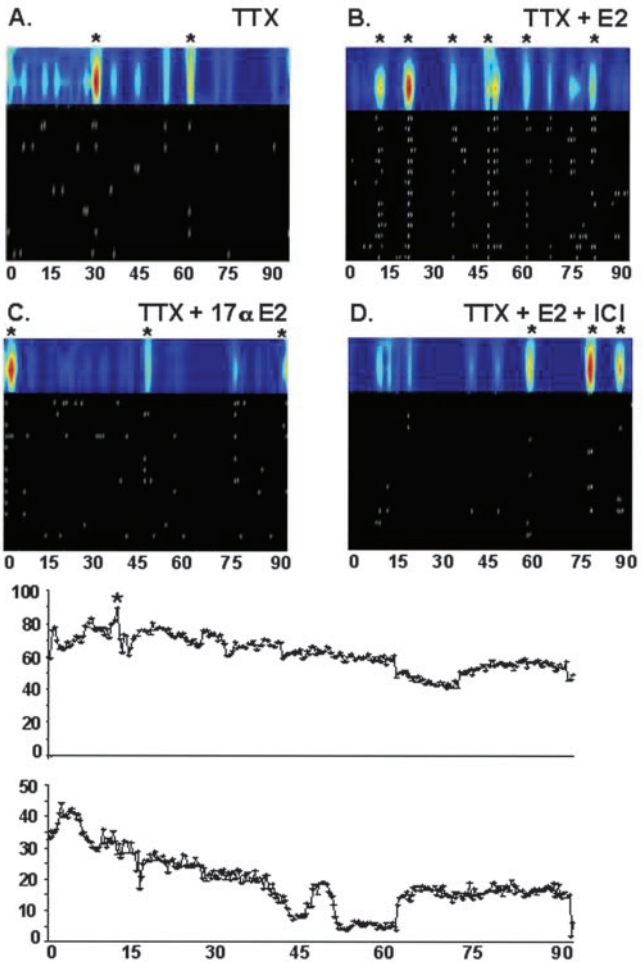

Figure 3. E2, but not 17α-estradiol, significantly increased GnRH-1 neuronal activity as well as the percentage of cells contributing to GnRH-1 pulses. Individual examples of calcium imaging traces of 7 div explants treated with TTX (*A*; $n = 8$ cells), TTX plus E2 (*B*; $n = 14$ cells), TTX plus 17α-estradiol (*C*; $n = 13$ cells), or TTX plus E2 plus ICI 182,780 (*D*; $n = 13$ cells). The *x*-axis is time (minutes). In the black and white section, each row is data from an individual cell across time, and the white lines represent the time at which a significant peak in intracellular calcium occurred. The colored trace above represents synchronized calcium oscillations across multiple cells (denoted with an asterisk). The more "intense" the pulsatile event, the closer to red the pulse appears. The bottom activity traces are individual examples of non-GnRH-1 neurons from an explant treated with TTX plus E2. The asterisk indicates a significant increase in intracellular calcium (peaks).

Results

Estradiol rapidly increases GnRH-1 neuronal activity

To determine whether E2 can act directly on GnRH-1 cells, four doses of E2 (2×10^{-10} to 2×10^{-6} M) were tested in the presence of TTX on 7 div explants. All doses increased GnRH-1 intracellular calcium above the level of TTX alone. However, the 2×10^{-9} and 2×10^{-7} M doses both increased the number of calcium peaks per cell as well as the percentage of cells with ≥10 pulses above the level of 2×10^{-6} and 2×10^{-10} M doses (Fig. 2). Based on these results, E2 at 2×10^{-7} M was used for all additional studies.

In explants at 7 div, E2 treatment in the presence of TTX significantly increased the number of calcium peaks/cell (more than twofold), the percentage of cells with ≥10 peaks (from 0 to 58%), the peak amplitude (>50%), and the percentage of cells contributing to each calcium pulse (25%) compared with TTX treatment alone ($p < 0.001$ for all parameters) (Table 2). Increases in these parameters were not found after treatment with 17α-estradiol (Fig. 3, Table 2) or by treatment with E2 in the absence of TTX (Table 1). In addition, increases in synchronous activity after E2 treatment were specific to the GnRH-1 neuronal population, because no synchronized activity and very few calcium peaks were observed in non-GnRH-1 neurons ($n = 8$) treated with TTX plus E2 (Fig. 3). GnRH-1 neurons in TTX-treated explants show a reduced neuronal activity compared with GnRH-1 neurons maintained in SFM alone (Tables 1, 2) (Moore et al., 2002). The E2-induced increase in GnRH-1 neurons activated in the presence of TTX resulted in many values similar to those observed in GnRH-1 neurons maintained in SFM alone. However, compared with GnRH-1 neurons maintained in SFM alone or TTX alone, the TTX plus E2 treatment significantly increased the percentage of GnRH-1 cells contributing to the calcium pulses (SFM, 41%; TTX, 40%; TTX plus E2, 66%). None of the treatments had an effect on the interpulse interval, which remained ~18 min (data not shown). In addition, no treatments affected the activity of non-GnRH-1 neurons, which exhibited small numbers of peaks (approximately one to two) and showed no intrinsic synchronous activity.

A time course analysis was conducted to determine the temporal rate of action of E2. In 7 div explants treated with TTX for 30 min then exposed to TTX plus E2, GnRH-1 neurons exhibited augmented synchronized calcium pulses (with a similar percentage as found for TTX plus E2; 66%) ~28 min after E2 application.

Mechanism of E2 action on GnRH-1 neurons

Time-dependent expression of ERβ in GnRH-1 neurons

It has previously been demonstrated that ERβ expression in GnRH-1 neurons in nasal explants is decreased at 21 div com-

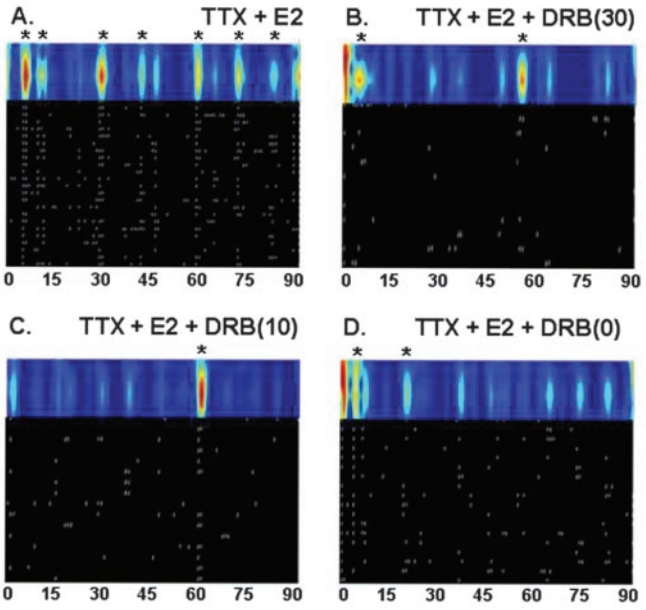

Figure 4. Inhibition of gene transcription reversed the effects of TTX plus E2 at three different time points. Individual examples of calcium imaging traces of 7 div explants treated with TTX plus E2 (*A*; $n = 26$ cells), TTX plus E2 plus DRB (*B*; 30 min before treatment; $n = 12$ cells), TTX plus E2 plus DRB (*C*; 10 min before treatment; $n = 16$ cells), or E2 plus TTX plus DRB (*D*; no pretreatment; $n = 18$ cells). The *x*-axis is time in minutes. In the black and white section, each row is data from an individual cell across time, and the white lines represent the time at which a significant peak in intracellular calcium occurred. The colored trace above represents synchronized calcium oscillations across multiple cells (denoted with an asterisk). The more "intense" the pulsatile event, the closer to red the pulse appears.

pared with 7 div. Thus, as a first step toward determining whether the action of E2 is mediated by ERβ, the effects of E2 on GnRH-1 neuronal activity in older explants were examined. Unlike GnRH-1 neurons in 7 div explants, E2 had no effect on GnRH-1 neuronal activity at 21 div (Table 2).

ER antagonist

In 7 div explants, the stimulatory effects of E2 were blocked when ICI 182,780, an ER antagonist, was added in conjunction with E2 and TTX. ICI 182,780 is a pure antagonist for both ERα and ERβ, but because GnRH-1 neurons do not contain ERα, it was used to block ERβ. However, it is unclear whether this compound can block the putative membrane ER. GnRH-1 neurons in TTX plus E2 plus ICI 182,780-treated explants had significantly fewer calcium peaks/cell when compared with GnRH-1 neurons in TTX plus E2-treated explants ($p \leq 0.001$) (Fig. 3). In addition, ICI 182,780 treatment significantly decreased the percentage of cells with $\geq$10 cells and peak amplitude ($p < 0.05$ and $p \leq 0.001$) (Table 2, Fig. 3).

Gene transcription

To determine whether the estrogenic effects observed involved gene transcription, 7 div explants were either pretreated (10 or 30 min) with DRB (an RNA polymerase II inhibitor), followed by either no drug or TTX plus E2, or were given DRB at the same time as TTX plus E2 (time 0). The DRB treatment alone had no inhibitory effects, but when DRB was combined with TTX plus E2, no estrogenic effects were observed, regardless of the duration of preincubation (Fig. 4, Table 3).

Membrane receptor

To determine whether the effects of E2 on GnRH-1 neuronal activity were mediated via a membrane receptor, explants were treated with E2 conjugated to BSA to prevent passage through the plasma membrane. TTX plus BSA-E2 treatment was not significantly different than TTX on most measures (Table 2). The BSA-E2 did, however, increase the percentage of cells contributing to each pulse ($p < 0.01$) and the peak amplitude ($p < 0.05$) compared with TTX alone. These effects were reversed by treatment with ICI 182,780 (the percentage of cells contributing to pulses was 41.3%, and the mean peak amplitude was 16.7 $\pm$ 0.8). Measurements of luciferase activity from MCF-7 cells transfected with an ERE–luciferase reporter construct and treated with E2, BSA-E2, or no hormone suggested that the effects of BSA-E2 can be attributed to a small amount of free E2. The E2 treatment resulted in a 240% increase in luciferase activity above the no hormone controls. However, the BSA-E2 treatment also yielded a modest (59%), but significant, increase in luciferase activity.

Discussion

This study investigated the action of E2 on GnRH-1 neuronal activity in an *in vitro* model system, devoid of humoral and CNS influences, in which many characteristics of GnRH-1 neurons *in vivo* are maintained. We show that in GnRH-1 cells, E2, in the presence of TTX, increased the number of calcium peaks/cell, the percentage of cells with $\geq$10 calcium peaks, the peak amplitude, and the percentage of cells contributing to each pulse in explants at 7 div, indicating a direct effect of E2 on GnRH-1 neuronal activity. E2 did not alter the GnRH-1 interpulse interval. A number of experiments were performed to examine the receptor transducing the E2 response. Changes in GnRH-1 neuronal activity did not occur when E2 was applied at 21 div, a time when few GnRH-1 neurons express ERβ, nor did any response occur at 7 div when 17α-estradiol was applied. When BSA-E2 was substituted for E2, no changes occurred in calcium peaks/cell or GnRH-1 cells with $\geq$10 calcium peaks. Increases in the percentage of cells contributing to pulses and the mean peak amplitude were observed but are attributable to a small amount of free E2 within the BSA-E2 preparation. Thus, only free E2 altered GnRH-1 neuronal activity. The effects of this E2 were reversed by treatment with ICI 182,780. Together, these data suggest E2 action on GnRH-1 neurons via ERβ. Classic E2-bound/ERβs act as

Table 3. Summary of calcium imaging analysis of GnRH-1 neurons after inhibition of gene transcription

	Number of cells with peaks (%)	Number of peaks/cell	Mean peak amplitude	Cells $\geq$10 peaks (%)	Percentage of cells contributing to each pulse
TTX plus E2	58/59 (98)	11.5 $\pm$ 0.8[a]	20.5 $\pm$ 0.6[a]	34/59 (58)[a]	65.8[a]
TTX plus DRB	26/29 (90)	5.2 $\pm$ 0.8	16.7 $\pm$ 0.7	3/29 (10)	45.2
TTX plus E2 plus DRB (30)	41/44 (93)	2.9 $\pm$ 0.3	14.1 $\pm$ 0.7	1/44 (2)	44.2
TTX plus E2 plus DRB (10)	38/38 (100)	3.9 $\pm$ 0.3	15.1 $\pm$ 0.6	0/38 (0)	35.4
TTX plus E2 plus DRB (0)	38/40 (95)	5.4 $\pm$ 0.5	18.1 $\pm$ 0.9	3/40 (8)	75.4[a]

[a]Significantly different from other treatments ($p < 0.05$ for ANOVA; $p < 0.01$ for χ^2).

DNA-binding proteins to alter transcription. When gene transcription was inhibited by administration of DRB, the effects of E2 on GnRH-1 neuronal activity were blocked. In summary, E2 can act directly on the GnRH-1 neuronal population to facilitate synchronization of neuronal activity. In addition, the effects of E2 on GnRH-1 neurons are rapid and mediated through a transcription-dependent mechanism.

Although commonly associated with reproduction, estrogen has actions throughout the nervous system and has been implicated as a neuroprotective agent (Wise, 2003). The mechanism(s) by which E2 regulates neuronal phenotypes is complex, involving both genomic and nongenomic pathways. The classical mechanism of estrogen action is for E2 to bind to intracellular ERs, which form dimers, bind to EREs, and directly regulate transcription of estrogen-sensitive genes. These transcriptional, or "genomic," events have long been thought to take hours to days (Barnea and Gorski, 1970). However, rapid effects of E2 have been described. Application of E2 to hippocampal cells, hypothalamic cells, and neostriatal cells leads to rapid alterations of membrane excitability (Wong and Moss, 1991; Mermelstein et al., 1996; Qiu et al., 2003). In addition, E2 activates multiple second messenger signaling pathways, including: phosphatidylinositol 3-kinase (Ivanova et al., 2002), tyrosine kinase (Bi et al., 2000), MAPK (mitogen-activated protein kinase) (Bi et al., 2000), and cAMP (Gu and Moss, 1996). These "nongenomic" effects occur within seconds to minutes and are proposed to be mediated by membrane-associated ERs (Kelly and Wagner, 1999). Finally, a third mechanism of E2 action has been described recently. Here, E2 can rapidly induce transcription of genes by activating second messenger pathways, such as MAPK and IP_3, which then go on to trigger DNA synthesis (Watters et al., 1997; Marino et al., 2001). These effects typically occur between 10 min and 1 hr and are also thought to occur through membrane ERs (Watters et al., 1997; Marino et al., 2001). Membrane action of E2 has been widely documented through use of BSA-E2 compounds. Using these membrane-insoluble E2s, studies have shown that ERα and ERβ, traditionally thought of as intracellular receptors, can be targeted to the membrane (Razandi et al., 1999). In addition, a novel membrane-associated ER, referred to as ERX, has been described recently (Toran-Allerand et al., 2002).

Several facts indicate that E2 is acting via ERβ, in the experiments reported here. First, ERα transcript has not been localized to GnRH-1 neurons *in vivo* (Herbison and Pape, 2001) and specifically not in GnRH-1 neurons maintained in the nasal explant system used in this study (Sharifi et al., 2002). Second, it has been demonstrated that ERβ can mediate rapid estrogenic actions within a subpopulation of GnRH-1 neurons (Abraham et al., 2003). Third, ERβ transcript expression in GnRH-1 neurons maintained in nasal explants directly correlates with the E2 response observed. At 7–14 div, ERβ was present in ~50% of the GnRH-1 neurons (Sharifi et al., 2002), corresponding to the time point when a response to E2 was noted. Between 14 and 28 div, the percentage of GnRH-1 neurons expressing ERβ decreased (Sharifi et al., 2002), coinciding with the decline in responsiveness to E2 in GnRH-1 neurons at 21 div. Thus, the primary action of E2 on a subpopulation of GnRH-1 neurons is likely mediated rapidly through ERβ.

Intracellular ERs (ERα and ERβ) have been implicated primarily in genomic actions of E2. However, recently, E2 was shown to increase the phosphorylation of cAMP response element-binding protein within GnRH-1 neurons within 15 min of administration (Abraham et al., 2003). This effect was dependent on intracellular ERβ, because it was absent in ERβ knockout (ERβKO) mice as well as when E2 was conjugated to BSA (Abraham et al., 2003). Based on their data, Abraham et al. (2003) suggested a nongenomic estrogen action via ERβ within the cytoplasm. Abraham et al. (2003) did not, however, specifically rule out the involvement of gene transcription. In this study, the action of E2 on GnRH-1 neuronal activity mimicked a nongenomic "fast" action, but inhibition of gene transcription eliminated the estrogenic effects. Importantly, compared with controls, DRB treatment alone did not alter the neuronal activity of GnRH-1 neurons in nasal explants. These data indicate that the rapid action of E2 on GnRH-1 neurons was genomic. Such a rapid change is consistent with E2-activating transcription of immediate early genes, which are then involved in increasing GnRH-1 neuronal activity. In general, immediate early genes are produced within 15 min after exposure to a stimulus (Mercier et al., 2001). Within GnRH-1 neurons, expression of *c-fos* (a well known member of the immediate early gene family) has been shown to significantly increase during the preovulatory luteinizing hormone (LH) surge, a time when GnRH-1 neuronal activity and E2 are simultaneously elevated (Lee et al., 1990). To date, however, *c-fos* expression in GnRH-1 neurons in nasal explants has not been detected (S. Wray, unpublished observations). Thus, the identity of the E2-induced transcript(s) in GnRH-1 neurons that is responsible for the estrogenic effects within the GnRH-1 neuronal population is still unknown.

Estrogenic regulation of GnRH-1 neuronal activity is critical for reproduction, especially in postpubertal females. The sex of the animal at E11.5 from which the explants were generated was not known. However, ERβ-positive and -negative GnRH-1 cells were found consistently within a single explant, indicating that the results obtained were not dependent on the sex of the animal (Sharifi et al., 2002). Therefore, prenatal GnRH-1 cells maintained *in vitro* in SFM are a heterogeneous population with respect to expression of ERβ transcript in both male- and female-derived explants. No sex differences have been found in coexpression of cholecystokinin in GnRH-1 cells in nasal explants (P. Giacobini and S. Wray, unpublished observations), although expression of this neuropeptide has been shown to be sexually dimorphic within the GnRH-1 neuronal population i*n vivo* (Ciofi, 2000). Similarly, there were no notable variations in the responses of the explants to E2 treatment in this study. Certainly, the time point at which the tissue is harvested (E11.5) is well before documented embryonic hormone surges that are thought to sexually differentiate the brain (MacLuskey and Naftolin, 1981). Thus, as with pulsatile GnRH-1 secretion, it appears that many properties of the GnRH-1 neuronal system are intrinsically programmed or established via nasal cues or both, and subsequently modified on entrance into the CNS.

One of the primary properties of GnRH-1 system regulation in the adult female is positive and negative feedback by E2. Because plasma E2 levels rise during the menstrual and estrous cycles, the GnRH-1 pulse frequency or amplitude increases as well (Freeman, 1994). Augmented release of GnRH-1 leads to a preovulatory LH surge (Freeman, 1994). Once ovulation occurs, E2 inhibits GnRH-1 pulsatility (Freeman, 1994). The negative feedback of E2 on GnRH-1 neurons requires ERα (Dorling et al., 2003; Wersinger et al., 1999). Because GnRH-1 neurons do not express ERα (Herbison and Pape, 2001), negative feedback is likely accomplished via interneuronal cell types (for review, see Petersen et al., 2003). ERβKO females are subfertile, with females getting pregnant ~20% of the time a sperm plug is found (Krege et al., 1998). Although the cause of this fertility deficit is still unclear, reproductive dysfunction in ERβKO mice is consistent

with deficits in E2-induced positive feedback. The identification of ERβ expression within GnRH-1 neurons (Hrabovsky et al., 2001; Kallo et al., 2001; Sharifi et al., 2002) provided a direct route for E2-positive feedback. Although the downstream signals induced after E2 exposure remain to be identified, the functional significance of E2 activation with respect to the GnRH-1 neuronal population is clear. E2 induced an upregulation of GnRH-1 neuronal activity and recruitment of an additional subpopulation of GnRH-1 neurons to the synchronized pulse. This would result in an increase in total GnRH-1 released in a single pulse, which could potentially mediate the rise in GnRH-1 that precedes the preovulatory LH surge.

In summary, this is the first report to show that E2 acts directly on a subpopulation of GnRH-1 neurons to increase neuronal activity and enhance the synchronicity of GnRH-1 pulses, an event associated with GnRH-1 secretion (Terasawa et al., 1999a; Moore and Wray, 2000; Moore et al., 2002). Furthermore, the data indicate that the actions of E2 are likely mediated via intracellular ERβ. Estradiol treatment recruited ~25% additional cells to contribute to each GnRH-1 pulse. In adult mice, ~20% of GnRH-1 neurons express ERβ (Skynner et al., 1999), and only a subset of GnRH-1 neurons appear to be involved in the generation of the preovulatory LH surge (Rajendren, 2001). The GnRH-1 cells examined in this study never entered the CNS and were maintained in a model system devoid of CNS cues, yet exhibit regulation by E2 that parallels observations made in reproductive-aged mice. Thus, based on our findings, we propose that *in vivo*, a subpopulation of ERβ-containing GnRH-1 neurons are recruited by rising levels of E2 before the preovulatory LH surge to mediate E2-induced positive feedback in the hypothalamic–pituitary–gonadal axis.

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