Behavioral/Systems/Cognitive

Differential Regulation of Gonadotropin-Releasing Hormone Neuron Activity and Membrane Properties by Acutely Applied Estradiol: Dependence on Dose and Estrogen Receptor Subtype

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Gonadotropin-releasing hormone (GnRH) neurons are critical to controlling fertility. *In vivo*, estradiol can inhibit or stimulate GnRH release depending on concentration and physiological state. We examined rapid, nongenomic effects of estradiol. Whole-cell recordings were made of GnRH neurons in brain slices from ovariectomized mice with ionotropic GABA and glutamate receptors blocked. Estradiol was bath applied and measurements completed within 15 min. Estradiol from high physiological (preovulatory) concentrations (100 pM) to 100 nM enhanced action potential firing, reduced afterhyperpolarizing potential (AHP) and increased slow afterdepolarization amplitudes (ADP), and reduced I_{AHP} and enhanced I_{ADP}. The reduction of I_{AHP} was occluded by previous blockade of calcium-activated potassium channels. These effects were mimicked by an estrogen receptor (ER) β-specific agonist and were blocked by the classical receptor antagonist ICI182780. ERα or GPR30 agonists had no effect. The acute stimulatory effect of high physiological estradiol on firing rate was dependent on signaling via protein kinase A. In contrast, low physiological levels of estradiol (10 pM) did not affect intrinsic properties. Without blockade of ionotropic GABA and glutamate receptors, however, 10 pM estradiol reduced firing of GnRH neurons; this was mimicked by an ERα agonist. ERα agonists reduced the frequency of GABA transmission to GnRH neurons; GABA can excite to these cells. In contrast, ERβ agonists increased GABA transmission and postsynaptic response. These data suggest rapid intrinsic and network modulation of GnRH neurons by estradiol is dependent on both dose and receptor subtype. In cooperation with genomic actions, nongenomic effects may play a role in feedback regulation of GnRH secretion.

Introduction

Gonadotropin-releasing hormone (GnRH) neurons form the final common pathway for the central regulation of fertility. GnRH is typically released in pulses of varying frequencies (Clarke and Cummins, 1982; Levine et al., 1982; Levine and Duffy, 1988; Moenter et al., 1991, 1992, 2003). In addition to episodic release, in females, a surge mode of GnRH release also exists in which neural activity and hormone release are elevated for hours (Döcke and Dörner, 1965; Moenter et al., 1991; Christian et al., 2005). The gonadal steroid estradiol is a critical feedback regulator of GnRH neurons and is involved in both suppression of episodic release (negative feedback) and induction of the surge mode of release (positive feedback) (Döcke and Dörner, 1965; Herbison, 1998). Estradiol feedback may involve both changes in synaptic transmission to GnRH neurons and altered intrinsic excitability (DeFazio et al., 2002; Nunemaker et al., 2002; Abe and Terasawa, 2005; Chu and Moenter, 2006; Wintermantel et al., 2006; Christian and Moenter, 2007; Romanò et al., 2008).

Estradiol may act via different receptors with different time courses and mechanisms (Filardo and Thomas, 2005; Heldring et al., 2007; Woolley, 2007). In the classical mechanism, estradiol binds to the α or β isoform of the estrogen receptor (ER), which binds to estrogen-response elements and alters gene transcription (Glass, 1994). Nonclassical action of ERα and ERβ are possible through alternative response elements in DNA (Sabbah et al., 1999; Kushner et al., 2000) or rapid changes in signaling cascades (Zhou et al., 1996; Nethrapalli et al., 2001). Alternative ERs have also been proposed, including a G-protein-coupled receptor, GPR30 (Revankar et al., 2005), ER-X (Toran-Allerand et al., 2002), and mER (Qiu et al., 2003). All of these mechanisms operate in the CNS.

The expression of estrogen receptors by GnRH neurons is controversial. Iodinated estradiol is concentrated by these cells, suggesting high affinity binding (Hrabovszky et al., 2000). In samples from brain, messenger RNA and protein for ERβ but not ERα have been detected in these cells (Skynner et al., 1999; Herbison et al., 2001; Hrabovszky et al., 2001). ERα transcript has been identified in immortalized GT1 GnRH neuronal cell lines and cultures of embryonic GnRH neurons (Roy et al., 1999; Na-

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varro et al., 2003). Both ER subtypes have important reproductive effects; ERα knock-out (KO) mice are infertile and ERβKO are subfertile (Couse and Korach, 1999; Wintermantel et al., 2006). Both ERα and ERβ have been implied in rapid effects of estradiol on GnRH neurons (Abrahám et al., 2003, 2004; Temple et al., 2004; Abe and Terasawa, 2005), although a recent report suggests a pure ERα/ERβ antagonist fails to block some rapid effects, indicating alternative mechanisms may exist (Abe et al., 2008).

Here, we examined how estradiol acutely affects GnRH neuron properties using whole-cell electrophysiology. We show that both stimulatory and inhibitory effects are elicited by rapid estradiol application, that the direction of response depends on dose and receptor subtype, and that different neurobiological mechanisms are engaged.

Materials and Methods

Animals. Adult (2–3 months of age) female mice expressing enhanced green fluorescent protein (GFP; Clontech) under the control of the GnRH promoter were used to facilitate identification of GnRH neurons (Suter et al., 2000). Mice were maintained under a 14 h light:10 h dark photoperiod with Harlan 2916 chow and water available *ad libitum*. To avoid the confounding effects of ovarian hormone changes during the estrous cycle, mice were ovariectomized (OVX) under isoflurane (Burns Veterinary Supply) anesthesia 5–9 d before experimentation; time after gonadectomy did not affect results. The long-acting local anesthetic bupivacaine (0.25%; Abbott Laboratories) was applied to surgical sites to minimize postoperative pain and distress. All mice were killed between 11 A.M. and 12 P.M. and all recordings performed between 2 P.M. and 7 P.M. time. All procedures were approved by the Animal Care and Use Committee of the University of Virginia.

Brain slice preparation. All chemicals were purchased from Sigma, unless noted. Brain slices were prepared as described previously (Nunemaker et al., 2002, 2003; Chu and Moenter, 2005). All solutions were bubbled with 95% O_2 and 5% CO_2 to maintain pH and oxygenation for at least 15 min before use and throughout experiments. In brief, brains were quickly removed and immersed immediately for 30–60 s in ice-cold sucrose buffer containing the following (in mM): 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 glucose, 1.25 Na_2HPO4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Sagittal brain slices (300 μM) through the preoptic area (POA) and hypothalamus were cut using a Vibratome 1000 or Vibratome 3000 (Technical Products International). Slices were immediately transferred into a holding chamber and incubated at 31–33°C for a 30 min recovery period in a mixture of 50% sucrose saline and 50% artificial CSF (ACSF) containing the following (in mM): 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 1.25 Na_2HPO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 10 D-glucose, pH 7.4. Slices were then transferred to 100% ACSF and maintained at room temperature (~21–23°C) until study (30 min to 8 h).

Data acquisition slices. Data acquisition slices were transferred to a recording chamber mounted on the stage of an upright microscope (Olympus BX50WI; Opelco) and stabilized in the chamber at least 5 min before recording. The chamber was continuously perfused with ACSF at a rate of 4–5 ml/min at 32°C. Pipettes (3–4 MΩ) were pulled from borosilicate glass capillaries (1.65 mm outer diameter; 1.12 mm inner diameter; World Precision Instruments) using a Flaming/Brown P-97 (Sutter Instrument). GnRH–GFP neurons from POA and ventral hypothalamus were identified by brief illumination at 470 nm. Data were acquired using one headstage of an EPC-10 dual amplifier (HEKA) controlled by PatchMaster (HEKA). Signals were low-pass filtered at 10 kHz. During whole-cell recordings, input resistance (R_{in}), series resistance (R_s), and membrane capacitance (C_m) were continually measured. Only recordings with stable R_{in} >500 MΩ and R_s <20 MΩ and stable C_m were used for analysis. Data were further examined to make sure changes in R_{in} or R_s within acceptable limits did not influence results. Calculated liquid junction potential error, estimated to be −13 mV, was not corrected (Barry, 1994).

Estradiol 17β (E), the main circulating estrogen, was used in all experiments. To examine the effects of E, baseline values for a parameter were first established, then estradiol (final concentration 10 pM–100 nM), vehicle (0.01% ethanol), or other treatment to be followed by estradiol was added to the bath; recording continued and measurements of estradiol effects began after a 5 min wash-in and were completed within 10–15 min of estradiol treatment initiation. In initial experiments, fast synaptic transmission to GnRH neurons was blocked by antagonists to ionotropic transmitter receptors [$GABA_A$ 20 μM bicuculline methiodide; AMPA 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX); NMDA 20 μM D(-)2-amino-5-phosphonovaleric acid (APV)]. Some studies omitted these blockers, and their presence or absence is specified in the Results section for each experiment.

Current-clamp recordings. Whole-cell current-clamp recordings were made using bridge balance and capacitance compensation. Pipettes were filled with a solution containing (in mM) the following: 120 K gluconate, 20 KCl, 10 HEPES, 5 EGTA, 4.0 MgATP, 0.4 NaGTP, 1.0 $CaCl_2$, pH 7.3, 290 mOsm. There was no difference in input resistance (973 ± 104 vs 994 ± 79 MΩ) or resting membrane potential (−63.4 ± 0.5 vs −63.9 ± 1.4) among cells during the control period even when different responses to estradiol based on dose were subsequently observed. All cells had an initial membrane potential negative to −55 mV without current injection and action potential amplitude of >90 mV. DC injection (< ±10 pA) was used to normalize membrane potential to facilitate comparison. Membrane properties were examined as follows. In some cells, action potential firing rate was monitored; changes in firing rate of at least 20% were used to classify cells as responding to treatment. Tetrodotoxin (TTX; 0.5 μM; Calbiochem) was added to some recordings to observe underlying changes in membrane potential. To study specific membrane properties, action potentials were elicited in some cells with short-duration (3 ms) current pulses (300 pA) as in Chu and Moenter, 2006. When multiple pulses were given, they were separated by 50 ms (20 Hz). Each protocol was run 10 times at 2 s intervals, and all 10 traces were averaged for analysis. Amplitude of the afterhyperpolarization (AHP) and slow afterdepolarization (sADP) that followed the action potential was measured from the prespike baseline. To examine effects of E on excitability, longer duration current injections (600 ms 0–50 pA) were given. Latency was defined as the time from start of the current injection (25–30 pA) to the peak of first action potential. The amount of current needed to initiate firing, spike threshold (defined as the point where the derivative of the trace exceeded 1 V/s), number of spikes, and spike amplitude (measured from spike threshold) were determined before and after estradiol treatment. The input resistance of GnRH neurons was determined from the steady-state voltage response to a depolarizing pulse (10–15 pA producing ~10 mV change in membrane potential). Membrane potential changes in response to current injection were determined during the plateau after current injection began and before action potential firing was initiated.

Voltage-clamp recordings. Whole-cell voltage-clamp was used to study currents underlying the AHP and sADP using the same pipette solution as current-clamp studies. Cells were held at −60 mV. Current response to single, double, or quadruple 2 ms square voltage pulses, or simulated action potentials, separated by 50 ms, was evaluated. Stimuli were repeated 50 times and traces averaged for analysis. Amplitude of the outward current (I_{AHP}) and inward current (I_{ADP}) was measured from the precommand baseline. For whole-cell recording of $GABA_A$ receptor-mediated postsynaptic currents (PSCs), cells were held at −60 mV, and pipettes were filled with high-chloride solution containing the following (in mM): 140 KCl, 10 HEPES, 5 EGTA, 4.0 MgATP, 0.4 NaGTP, 1.0 $CaCl_2$, pH 7.3, 290 mOsm.

Cell culture and Western blot. To confirm the efficacy of the G_1 agonist, its ability to stimulate mitogen-activated protein kinase (MAPK) (ERK1/2) activity was measured. Ishikawa endometrial carcinoma cells, which respond to G_1, were plated in 35 mm wells (10^6 cells per well) as described previously (Bryant et al., 2005; Vivacqua et al., 2006). After serum starvation overnight, cells were treated in serum-free, phenol-red-free Dulbecco's Essential Medium containing either ethanol vehicle (0.01%), 10 nM E2, and either 100 or 500 nM G_1 for 15 min. After treatment, cells were collected in SDS-containing gel buffer; 10 μg total protein was subjected to electrophoresis, transferred to nitrocellulose mem-

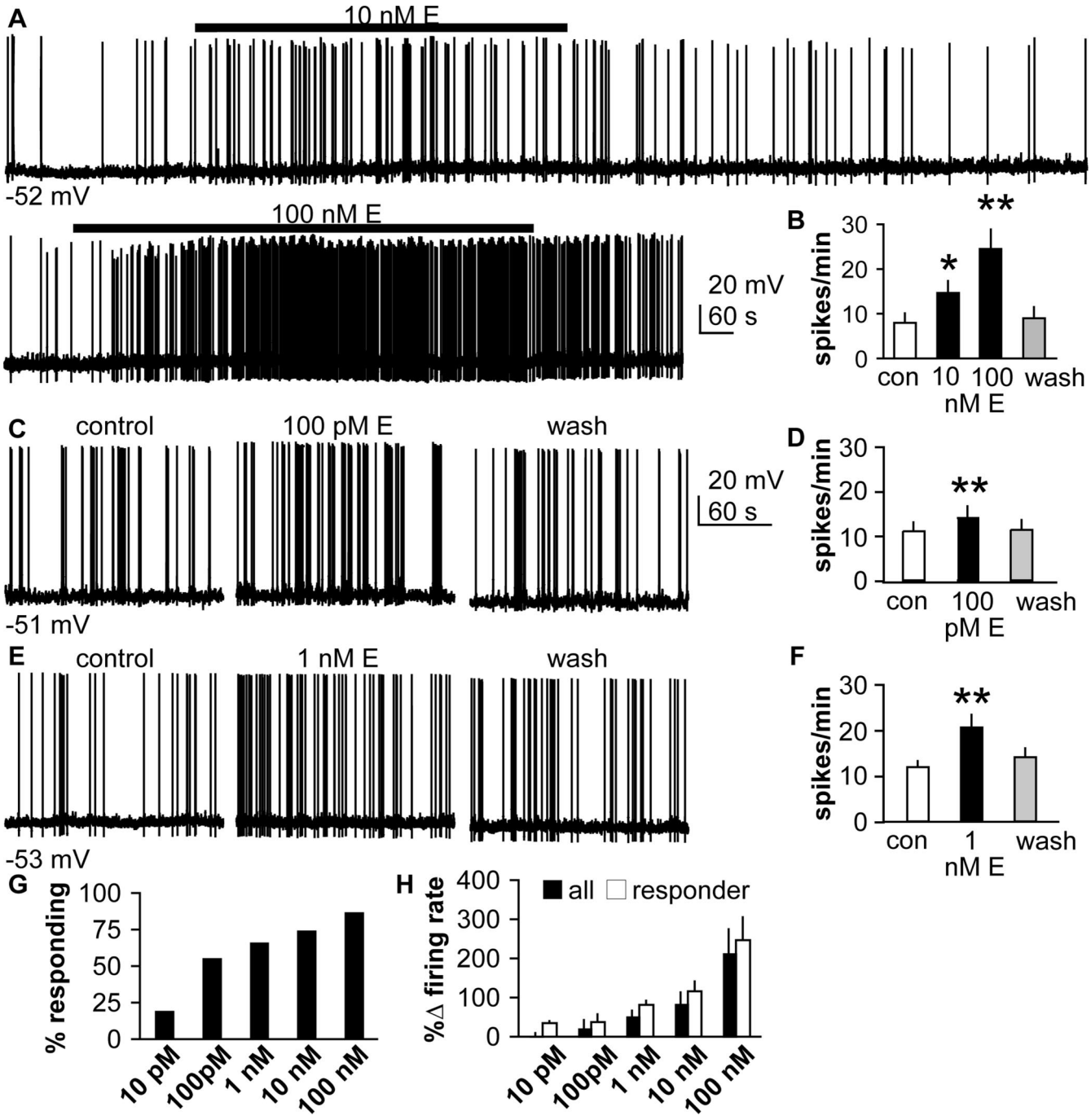

Figure 1. Estradiol (100 pM to 100 nM) increases firing rate of GnRH neurons. ***A***, Representative continuous current-clamp recording of spontaneous firing in a GnRH neuron showing the excitatory effect of 10 nM estradiol and wash-out; second line is the same neuron 10 min later at start of trace showing response to 100 nM estradiol. ***B***, Summary of spikes per minute in different conditions (10 nM, $n = 20$; 100 nM, $n = 8$). ***C***, Representative response to 100 pM estradiol. ***D***, Mean ± SEM response to 100 pM estradiol ($n = 16$). ***E***, Representative response to 1 nM estradiol. ***F***, Mean ± SEM response to 1 nM estradiol ($n = 18$). ***G***, Summary of percentage of cells responding by dose. ***H***, Percentage change in firing rate when all cells are quantified (black bars) or just responding cells are quantified (white bars). *$p < 0.01$, **$p < 0.001$.

branes, and assayed for phosphorylated (activated) and total ERK1/2 with specific antibodies (Cell Signaling Technology) as described previously (Song et al., 2002; Bryant et al., 2005).

Drug treatments. The following treatments were bath applied. The G-protein-coupled estradiol receptor GPR30 (Revankar et al., 2005) was activated with the G_1 agonist (100–500 μM) (Bologa et al., 2006). The pure nuclear estrogen receptor antagonist ICI182780 (1 μM) was used to block effects through ERα and ERβ. The ERα-specific agonist propylpyrazoletriol (PPT) and the ERβ-specific agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) were both used at 10 nM (Harrington et al., 2003; Sun et al., 2003). Small conductance calcium-activated potassium currents (I_{KCa}) were blocked with 200 nM apamin; large conductance I_{KCa} were blocked with 100 nM iberiotoxin (IbTx). Protein kinase A (PKA) was blocked with 100 nM KT5720.

Statistical analyses. Statistical analyses were performed using Instat or Prism (Graphpad Software). Responding cells were defined as those with a >20% change in firing rate. Values for electrophysiological data are expressed as mean ± SEM. Each cell served as its own control. All statistical comparisons were made using paired two-tailed Student's *t* test. Significance was set at $p < 0.05$; all nonsignificant *p* values were >0.1, unless specified.

Results

High physiological (100 pM) through nanomolar concentrations of estradiol increase GnRH neuron activity

To test if estradiol acutely alters GnRH neuron activity, current-clamp recordings were made of GnRH neurons before, during, and after treatment with estradiol (10–100 nM). Fast synaptic transmission via ionotropic GABA and glutamate receptors was blocked in all recordings, unless indicated. After establishing baseline values, estradiol was added to the bath; effects were assessed after a 5 min wash-in, and measurements were complete within 10–15 min of treatment initiation. Estradiol (10–100 nM) rapidly increased the firing rate of 70% of recorded GnRH neurons (10 nM, 14 of 20 cells; 100 nM, 7 of 8 cells) by an average of twofold to threefold in a dose-dependent manner (Fig. 1*A*,*B*) (10 nM estradiol, $p < 0.001$, $n = 20$; 100 nM estradiol, $p < 0.001$, $n = 8$). This effect was reversible, with action potential firing returning to pretreatment levels within 15–20 min (Fig. 1*A*,*B*). Vehicle (0.01% ethanol) had no effect (data not shown).

Although estradiol doses of 10–100 nM are commonly used, particularly in brain slice preparations in which higher concentrations are needed for slice penetration, these levels are higher than those achieved during the preovulatory peak of estradiol *in vivo*. We thus examined the response to 100 pM, which is in the high end of the physiological range, and 1 nM estradiol; these concentrations also increased firing in GnRH neurons (Fig. 1*C–F*) (100 pM, $p < 0.01$, $n = 16$; 1 nM, $p < 0.001$, $n = 18$). Membrane depolarization preceding increased firing was observed at the 100 nM dose in some cells (5 of 8 cells; mean depolarization, 7.2 ± 1.2 mV); at other doses, changes in this parameter were often within the range of noise of the baseline (data not shown). Increasing concentrations of estradiol increased both the percentage of cells responding to treatment and the change in firing rate (Fig. 1*G*,*H*). Changes in firing rate and membrane polarization were observable within 5 min of the addition of estradiol to the bath, strongly suggesting nongenomic mechanisms were engaged.

We next identified the estrogen receptor subtype mediating these effects. The pure classical estrogen receptor antagonist ICI 182780 blocks signaling through both known intracellular forms of the estrogen receptor, ERα and ERβ. ICI182780 had no effect on its own (Fig. 2*A*) ($n = 8$) but completely abolished the ability of nanomolar estradiol to increase firing of GnRH neurons (Fig. 2*A*,*F*) ($n = 8$). The ERβ agonist DPN (10 nM) increased GnRH neuron firing in a manner similar to the native ligand in a majority of cells (Fig. 2*B*,*F*) ($n = 12$, $p < 0.01$). In contrast, the ERα agonist PPT (10 nM) had no effect on GnRH neuron activity (Fig. 2*C*,*F*) ($n = 6$). Consistent with the blockade of estradiol action by ICI182780, G_1, an agonist of GPR30, a putative estrogen receptor, also had no effect on GnRH neuron firing (Fig. 2*D*,*F*) ($n = 6$). G_1 was shown to be active in a Western blot of Ishikawa human endometrial carcinoma cells, demonstrating phosphorylation of extracellularly regulated kinase (ERK1/2) (Fig. 2*E*). Furthermore, as ICI182780 had no effect on its own and has been shown to activate G_1 (Prossnitz et al., 2008), this further indicates that a classical estrogen receptor mediates the effects. Together, these data suggest nanomolar levels of estradiol activate GnRH neuron firing via ERβ.

Nanomolar estradiol increases excitability and alters the AHP and sADP and their underlying currents

To begin to understand the mechanism by which acutely applied estradiol increases GnRH neuron firing, we examined excitability (Fig. 3). DC current (< ±10 pA) was injected in some cells to bring the membrane potential to approximately −60 mV, then depolarizing current injection steps were given. Estradiol (10 nM) increased the excitability of GnRH neurons manifested as a shorter latency to action potential initiation (control, 294 ± 35 ms vs estradiol, 162 ± 26 ms, $p < 0.05$), hyperpolarized threshold potential for action potential initiation (control, −33.4 ± 1.6 mV vs estradiol, −37 ± 1.7 mV, $p < 0.05$), increased input resistance (control, 973 ± 104 MΩ vs estradiol, 1050 ± 106 MΩ, $p < 0.05$), increased membrane depolarization in response to current injection and greater number of spikes fired at each current injection

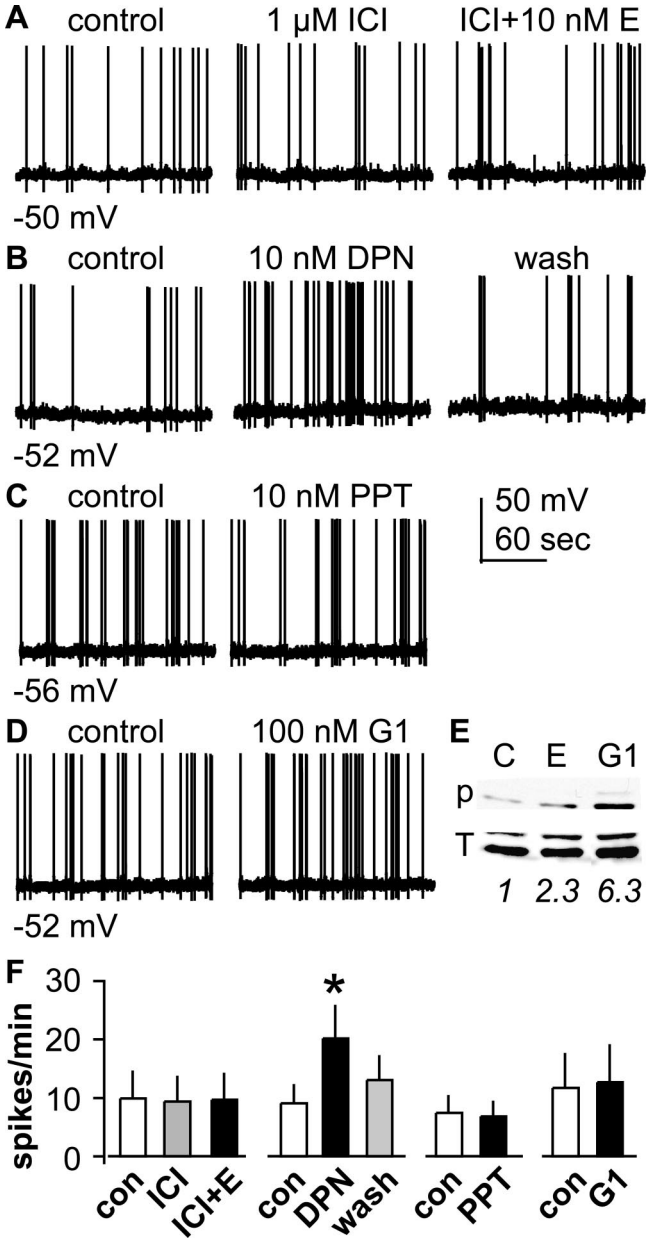

Figure 2. Classical ERβ mediates the acute effects of nanomolar estradiol on GnRH neurons. ***A–D***, Representative 3 min segments of continuous current-clamp recordings before and after treatment, followed by wash or subsequent treatment. ***A***, ICI182780 has no effect on its own but blocks the stimulatory effect of 10 nM estradiol. ***B***, The ERβ agonist DPN reversibly increases GnRH neuron firing. ***C***, The ERα agonist DPN has no effect. ***D***, The GPR30 agonist G_1 has no effect. ***E***, Western blot of Ishikawa cells showing phosphorylation of ERK relative to control (C) induced by 10 nM estradiol (E) or 100 nM G_1 (p) and total ERK 1/2 (T). Numbers in italics below lanes indicate fold induction of phospho ERK. ***F***, Summary of spikes per minute (ICI, $n = 8$; DPN, $n = 12$; PPT, $n = 6$; G_1, $n = 6$). *$p < 0.01$.

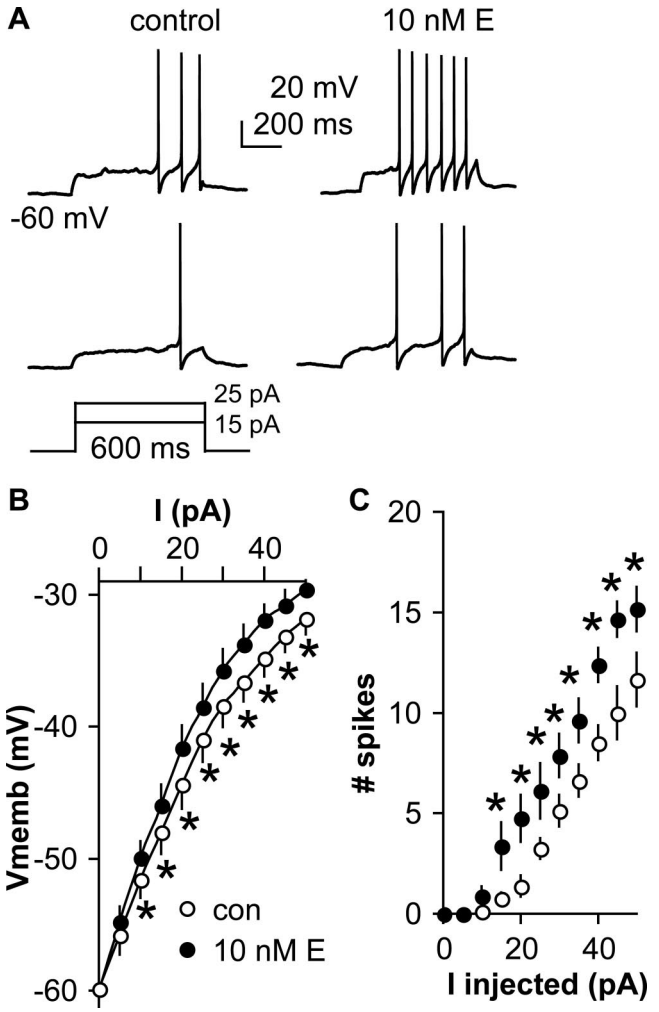

Figure 3. Nanomolar estradiol increases GnRH neuron excitability. ***A***, Bottom, Current injection protocol; top, resulting membrane response before (left) and after (right) bath application of 10 nM estradiol. Just two steps are shown for clarity. ***B***, Mean ± SEM membrane potential change in response to current injection. ***C***, Mean ± SEM number of spike elicited during the control and 10 nM estradiol treatment periods for each current injection. $*p < 0.05$.

but did not change spike amplitude (control, 79 ± 3 mV vs estradiol, 81 ± 2 mV).

To more closely examine action potential waveform, brief current injections (300 pA, 3 ms) were applied to induce single action potentials or pairs separated by 50 ms (Chu and Moenter, 2006). Estradiol (10 nM) markedly reduced the amplitude of the AHP (Fig. 4*A*,*B*) ($n = 14$, $p < 0.01$). The sADP characteristic of GnRH neurons was also enhanced (Fig. 4*A*) ($n = 14$, $p < 0.01$). This latter finding may be a consequence of the former as the absolute amplitude from the peak of the AHP to peak of the ADP did not change (control, 10.1 ± 0.5 mV; estradiol, 10.0 ± 0.5 mV), suggesting that a reduction in the outward current underlying the AHP may have caused a depolarizing shift in the entire after-spike waveform. Blockade of both large- and small-conductance I_{KCa} with iberiotoxin and apamin reduced the AHP and enhanced the sADP to similar extents as nanomolar estradiol and occluded further response to estradiol, consistent with this postulate (Fig. 4*E*,*F*) ($n = 9$, $p < 0.01$). ICI182780 blocked the effects of estradiol (Fig. 4*C*,*E*,*F*) ($n = 6$), whereas G_1 and vehicle were without effect (Fig. 4*D–F*) ($n = 6$ and 8, respectively).

To begin to examine the mechanisms underlying the changes in membrane properties, we performed whole-cell voltage-clamp

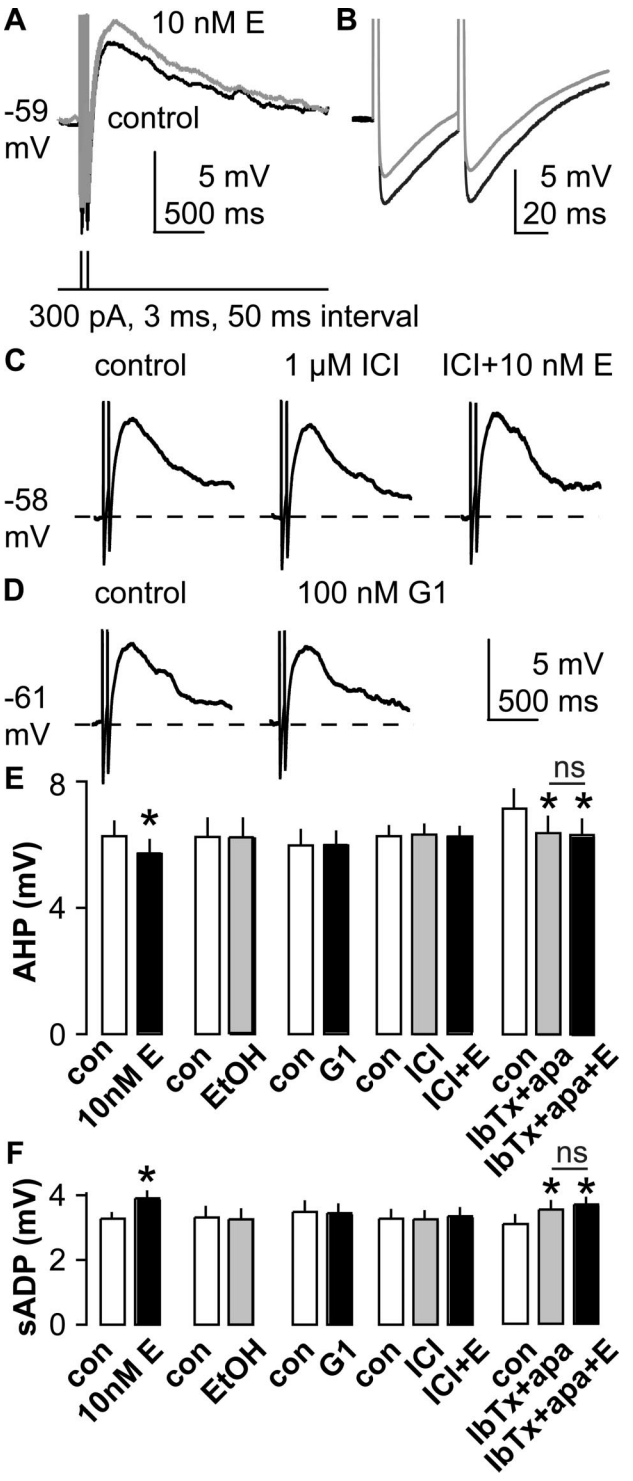

Figure 4. Nanomolar estradiol alters AHP and sADP. All action potential spikes are truncated in this figure. ***A***, Current-clamp trace showing action potentials elicited by brief current injections (bottom). Black line shows control and gray line after 10 nM estradiol. ***B***, Expanded time scale to better illustrate AHP. ***C***, ICI182780 blocks the effects of estradiol on the AHP and sADP. ***D***, G_1 has no effect on ADP or AHP. ***E***, ***F***, Summary of estradiol effects on sADP and AHP, respectively [10 nM E, $n = 14$; ethanol (EtOH), $n = 8$; G_1, $n = 6$; ICI, $n = 6$; IbTx plus apa, $n = 9$]. $*p < 0.01$; ns, nonsignificant.

studies. From a holding potential of −60 mV, cells were depolarized to +20 mV for 2 ms, to mimic the depolarization of an action potential. After the current artifacts of the voltage step, a fast outward current was observed, followed by a slower inward cur-

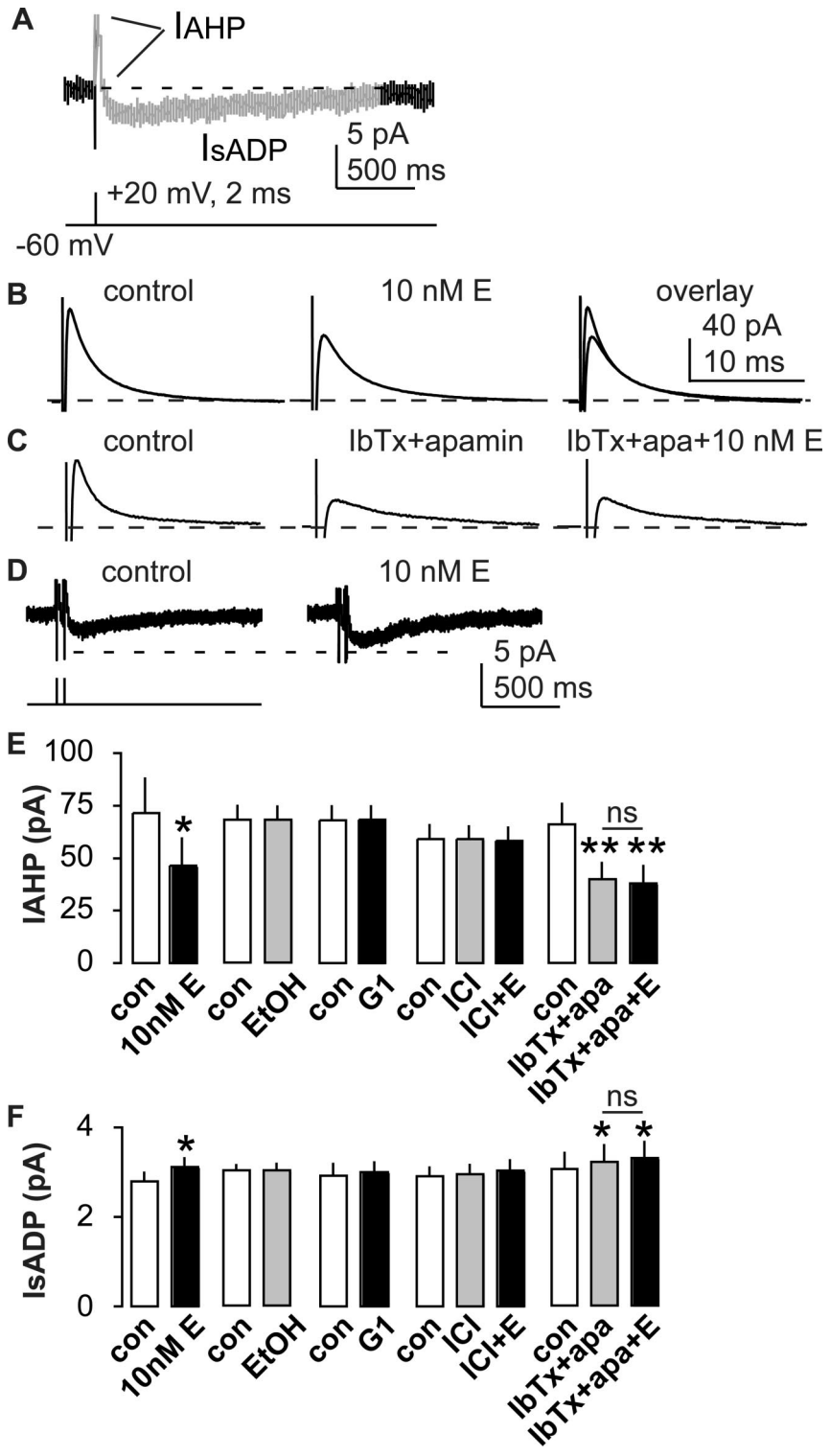

Figure 5. Nanomolar estradiol acutely alters the currents underlying the AHP and sADP. ***A***, Voltage-clamp recording of total membrane current (top) in response to spike depolarization (bottom), showing I_{AHP} (above dashed line) and I_{ADP} (below dashed line) in gray. ***B***, Estradiol decreases I_{AHP} amplitude. ***C***, IbTx and apamin (apa) reduce the amplitude of the I_{AHP} and occlude the response to estradiol. ***D***, Estradiol increases I_{ADP}. ***E***, ***F***, Summary of estradiol effects on I_{ADP} and I_{AHP}, respectively [10 nM E, $n = 11$; ethanol (EtOH), $n = 7$; G_1, $n = 7$; ICI, $n = 8$; IbTx plus apa, $n = 7$]. *$p < 0.05$ and **$p < 0.01$; ns, nonsignificant.

rent (Fig. 5*A*). The former is the current underlying the AHP (I_{AHP}) and the latter that underlying the sADP (I_{ADP}). Estradiol (10 nM) acutely reduced the I_{AHP} (Fig. 5*B*,*E*) ($n = 11$, $p < 0.01$) and enhanced the I_{ADP} (Fig. 5*D*,*F*) ($n = 11$, $p < 0.05$). As with membrane properties, these changes were blocked by ICI182780, with G_1 and the vehicle having no effect (Fig. 5*E*,*F*).

In previous work, the I_{ADP} was shown to be conducted via a TTX-sensitive sodium channel and modulated by *in vivo* treatment with estradiol (Chu and Moenter, 2006). We thus focused on the ionic basis of the I_{AHP}. Further analysis of these published data revealed that *in vivo* treatment with estradiol reduced the I_{AHP} (OVX, 63.9 ± 9.4 pA vs OVX plus E, 40.1 ± 6.6 pA, $n = 14$, $p < 0.05$) to a similar extent as observed for acute estradiol in the present study (Fig. 5). In the present study, pharmacological blockade of I_{KCa} with iberiotoxin and apamin markedly reduced the amplitude of the I_{AHP} (Fig. 5*C*,*E*) ($n = 7$, $p < 0.01$). Moreover, blockade of I_{KCa} occluded further response to estradiol ($p > 0.3$ vs iberiotoxin/apamin alone).

To begin to examine the signaling mechanism engaged by estradiol, we examined the ability of blocking signaling via PKA on the response to estradiol. PKA was chosen because it is one upstream activator of the cAMP response element-binding protein (CREB), which has been implicated in the rapid actions of estradiol in GnRH neurons by *in vivo* studies (Abrahám et al., 2003). The cell permeant PKA inhibitor KT5720 eliminated the excitatory effects of 1 nM estradiol on spontaneous firing rate (Fig. 6). These data suggest that estradiol activates PKA to modulate I_{AHP} by reducing conductance through calcium-activated potassium channels, consistent with the increase in input resistance observed with acute estradiol treatment (Fig. 3*C*).

Low physiological concentrations of estradiol (10 pM) do not affect GnRH neuron activity or intrinsic properties when fast synaptic transmission is blocked

Estradiol has biphasic and dose-dependent effects on GnRH neuron activity and release. Lower levels of estradiol typically provide homeostatic (negative) feedback, whereas during the female reproductive cycle, exposure to the sustained high level of estradiol in the circulation at the end of the follicular phase elicits a neurobiological switch to positive feedback action, inducing a massive surge in GnRH neuron activity that serves as a central initiator of the ovulatory process. Because of the dose dependence, we examined the effects of low levels of estradiol to determine if the response of GnRH neurons was similar. Estradiol (10 pM, $n = 10$) had no effect on GnRH neuron firing in the same recording conditions in which all of the above recordings were made, with fast synaptic transmission via ionotropic GABA and glutamate receptors blocked (Fig. 7*A*,*B*).

Figure 6. The excitatory response to 1 nM estradiol is eliminated by blocking PKA activity. ***A***, Representative recording during control, 100 nM KT5720 and 100 nM KT5720 plus 1 nM estradiol. ***B***, Mean ± SEM spikes per minute ($n = 14$).

Low physiological (10 pM) estradiol decreases GnRH neuron activity when synaptic transmission within the brain slice is left intact

Much of the feedback action of estradiol is thought to require ERα (Wintermantel et al., 2006). Because the expression of ERα by GnRH neurons is controversial and because feedback actions of estradiol have been shown to be at least in part via presynaptic mechanisms (Couse and Korach, 1999; Wintermantel et al., 2006; Christian and Moenter, 2007; Christian and Moenter, 2008b), we examined the effect of both 10 nM and 10 pM estradiol on GnRH neurons in brain slices in which fast synaptic transmission was operable. Importantly, there was no change in action potential properties of GnRH neurons between slices that were treated with APV, CNQX, and bicuculline and those that were not, indicating no confounding effects of potential nonspecific actions (data not shown, although compare Figs. 3*A* and 8*A*). The 10 nM dose of estradiol had a stimulatory effect on approximately half (9 of 17) of GnRH neurons studied (for all cells studied: control, 12.4 ± 3.0; estradiol, 15.7 ± 3.5 spikes/min, $n = 17$, $p < 0.05$; for responding cells: control, 10.0 ± 2.7; estradiol, 15.9 ± 3.7 spikes/min, $n = 9$, $p < 0.01$). In marked contrast, both to the lack of effect of 10 pM estradiol in blocked slices and the stimulatory effect of nanomolar estradiol in blocked and unblocked slices, 10 pM estradiol inhibited GnRH neuron firing in 9 of 16 cells (Fig. 7*C*,*G*) ($n = 16$, $p < 0.01$). This effect was blocked by ICI182780 (Fig. 7*D*) ($n = 6$). The ERβ agonist DPN had no effect on GnRH neuron firing when fast synaptic transmission was not blocked (Fig. 7*E*). In contrast, the ERα agonist PPT (10 nM) reversibly inhibited GnRH neuron firing in slices in which fast synaptic transmission was not blocked (Fig. 7*F*) ($n = 6$ of 17 cells tested, $p < 0.01$ for $n = 6$ responding cells, $p < 0.07$ for $n = 17$ cells studied). There was no effect of 10 pM estradiol on input resistance (control, 994 ± 79 MΩ; estradiol, 980 ± 83 MΩ, $n = 7$), sADP amplitude (control, 3.5 ± 0.2 mV vs estradiol, 3.3 ± 0.2 mV), AHP amplitude (control, 7.7 ± 0.6 mV vs estradiol, 7.7 ± 0.6 mV), I_{ADP} (control, 3.0 ± 0.2 pA vs estradiol, 3.0 ± 0.2 pA), I_{AHP} (control, 63.6 ± 12.0 pA vs estradiol, 61.9 ± 11.2 pA, all $n =$ 11–12) or other intrinsic membrane properties examined (Fig. 8*A–C*) ($n = 7$). Together, these data suggest picomolar estradiol decreases GnRH neuron activity via a presynaptic mechanism(s).

GABA transmission to GnRH neurons is acutely altered in an estradiol receptor subtype-dependent manner

To begin to explore transsynaptic mechanisms for acute estradiol action, we examined GABA transmission in unblocked slices. GABA was chosen as it is a major mediator of fast synaptic transmission on GnRH neurons; although glutamatergic transmission is observed (Suter, 2004; Christian et al., 2009), it is of much lower frequency. Indeed, blockade of $GABA_A$ receptors with bicuculline eliminates most spontaneous fast synaptic transmission detectable at the cell body (Fig. 9*A*) (ionotropic glutamate receptors are not blocked in these recordings). Of note, GnRH neurons have been reported to maintain elevated chloride levels in adult animals (DeFazio et al., 2002; Yin et al., 2008), and thus can be depolarized and even excited by GABA, although this remains an area of controversy (Han et al., 2004; Moenter and DeFazio, 2005). The ERα agonist PPT significantly reduced the frequency of GABA transmission to GnRH neurons (Fig. 9*B*,*F*) ($n = 17$, $p < 0.001$) but had no effect on amplitude (Fig. 9*D*,*G*), consistent with an inhibitory network effect of low-dose estradiol via ERα. In contrast, the ERβ agonist DPN increased frequency of GABA transmission (Fig. 9*C*,*F*) ($n = 14$, $p < 0.001$) as well as amplitude of GABAergic PSCs (Fig. 9*E*,*G*) ($p < 0.001$). These data provide proof-of-principle that estradiol can act rapidly via both classical receptor subtypes to change transmission to GnRH neurons in a receptor subtype-dependent manner.

Discussion

The feedback actions of estradiol to regulate GnRH release are complicated. During the reproductive cycle, low levels of estradiol are typically associated with suppression of GnRH release via homeostatic negative feedback, whereas sustained exposure to higher levels at the end of the follicular phase triggers one of the rare positive feedback events in physiology, the GnRH surge, which triggers generation of the pituitary signal for ovulation. Although these whole-organism responses have long been recognized, the underlying mechanisms are less understood. There is now appreciation that steroids, including estradiol, can signal through both genomic mechanisms and rapid changes in signaling cascades. We examined the effects of acutely applied estradiol on GnRH neurons in brain slices. The short latency (<5 min) of responses strongly argues for a mechanism that does not rely on changes in gene expression via classical or nonclassical mechanisms. The direction of response to acutely applied estradiol varies with dose, and stimulatory versus inhibitory actions appear to involve different receptor subtypes and different neurobiological mechanisms.

High physiological and greater doses of estradiol (100 pM to 100 nM) depolarized GnRH neurons, increasing firing activity. Depolarization was observed after blocking fast synaptic transmission and action potential generation, which minimizes neu-

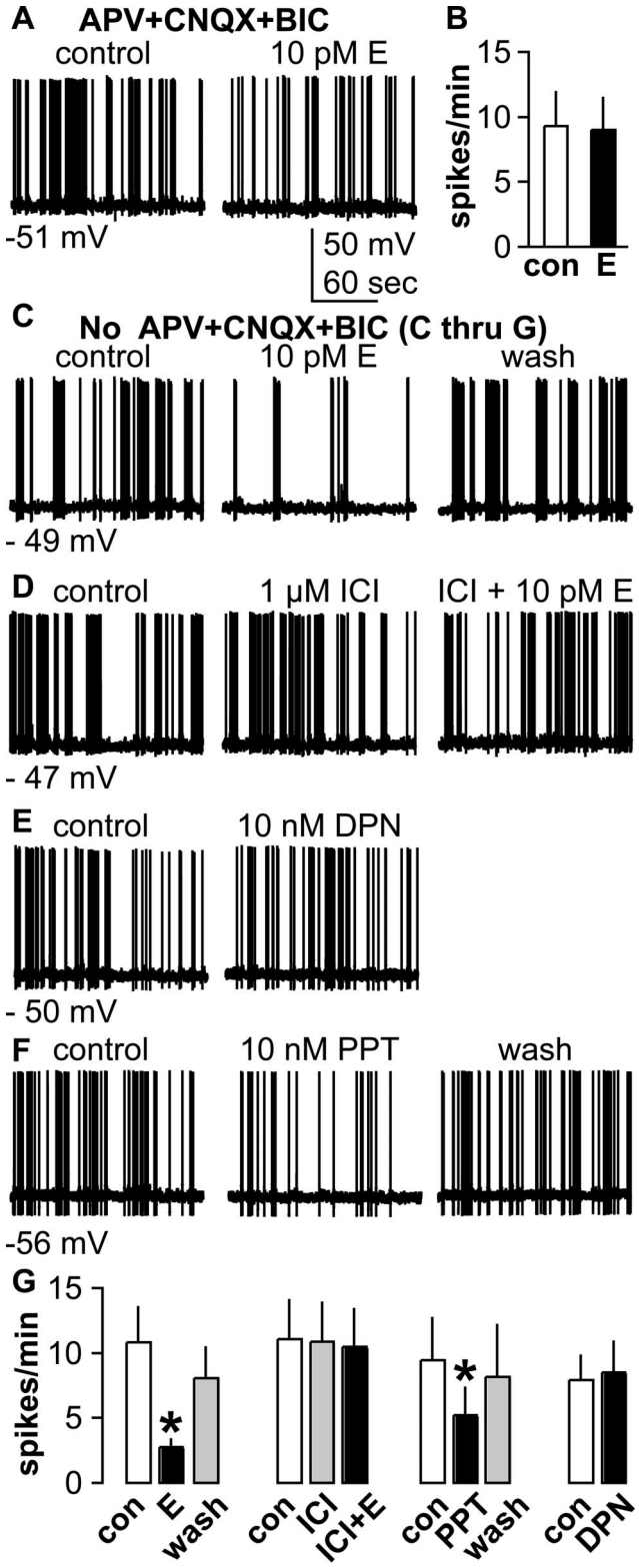

Figure 7. Picomolar concentrations of estradiol inhibit GnRH neuron firing via the presynaptic network in an ERα-dependent manner. ***A***, In the presence of blockers of ionotropic GABA and glutamate receptors, representative 3 min segments of continuous current-clamp recording before and during treatment with 10 pM estradiol. ***B***, Summary of the effects of picomolar estradiol on firing. ***C***, In absence of blockers of ionotropic GABA and glutamate receptors, 10 pM estradiol inhibits GnRH neuron firing in a subpopulation of cells. ***D***, ICI182780 blocks the inhibition in firing. ***E***, The ERβ agonist DPN has no effect on firing. ***F***, The ERα agonist PPT reversibly inhibits firing in a subpopulation of GnRH neurons. ***G***, Summary of spikes per minute responding cells (10 pM E, $n = 9$; ICI, $n = 6$; PPT, $n = 6$; DPN, $n = 10$).*$p < 0.01$.

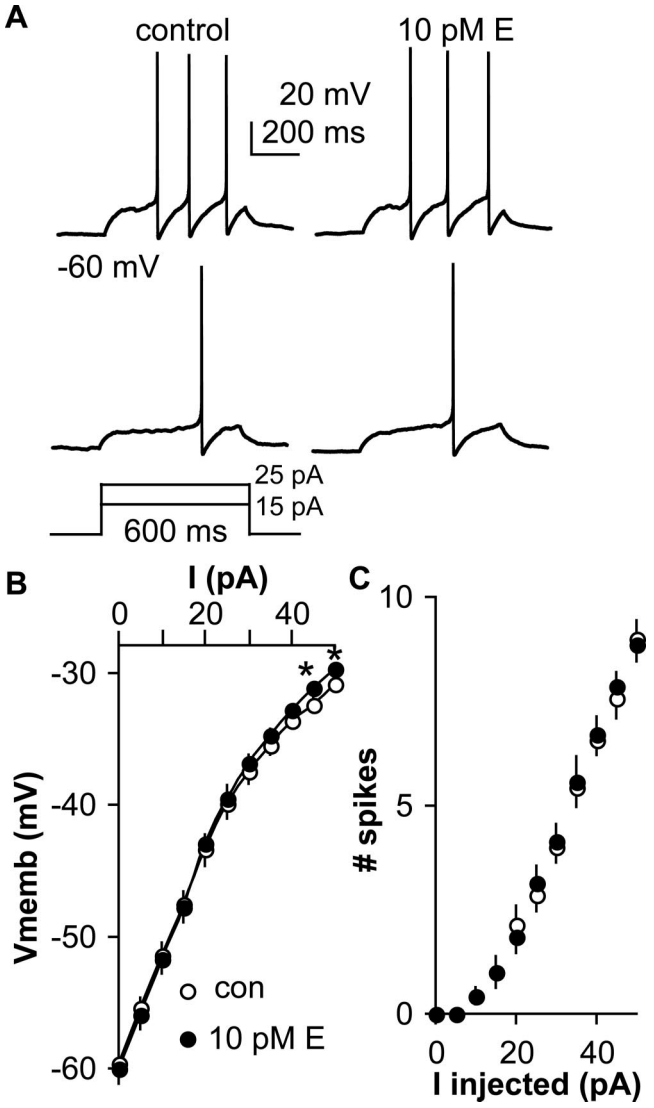

Figure 8. Picomolar concentrations of estradiol have no effect on intrinsic properties of GnRH neurons. ***A***, Representative current-clamp recordings showing no effect of 10 pM estradiol on GnRH neuron firing in response to injected current. ***B***, Mean ± SEM membrane potential change in response to current injection ($n = 8$). ***C***, Mean ± SEM number of spike elicited during the control and 10 pM estradiol treatment periods for each current injection ($n = 8$).

romodulator release, indicating an intrinsic mechanism. Consistent with an intrinsic effect, an ERβ agonist mimicked the effect of nanomolar estradiol. ERβ, specifically ERβ2, is expressed in GnRH neurons, whereas expression of ERα in GnRH neurons is controversial (Radovick et al., 1991; Herbison and Pape, 2001; Hrabovszky et al., 2001; Navarro et al., 2003; Chung et al., 2007). The present data provide further evidence that intrinsic properties are not affected by this isoform. The percentage of GnRH neurons responding to nanomolar estradiol in the present study (70%) is similar to that expressing ERβ in the rat (67–73%) (Hrabovszky et al., 2001). Estradiol (1 nM) increased firing rate of cultured embryonic primate GnRH neurons with a similar time course in a manner dependent on classical estrogen receptors (Abe and Terasawa, 2005). In the same model, increased frequency and synchronization of oscillations in intracellular calcium concentrations were reported, but interestingly, these actions were not blocked by ICI182780 and were attributed to an integral membrane protein such as GPR30 (Abe et al., 2008).

Similar to the present study, changes in intracellular calcium dynamics in cultured embryonic murine GnRH neurons and phosphorylation of CREB in GnRH neurons were dependent on ERβ, although the former, measured at 30 min, was transcription dependent (Abrahám et al., 2003; Temple et al., 2004). These differences may be attributed to species, for example differences in the propagation of calcium waves (Moore et al., 2002; Abe et al., 2008) or to the different developmental stages of the cells (embryonic vs adult). In contrast to the excitatory responses in the above and present studies, 1–10 nM estradiol acutely hyperpolarized GnRH neurons and other hypothalamic cells in guinea pigs by opening an inwardly rectifying potassium channel (Kelly et al., 1984; Lagrange et al., 1995).

Mechanistically, 10 nM estradiol increased excitability, hyperpolarized action potential threshold, and inhibited the AHP and its underlying current. The effects on the AHP and I_{AHP} were occluded by previous blockade of I_{KCa}, suggesting acute estradiol either inhibits calcium-activated potassium currents in GnRH neurons, as in the hippocampus and hypothalamus (Kelly et al., 2002; Carrer et al., 2003), or that estradiol decreases calcium conductances that subsequently activate I_{KCa} (Kumar and Foster, 2002). GnRH neurons (Jasoni et al., 2007) and GT1 GnRH neurons (Spergel, 2007) exhibit I_{KCa}. Acute estradiol (1 nM) had no effect on I_{KCa} in GT1 cells, whereas a 3 d exposure increased expression of these channels and resulting current in an ERβ-dependent manner (Nishimura et al., 2008). This difference may be attributed to mechanistic differences, with the longer treatment enabling changes in gene expression that alter current in the opposite direction of the nongenomic actions of an acute treatment. In this regard, however, *in vivo* treatment with estradiol for 2–4 d reduced the AHP in GnRH neurons to a similar extent as acute estradiol. Alternatively, the transformed and/or immature state of GT1 cells may alter response to estradiol. Here, 10 nM estradiol increased input resistance, further supporting a mechanism involving reduced membrane conductance.

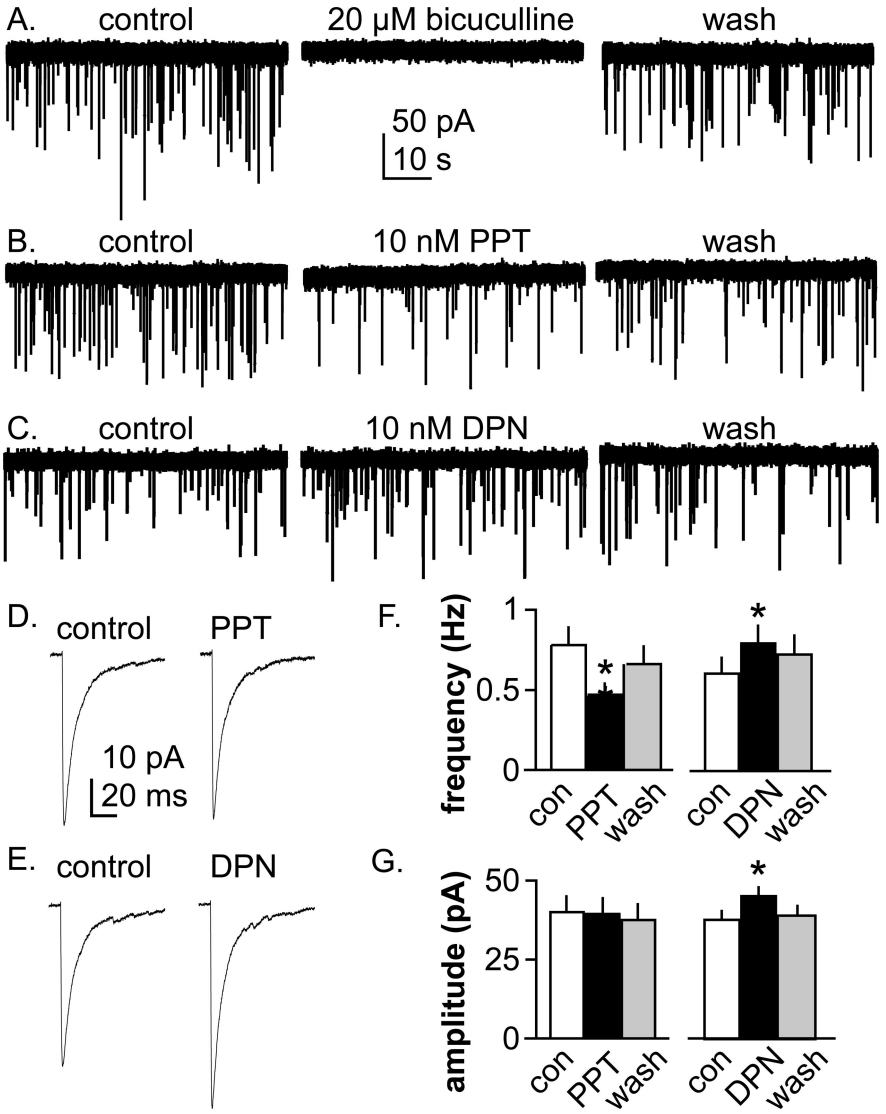

Figure 9. Estradiol receptor activation acutely alters GABA transmission to GnRH neurons in a subtype-dependent manner. Recordings made without blockers of ionotropic glutamate receptors present. ***A***, Bicuculline blocks the majority of fast synaptic currents to GnRH neurons, demonstrating they are GABAergic. ***B***, The ERa agonist PPT reduces GABA transmission to GnRH neurons. ***C***, The ERβ agonist DPN increases GABA transmission to GnRH neurons. ***D***, ***E***, Averaged PSC traces showing lack of effect of PPT on PSC amplitude (***D***) and increased amplitude in response to DPN (***E***). ***F***, ***G***, Mean ± SEM frequency and amplitude of GABAergic PSCs (PPT, $n = 17$; DPN, $n = 14$). *$p < 0.001$.

In contrast to the marked excitatory effects of 10 nM estradiol, low physiological doses of estradiol (10 pM) did not affect GnRH neuron firing when ionotropic GABA and glutamate receptors were blocked to reduce slice network interactions. When network interactions were left intact, however, a subpopulation of GnRH neurons was inhibited by 10 pM estradiol in an ERα-mediated response. The relative lack of evidence for ERα expression in adult GnRH neurons, the lack of effect of 10 pM estradiol on any examined intrinsic properties of GnRH neurons, and the need for network interactions for a response suggest this inhibitory action of 10 pM estradiol is synaptically mediated. Consistent with this, stimulatory drive via GABAergic transmission was acutely inhibited by an ERα agonist. Interestingly, 100 nM estradiol increased GABA transmission in ERβKO mice, implying involvement of ERα (Romanò et al., 2008). A small percentage of GnRH neurons were affected in that study, and the timing was delayed relative to the present study, but this illustrates the complex temporal nature of estradiol regulation of GnRH neurons, even in acute *in vitro* studies.

With network interactions intact, approximately half of GnRH neurons were still excited by 10 nM estradiol. An ERβ agonist had no overall effect on firing rate in this paradigm but did increase GABA transmission and amplitude of GABAergic PSCs, which, like the ERβ-mediated effects on intrinsic properties, should be stimulatory. This dissociation between the effect of 10 nM estradiol and the ERβ-specific agonist suggests ERβ may alter additional network interactions that inhibit GnRH neurons.

Estradiol alters spinyness synaptic transmission (Woolley and McEwen, 1992; Gu and Moss, 1996; Rudick and Woolley, 2003) and may act directly on secretory vesicles (Hart et al., 2007).

A pure classical estrogen receptor antagonist blocked both inhibitory and stimulatory effects of acute estradiol. Furthermore, these effects were mimicked by agonists of ERα and ERβ, respectively, but not by a GPR30 agonist, indicating rapid estradiol effects on murine GnRH neurons are mediated by classical receptors, rather than GPR30, ERX, or other membrane estrogen receptors (Toran-Allerand et al., 2002; Qiu et al., 2003; Bologa et al., 2006). The pure estrogen ERα/β ICI antagonist had no stimulatory effect alone, further supporting the lack of a role of GPR30 in this system (Prossnitz et al., 2008). In neuronal systems, estradiol initiates signaling via kinase cascades, including PKA, MAPK, and phospholipase C-protein kinase C (Gu and Moss, 1996; Zhou et al., 1996; Watters et al., 1997; Qiu et al., 2003). In some neurons, rapid actions of estradiol appear to involve activation of type-1 metabotropic glutamate receptors (mGluR1s) (Boulware et al., 2005). In cultured embryonic rat GnRH neurons and GT1 cells, picomolar estradiol acted via ERα to reduce cAMP accumulation, whereas nanomolar estradiol activated cAMP accumulation, a dose dependence with interesting parallels to the present results (Navarro et al., 2003). Our initial studies indicate blocking PKA eliminated the excitatory effects of 1 nM estradiol in GnRH neurons, suggesting involvement of PKA, but not excluding participation of other signaling pathways. Future work will determine if mGluRs are involved; although previous work indicated no direct effect of mGluR1 agonists in GnRH neurons, parameters examined were related to the response to GABAergic transmission rather than other intrinsic properties (Chu and Moenter, 2005).

ERβ, which mediates the direct effects estradiol on GnRH neurons, has been considered to be less critical to reproductive success than ERα (Couse and Korach, 1999; Wintermantel et al., 2006). ERβ mice, however, are suboptimally fertile, and although ovarian effects are often cited as the reason for this, central modulatory roles for ERβ that are important for full fertility may exist. Although interaction of ERα with estrogen-response elements appears essential for generating the increased activity of GnRH neurons during positive feedback (Christian et al., 2008), this does not preclude a supporting role for ERβ to further increase GnRH neuron activity during a time when many parallel excitatory pathways appear to be (Christian et al., 2005; Christian and Moenter, 2008a,b; Clarkson et al., 2008). Another consideration is that ERβ activation and thus GnRH neuron activity may be enhanced by increased central synthesis of estradiol (Hojo et al., 2004; Toran-Allerand et al., 2005). Of interest, circulating estrogen is elevated in women with polycystic ovary syndrome, a pathology associated with increased GnRH neuron activity (Yen et al., 1976; Eagleson et al., 2003). This concept of changes in local synthesis of estradiol opens a novel and intriguing mechanism to take into consideration in future studies. Circulating estradiol changes during the female reproductive cycle, peaking at high picomolar levels during the preovulatory period, and remaining at low picomolar levels during the majority of the cycle. Our finding that the rapid response to estradiol changes direction from inhibitory to excitatory within the physiological range of this steroid by engaging different receptor subtypes and neurobiological mechanisms, suggests these rapid actions are poised to play a contributing role to the overall feedback action of estradiol.

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