

Physiologic Regulation of a Tetrodotoxin-Sensitive Sodium Influx That Mediates a Slow Afterdepolarization Potential in Gonadotropin-Releasing Hormone Neurons: Possible Implications for the Central Regulation of Fertility

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The brain controls fertility through release of gonadotropin-releasing hormone (GnRH), but the mechanisms underlying action potential patterning and GnRH release are not understood. We investigated whether GnRH neurons exhibit afterdepolarizing potentials (ADPs) and whether these are modified by reproductive state. Whole-cell current-clamp recordings of GnRH neurons in brain slices from ovariectomized mice revealed a slow ADP (sADP) after action potentials generated by brief current injection. Generating two or four spikes enhanced sADP amplitude and duration. sADP amplitude was not affected by blocking selected neurotransmitter/neuromodulator receptors, delayed-rectifier potassium channels, calcium-dependent cation channels, or hyperpolarization-activated cation channels but was halved by the calcium channel blocker cadmium and abolished by tetrodotoxin. Cadmium also reduced peak latency. Intrinsic mechanisms underlying the sADP were investigated using voltage-clamp protocols simulating action potential waveforms. A single action potential produced an inward current, which increased after double and quadruple stimulation. Cadmium did not affect current amplitude but reduced peak latency. Pretreatment with blockers of calcium-activated potassium currents (I_{KCa}) reproduced this shift and blocked subsequent cadmium-induced changes, suggesting cadmium changes latency indirectly by blocking I_{KCa} . Tetrodotoxin abolished the inward current, suggesting that it is carried by sodium. In contrast, I_{KCa} blockers increased the inward current, indicating that I_{KCa} may oppose generation of the sADP. Strong sADPs were suprathreshold, generating repetitive spontaneous firing. I_{ADP} , sADP, and excitability were enhanced by *in vivo* estradiol, which triggers a preovulatory surge of GnRH release. Physiological feedback modification of this inward current and resulting sADP may modulate action potential firing and subsequent GnRH release.

Key words: GnRH; intrinsic activity; sodium current; excitability; neuroendocrine; afterdepolarizing potential; feedback regulation

Introduction

Gonadotropin-releasing hormone (GnRH) neurons form the final common pathway for the central regulation of fertility. In males, and during most of the female reproductive cycle, GnRH is released in pulses of varying frequencies, altering downstream function (Clarke and Cummins, 1982; Levine and Duffy, 1988; Moenter et al., 1992). Pulse generation appears intrinsic to GnRH neuronal networks, but the mechanisms are not well understood (Moenter et al., 2003). Immortalized GnRH neurons release GnRH in pulses (Krsmanovic et al., 1992; Martinez de la Escalera et al., 1992; Pitts et al., 2001). Green fluorescent protein (GFP)-identified GnRH neurons in brain slices fire spontaneously (Spergel et al., 1999; Suter et al., 2000b; Han et al., 2004) even

when fast synaptic transmission is blocked (Nunemaker et al., 2002), and spontaneous firing persists in isolated GnRH neurons (Kuehl-Kovarik et al., 2002). In females, a surge mode of continuous GnRH release also exists; this is induced by estradiol-positive feedback and provides the central signal initiating ovulation (Docke and Dorner, 1965; Moenter et al., 1991). Firing activity of GnRH neurons in brain slices during surge mode is increased relative to that observed during negative feedback in an animal model in which constant estradiol administration induces daily switching between these feedback modes (Christian et al., 2005). This altered activity may be attributable to changes in synaptic transmission to GnRH neurons and/or enhanced intrinsic excitability to increase both response to synaptic changes and spontaneous endogenous activity.

Action potentials, or extracellularly recorded action currents, of GnRH neurons often occur in groupings of two to several (Suter et al., 2000b; Kuehl-Kovarik et al., 2002; Nunemaker et al., 2003b; Abe and Terasawa, 2005). Such burst firing increases hormone release from magnocellular neuroendocrine neurons (Dutton and Dyball, 1979; Poulain and Wakerley, 1982). The mechanisms underlying burst firing in GnRH neurons are unknown. In magnocellular neuroendocrine neurons and neurons

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of the hippocampus and cortex, action potential firing can be followed by an afterdepolarization potential (ADP) or depolarizing afterpotential (DAP) (Kandel and Spencer, 1961; Fujita, 1975; Connors et al., 1982; Andrew and Dudek, 1984; Azouz et al., 1996; Hestrin and Armstrong, 1996; Li and Hatton, 1997a,b; Armstrong and Stern, 1998; Chu et al., 2003; Metz et al., 2005). ADPs can contribute to intrinsically repetitive burst firing (Andrew and Dudek, 1983, 1984; Bourque, 1986; Jensen et al., 1996; Bourque et al., 1998; Metz et al., 2005). Several ionic mechanisms may underlie the ADP, including low- and high-voltage-activated calcium currents (Li and Hatton, 1997a; Metz et al., 2005), hyperpolarization-activated currents (I_h) (Erickson et al., 1993; Luthi et al., 1998), persistent sodium current (Azouz et al., 1996; Su et al., 2001; Yue et al., 2005), calcium-activated nonselective cation currents (Greene et al., 1994), or reduced outward potassium currents (Li and Hatton, 1997b; Yue and Yaari, 2004).

GnRH neurons exhibit action potential-dependent ADPs of unknown mechanism (Spergel et al., 1999; Sim et al., 2001; Kuehl-Kovarik et al., 2002, 2005). We investigated mechanisms underlying ADP generation, function, and estradiol regulation. The data suggest that ADPs contribute to repetitive firing in GnRH neurons and are steroid sensitive.

Materials and Methods

Animals. Adult (2–3 months of age) female mice expressing enhanced GFP (Clontech, Palo Alto, CA) under the control of the GnRH promoter were used to facilitate identification of GnRH neurons (Suter et al., 2000a). Mice were maintained under a 14/10 h light/dark photoperiod with Harlan (Indianapolis, IN) 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 Metofane (Janssen Pharmaceuticals, Toronto, Ontario, Canada) or isoflurane (Burns Veterinary Supply, Westbury, NY) anesthesia 5–9 d before experimentation; time after gonadectomy did not affect results. Estradiol feedback was studied using a previously characterized model in which treating OVX females with a constant-release estradiol implant produces daily switches between negative and positive feedback that persist in the brain slice preparation (Christian et al., 2005). The long-acting local anesthetic bupivacaine (0.25%; Abbot Labs, North Chicago, IL) was applied to surgical sites to minimize postoperative pain and distress. 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 (St. Louis, MO) unless noted. Brain slices were prepared as described previously (Nunemaker et al., 2002, 2003a; 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, mice were decapitated, and 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 NaH_2PO_4 , 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, St. Louis, MO). 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% normal saline, containing the following (in mM): 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4 , 2.5 $CaCl_2$, 1.2 $MgSO_4$, 10 D-glucose, pH 7.4. Slices were then transferred to 100% normal saline and maintained at room temperature (~21–23°C) until study (30 min to 8 h).

Data acquisition. Slices were transferred to a recording chamber mounted on the stage of an upright microscope (Olympus Optical BX50WI; Opelco, Dulles, VA) and stabilized in the chamber at least 5 min before recording. The chamber was continuously perfused with normal saline at a rate of 4–5 ml/min at 32°C. Pipettes (3–4 M Ω) were pulled

from borosilicate glass capillaries (1.65 mm outer diameter; 1.12 mm inner diameter; World Precision Instruments, Sarasota, FL) using a Narishige model pp-830 (Pacer Scientific, Los Angeles, CA) or Flaming/Brown P-97 (Sutter Instruments, Novato, CA). Pipettes were filled with a solution containing the following (in mM): 125 K-gluconate, 20 KCl, 10 HEPES, 5 EGTA, 4.0 MgATP, 0.4 NaGTP, and 0.1 $CaCl_2$, pH 7.2 (300 mOsm). GnRH-GFP neurons from POA and ventral hypothalamus were identified by brief illumination at 470 nm. Data were acquired and digitized using either an EPC-8 (HEKA Elektronik, Mahone Bay, Nova Scotia, Canada) with an ITC-18 interface (InstruTech, Port Washington, NY) controlled by the PulseControl XOP (InstruTech) running in Igor-Pro (WaveMetrics, Lake Oswego, OR) or using one head stage of an EPC-10 dual amplifier (HEKA Elektronik) controlled by PatchMaster (HEKA Elektronik). There were no differences attributable to acquisition system. 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 $R_{in} > 500$ M Ω and $R_s < 20$ M Ω 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). Neurobiotin or biocytin (0.5%) was included in some cells for morphological study; no differences were observed between cells with and without these compounds.

Current-clamp recordings. Whole-cell current-clamp recordings were made using bridge balance and capacitance compensation. All cells had an initial membrane potential negative to –55 mV without current injection and action potential amplitude of >90 mV; direct current (DC) injection (<20 pA) was used to normalize membrane potential to the range of –55 to –65 mV to facilitate comparison. Action potentials were elicited with short-duration (3–5 ms) current pulses (200–300 pA). 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 slow afterdepolarization (sADP) that followed the action potential was measured from the prespike baseline. Latency to peak of the sADP was measured from the action potential threshold (defined as 1 V/s); threshold of the final spike in a train was used if multiple spikes were initiated. sADP duration was the length of time the membrane voltage exceeded prestimulus baseline levels.

Voltage-clamp recordings. Whole-cell voltage-clamp was used to study currents underlying the sADP. 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 were averaged for analysis. To assess changes in conductance during the sADP, small hyperpolarizing voltage steps (10 mV, 50 ms duration) were given before, during, and after the peak of the inward current; input resistance was the quotient of the magnitude of the voltage step divided by the steady-state current. Amplitude of the inward current (I_{ADP}) was measured from the precommand baseline. Latency to peak of I_{ADP} was measured from the onset of the final depolarizing step of the voltage command.

Drug treatments. The following treatments were bath applied. Ionotropic glutamate receptors (iGluRs) were blocked with 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 20 μM D(-)-2-amino-5-phosphonovaleic acid (D-APV). GABA $_A$ receptors were blocked with 20 μM bicuculline methiodide. Sodium channels were blocked with tetrodotoxin (TTX) (0.5 μM ; Calbiochem, San Diego, CA). GnRH receptors were blocked with D-pGlu1, D-Phe2, D-Trp3,6 (D-P-Glu) (20–40 nM; Bachem, Torrance, CA). Galanin receptors were blocked with galantide (100 nM; Bachem). Metabotropic glutamate receptors (mGluRs) were blocked with (RS)- α -methyl-4-carboxyphenylglycine (1 mM) and (RS)- α -cyclopropyl-4-phosphonophenylglycine (200 μM). P2 purinergic receptors were blocked with 100 μM suramin. Calcium channels were blocked with 200 μM Cd $^{2+}$. Hyperpolarization-activated cation currents (I_h) were blocked with 40 μM ZD7288 (4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride). Delayed rectifier potassium currents (I_K) were blocked with 10 mM tetraethylammonium (TEA). Small-conductance calcium-activated potassium currents (I_{KCa}) were blocked with 200 nM apamin, large-conductance I_{KCa} were blocked

with 100 nM iberiotoxin, persistent sodium current (I_{NaP}) was blocked with 5–10 μM riluzole, and calcium-dependent nonselective cation channels were blocked with Cs^+ (3 μM) or flufenamic acid (200 μM).

Statistical analyses. The values are expressed as mean \pm SEM. Statistical comparisons were made using paired parametric or nonparametric tests, as dictated by data distribution. All tests were two tailed, and $p < 0.05$ was considered significant.

Results

GnRH neurons exhibit homogeneous firing properties

Whole-cell current clamp was used to investigate the firing properties of GnRH neurons identified by expression of green fluorescent protein (Fig. 1A). All recorded cells were located in the preoptic area and anterior hypothalamus. Small amounts of positive current consistently evoked repeated action potentials (Fig. 1B). The small amount of current required to initiate action potential firing is consistent with the high input resistance of these cells (Kusano et al., 1995; Spergel et al., 1999; Suter et al., 2000b; DeFazio and Moenter, 2002; Kuehl-Kovarik et al., 2002). There was little evidence of spike frequency adaptation over the 500 ms injection, although a slight decrement in spike amplitude was routinely observed. Similarly, the prominent afterhyperpolarization (AHP) was reduced in amplitude as an evoked firing train proceeded. GnRH neurons were very homogeneous with regard to these firing properties.

GnRH neurons exhibit a prominent sADP

Action potential generation entails regenerative depolarization followed by a restorative hyperpolarization. These two phases are mediated by fast voltage-dependent sodium current and delayed voltage-dependent potassium current, respectively (Hodgkin and Huxley, 1952; Connors et al., 1982). In examining the membrane potential of GnRH neurons in current clamp (Fig. 1C), we noticed that spontaneous action potentials were often followed by ADPs. To study ADPs under consistent conditions, action potentials were induced in current-clamp mode by injection of a brief depolarization current pulse (200–300 pA, 3–5 ms duration). Brief injections were used because they are completed before much of the action potential waveform occurs (Fig. 1D). The changes in membrane potential observed are thus more likely attributable to native conductances, not injected current.

GnRH neurons typically responded to this current injection with a single spike, followed immediately by an AHP, and then an ADP that was slow to develop and decay, hence termed a sADP (Fig. 1E). The sADP is the prominent and prolonged period during which the membrane potential of the cell exceeds the prespike potential. Most GnRH neurons (87%, 114 of 131 cells) in the preoptic area and ventral hypothalamus exhibited sADPs after action potential generation. sADPs were also evident after more prolonged, lower-amplitude current injections that generated action potential firing (Fig. 1B), but the remainder of the characterization of this phenomenon was performed with the above-described brief current injections. Six unidentified neurons in the vicinity of GnRH neurons failed to show sADPs after brief current injections to induce action potential firing (data not shown).

sADP amplitude depends on the number of spikes and membrane potential

When GnRH neurons are recorded extracellularly, bursts of action currents reflecting action potential firing are observed from most cells (Kuehl-Kovarik et al., 2002; Nunemaker et al., 2002, 2003b; Abe and Terasawa, 2005; Chu and Moenter, 2005; Pielecka and Moenter, 2006; Pielecka et al., 2006). These are often

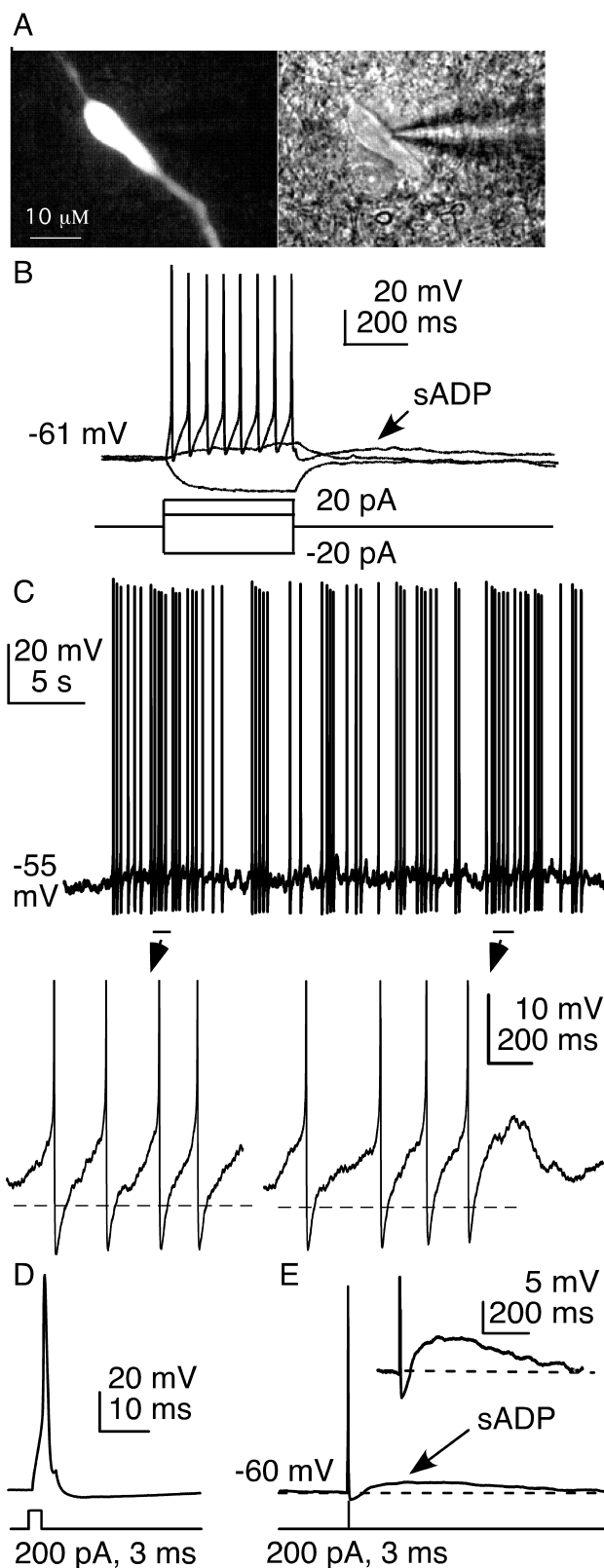


Figure 1. Firing properties and afterdepolarization in GnRH neurons. **A**, GFP signal (left) and infrared differential interference contrast image (right) of a GFP-identified GnRH neuron. **B**, Stereotypical response of a GnRH neuron to current injection between pulse and surge modes of secretion that is critical to reproductive success. **C**, Spontaneous action potentials are followed by ADPs (below, spikes truncated). Dashed line indicates prespike baseline. **D**, Brief current injections are terminated before the evoked spike begins. Bottom, Current injection protocol; top, membrane potential response. **E**, Brief current injection triggers action potentials followed by an sADP; inset shows expanded scale (spikes truncated in inset).

grouped so that approximately two to six or more action currents occur over a few hundred milliseconds. We thus examined the effects of multiple induced spikes on the sADP. One, two, four, or eight spikes were induced by 3 ms, 200–300 pA current injection at 50 ms intervals (Fig. 2*A, B*). sADP amplitude increased ($p < 0.01$, ANOVA with Bonferroni's *post hoc* test) from 2.9 ± 0.1 to 3.7 ± 0.1 to 4.5 ± 0.1 mV from one ($n = 44$) to two ($n = 44$) to four ($n = 44$) spikes, respectively, but was not further increased by eight spikes (4.5 ± 0.3 mV; $n = 17$) (Fig. 2*C*). Likewise, duration of the sADP was increased from 1.08 ± 0.2 s with one spike ($n = 30$) to 1.38 ± 0.03 s with two spikes ($n = 30$; $p < 0.001$) to 1.71 ± 0.03 s with four spikes ($n = 30$; $p < 0.001$) but was not further increased by eight spikes (1.86 ± 0.04 s; $n = 10$; Kruskal–Wallis with Dunn's *post hoc* test) (Fig. 2*D*). Latency to peak of the sADP from the threshold of the final spike was not affected by spike number (191 ± 7 ms, $n = 30$; 194 ± 7 ms, $n = 30$; 199 ± 7 ms, $n = 30$; and 185 ± 7 ms, $n = 17$, respectively) (Fig. 2*E*). This suggests that the magnitude, but not the onset, of sADP is dependent on the preceding stimulus up to a point at which amplitude is maximized. Of interest, many extracellularly recorded bursts consist of fewer than eight action currents, suggesting that the regulation of sADP parameters occurs over the physiological range of short-term firing patterns of GnRH neurons.

The voltage dependence of the sADP was investigated next. The membrane potential of GnRH neurons was maintained from -90 to -50 mV at ~ 10 mV intervals by injecting negative or positive DC. At each potential, a pair of spikes was initiated (200–350 pA, 3 ms, 50 ms interval), and the sADP amplitude was determined 200 ms after the termination of the final spike. The amplitude of current injection was varied to the minimum needed to induce spikes to avoid overstimulation. A representative example of sADPs from a GnRH neuron at different membrane potentials is illustrated in Figure 2*F*; data from all 10 cells are plotted in Figure 2*G*. Amplitude exhibited clear voltage dependence, peaking between -65 and -70 mV. The sADP is less evident at very hyperpolarized potentials near E_K . Amplitude of the sADP was also reduced at more depolarized potentials (e.g., near -50 mV). With regard to the latter, it should be noted that spontaneous firing sometimes precluded measurement of the sADP at more depolarized potentials. The peak is near to slightly negative to the membrane potentials typical of quiescent GnRH neurons, suggesting an early net inward postspike current as a possible mechanism for the sADP. The voltage dependence of the sADP indicates that it is poised to alter the function of these cells under physiological conditions.

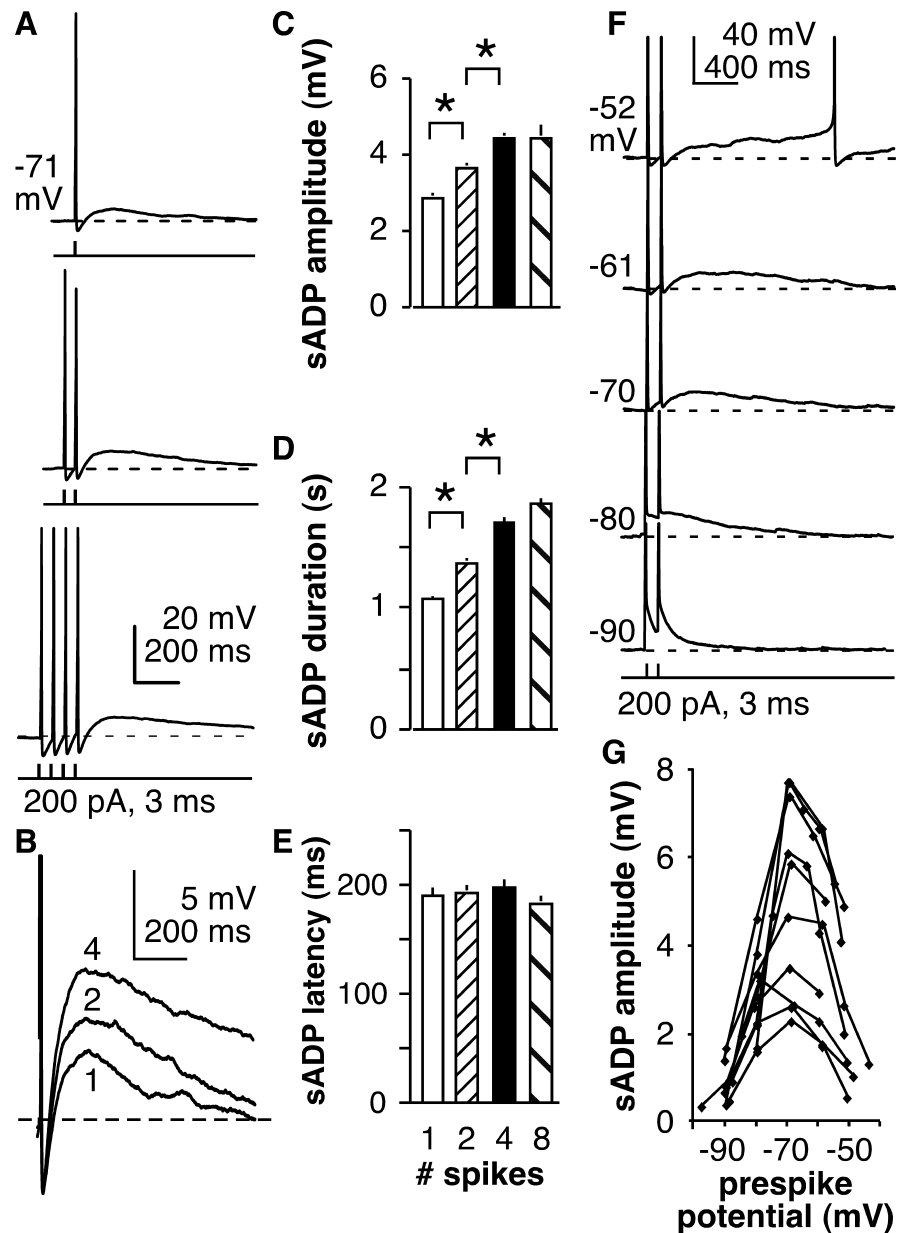


Figure 2. sADPs are dependent on the number of preceding spikes and membrane potential. *A*, Representative example of sADP after one (top), two (middle), or four (bottom) preceding spikes. Data are aligned by last induced spike. Dashed line shows prespike baseline. *B*, Expansion of sADP from *A*; spikes are truncated. The number near the trace indicates number of preceding spikes. *C–E*, Mean \pm SEM sADP amplitude, duration, and latency, respectively, as a function of spike number. $*p < 0.05$. *F*, Representative example of sADP after two spikes in an individual cell in which membrane potential was adjusted to the values shown on the left by current injection. Spikes are truncated; dashed lines show prespike baseline. *G*, Plot of sADP amplitude versus membrane potential for all 10 cells tested.

sADP is not altered by blockade of neurotransmitter/neuromodulator receptors

A net inward current might arise from intrinsic properties of the neuron or from synaptic inputs. We first examined synaptic mechanisms by testing the effects of blocking the receptors for a variety of neurotransmitters and neuromodulators thought to be key inputs to GnRH neurons. GnRH neurons are spiny (Witkin, 1989; Campbell et al., 2005) and express functional iGluRs (Brann and Mahesh, 1994; Eyigor and Jennes, 1997; Adams et al., 1999; Suter, 2004). Recent evidence indicates that they express vesicular glutamate transporter 2 (Hrabovszky et al., 2004) and are functionally glutamatergic (Chu and Moenter, 2005).

mGluRs modulate the ADP in CA3 hippocampal neurons (Young et al., 2004). Blockade of either iGluRs ($n = 5$) or mGluRs ($n = 8$), however, had no effect on the sADP (Fig. 3A, iGluR raw data, D, summary). A main input to GnRH neurons is GABAergic via the GABA_A receptor (Sim et al., 2000; Jansen et al., 2003; Pompolo et al., 2003; Sullivan et al., 2003; Sullivan and Moenter, 2004a,b, 2005), but blocking GABA_A receptors did not alter the sADP ($n = 7$) (Fig. 3B,D). GnRH peptide has been reported to have both stimulatory and inhibitory effects on GnRH neurons and on the anterior pituitary (Tse et al., 1994; Xu et al., 2004), but blocking receptors for this hormone did not alter the sADP ($n = 5$) (Fig. 3C,D). A high percentage of GnRH neurons coexpress galanin (Merchenthaler et al., 1990; Hohmann et al., 1998) and galanin receptor is also expressed in these neurons (Mitchell et al., 1999), but blocking galanin receptors was also without effect on the sADP ($n = 8$) (Fig. 3D). ATP can mediate fast synaptic transmission (Edwards et al., 1992; Evans et al., 1992), and a role for purinergic receptors in GnRH release and in synchronizing calcium oscillations in GnRH neurons has been suggested recently (Terasawa et al., 2005). The broadspectrum purinergic receptor antagonist suramin had no effect on the sADP in GnRH neurons ($n = 6$) (Fig. 3D). Although this list is not exhaustive, the lack of effect of these receptor blockers suggests that the sADP may be intrinsically generated. Further supporting this notion, the sADP persists in isolated GnRH neurons (Kuehl-Kovarik et al., 2005).

Blocking specific conductances can block the sADP

To explore possible intrinsic mechanisms underlying the sADP, we individually blocked candidate ion channels. Hyperpolarization-activated currents (I_h) are involved in pacemaking and recovery from hyperpolarization in some neurons (Robinson and Siegelbaum, 2003). GnRH neurons have been reported to express I_h in guinea pigs and mice (Kelly and Wagner, 2002) (Z. Chu, H. Takagi, and S. M. Moenter, unpublished observation). ZD7288, a fairly specific inhibitor of the HCN channels through which I_h is conducted, did not significantly alter sADP amplitude in GnRH neurons ($n = 6$) (Figs. 4A, 5F). An sADP with similar kinetics is observed in magnocellular neuroendocrine neurons (Andrew and Dudek, 1983, 1984; Li and Hatton, 1996); it has been proposed that a reduction in outward TEA-sensitive I_K generates this sADP (Li and Hatton, 1997b). The sADP of GnRH neurons, however, was not affected by TEA ($n = 7$) (Figs. 4B, 5F). Similarly, blockade of calcium-dependent cation channels with either Cs⁺ (which also blocks I_h and some I_K) or flufenamic acid had no effect on the sADP (Fig. 5F) (Ghamari-Langroudi and Bourque, 1998, 2002).

Calcium channels can mediate depolarization either near the resting potential (low-voltage-activated) or as a consequence of activation during action potentials (high-voltage-activated) (Perez-Reyes, 2003; Lacinova, 2005). Elevations in intracellular calcium from a variety of sources are critical in neurotransmitter and hormone release. To test for a role of calcium in mediating the sADP, the broadspectrum calcium channel blocker cadmium (200 μM) was used. Cadmium reduced the amplitude of the sADP by ~60% ($p < 0.001$; $n = 7$) (Figs. 4C–E, 5F). Similarly, removal of calcium from the external solution also reduced the amplitude of the sADP ($p < 0.05$; $n = 4$; data not shown). Cadmium also reduced the latency to peak of the sADP ($p < 0.002$) (Fig. 4F) and increased the amplitude of the AHP ($p < 0.05$) (Fig. 4G). Overall magnitude of the sADP measured from the most hyperpolarized potential of the AHP to the peak of the sADP was not altered by cadmium (9.3 ± 0.7 mV; control, $9.6 \pm$

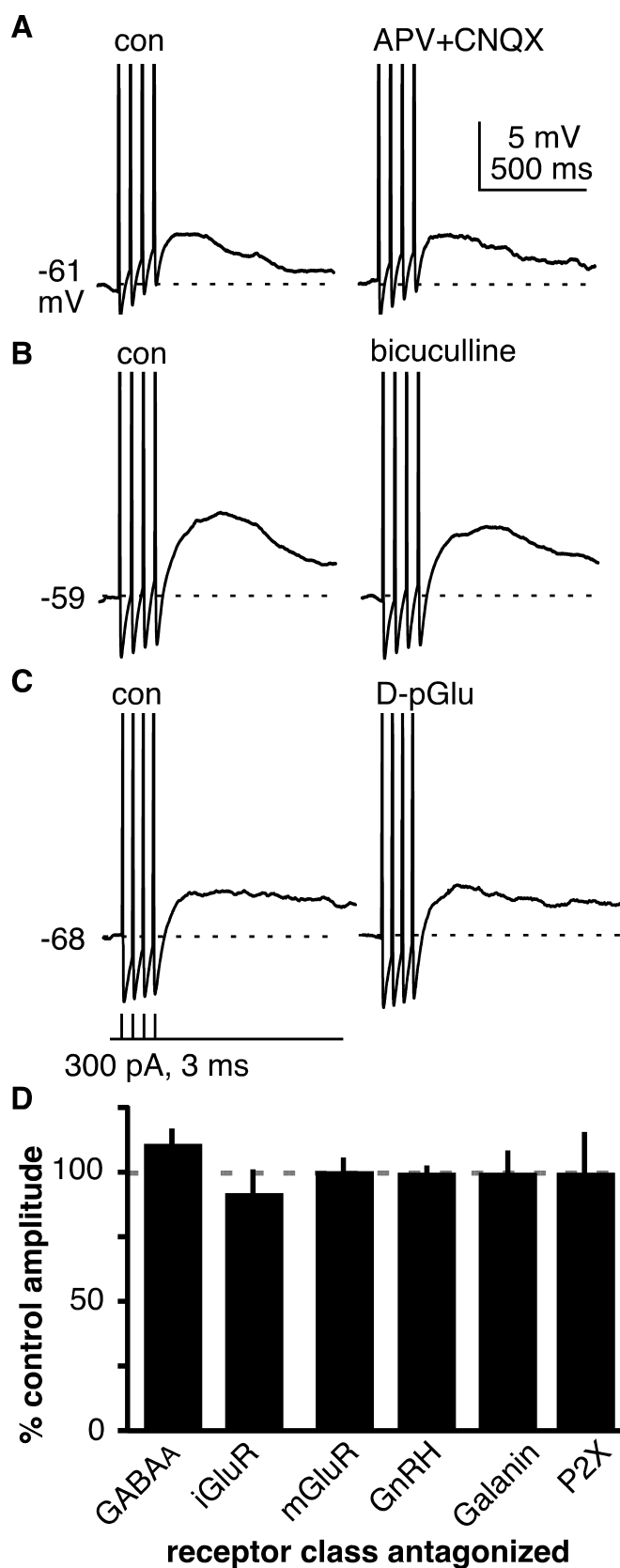


Figure 3. sADPs are not altered by selected neurotransmitter and neuromodulator receptor blockers. **A–C**, Representative examples of sADP before and after treatment with receptor blockers (**A**, 20 μM APV plus 20 μM CNQX; **B**, 20 μM bicuculline; **C**, 20–40 nM D-P-Glu). Spikes are truncated; dashed lines show prespike baseline. **D**, Mean ± SEM change in sADP amplitude in response to receptor blockade. Con, Control.

0.6 mV). Together, these studies suggest that blocking calcium channels alters the time course of the sADP, perhaps by blocking the influence of calcium-activated potassium currents, but do not contribute to the depolarization of the sADP itself.

Voltage-dependent sodium channels play a major role in determining neuronal activity. Persistent and resurgent sodium currents can contribute to regenerative spiking (Crill, 1996; Raman and Bean, 1997; Agrawal et al., 2001). TTX ($n = 6$; $0.5 \mu\text{M}$) reversibly abolished the sADP in GnRH neurons after induced spikes ($p < 0.0001$) (Fig. 5*A, B, F*). An obvious caveat of this experiment is that, in the presence of TTX, the membrane does not go through the same changes in potential as during an action potential. A loss of activation of voltage-gated conductances that occurs secondary to loss of the action potential could thus explain the lack of sADP after blocking sodium channels. To correct for this, we increased the amplitude of the current injection so that the capacitive changes in membrane potential were of similar magnitude to the action potential (Swensen and Bean, 2003). Under these conditions, TTX still eliminated the sADP ($n = 4$) (Fig. 5*C, D*). The slow kinetics of the sADP suggest that it may be mediated by a persistent sodium current (I_{NaP}). Riluzole is reported to block I_{NaP} , but it failed to block the sADP, even at high concentrations ($n = 7$) (Fig. 5*E*). As a control, we showed that these concentrations of riluzole blocked a majority of the inward persistent sodium current generated by a voltage ramp (-80 to -20 mV at 50 mV/s) in CA1 pyramidal neurons as reported previously (Yue et al., 2005) (data not shown). These data suggest that current through TTX-sensitive voltage-dependent sodium channels is a key mechanism underlying the sADP.

Intrinsic mechanisms underlying the sADP

Voltage clamp was used to explore the current that underlies the sADP (I_{ADP}). Two types of voltage commands were used. Short square-wave depolarizing steps (2 ms, -60 to $+20$ mV) (Fig. 6*A*, left) approximate the depolarization of an action potential. Because a square wave does not include the afterhyperpolarization, which is typically pronounced in GnRH neurons, a voltage command more closely replicating an action potential was also used (Fig. 6*A*, middle, right). Both regimens yielded a similar inward current (I_{ADP}); data from these two commands were pooled. Latency to the peak of I_{ADP} after a single simulated action potential was 95.5 ± 4.3 ms ($n = 24$), which is shorter than the latency to the peak of the sADP. This advancement in the peak of the current relative to the sADP would be expected by the time required to charge the membrane capacitance in current clamp.

In current-clamp experiments, inducing multiple spikes in-

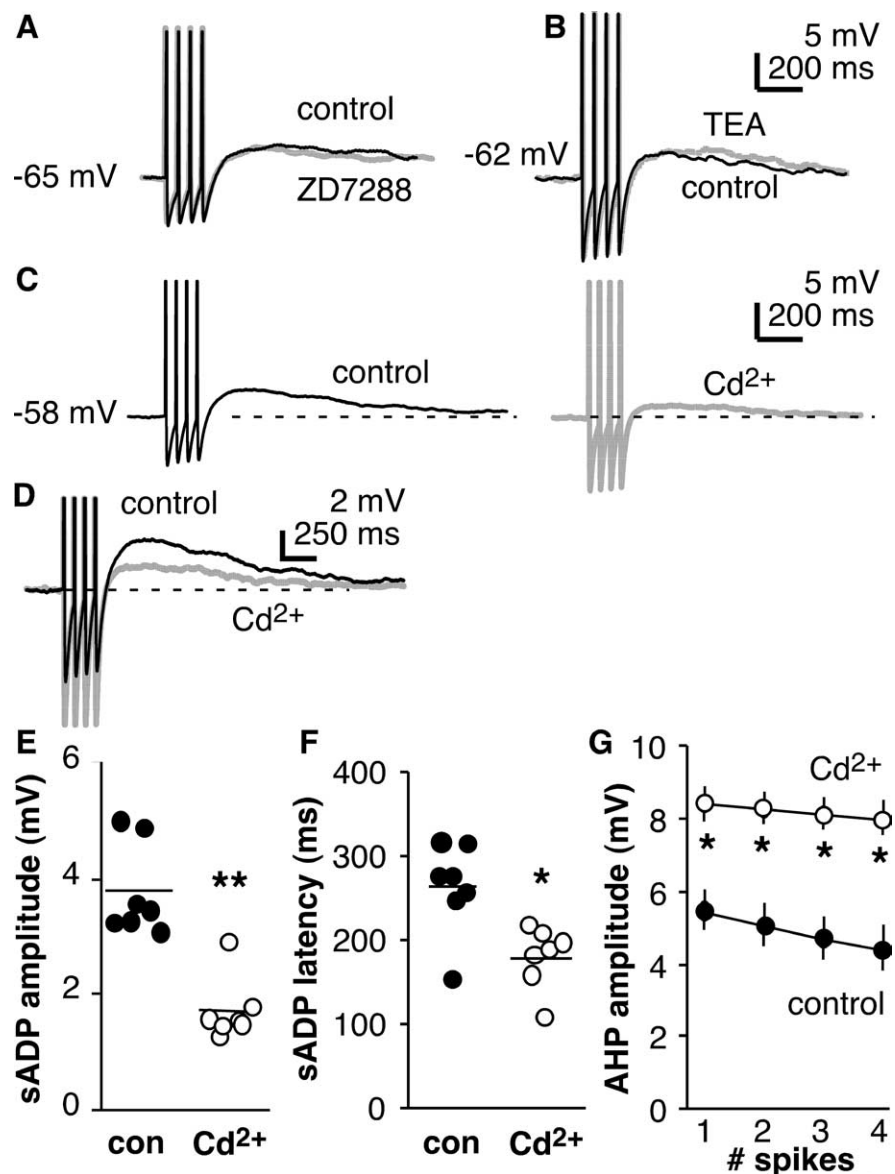


Figure 4. Effect of channel blockers on the sADP. *A*, sADP recorded before (black) and after (gray) blocking I_h with ZD7288 ($40 \mu\text{M}$). *B*, sADP recorded before (black) and after (gray) blocking I_K with TEA ($10 \mu\text{M}$). *C, D*, Absolute amplitude of the sADP is decreased by application of cadmium (Cd^{2+} , $200 \mu\text{M}$) to block calcium channels. *E, F*, Cadmium reduces ADP amplitude (*E*) and latency (*F*); each individual value is shown with horizontal line indicating mean. *G*, Cadmium also increases amplitude of the AHP relative to prespike baseline. Spikes are truncated; dashed lines show prespike baseline. $*p < 0.05$; $**p < 0.001$. con, Control.

creased the amplitude of the sADP (Fig. 2). To test whether I_{ADP} had a similar dependence, one, two, or four action potential clamp commands were given at 50 ms intervals (Fig. 6*B*). A single command produced an inward current of 2.7 ± 0.2 pA; this current increases to 3.5 ± 0.2 pA ($p < 0.001$) after double stimulation and to 4.1 ± 0.2 pA after quadruple stimulation ($p < 0.001$; $n = 24$). Both the timing of the inward current and its dependence on the number of preceding stimuli are consistent with the hypothesis that this inward current mediates the sADP.

Previous studies of DAPs in magnocellular neuroendocrine neurons had proposed a reduction in potassium conductance via delayed rectifier channels as the mediator of the inward current (Li and Hatton, 1997b). The voltage dependence of the sADP in GnRH neurons is perhaps consistent with this notion because the sADP was minimal near the reversal potential for potassium (Fig. 2). We thus explored the change in overall membrane conduc-

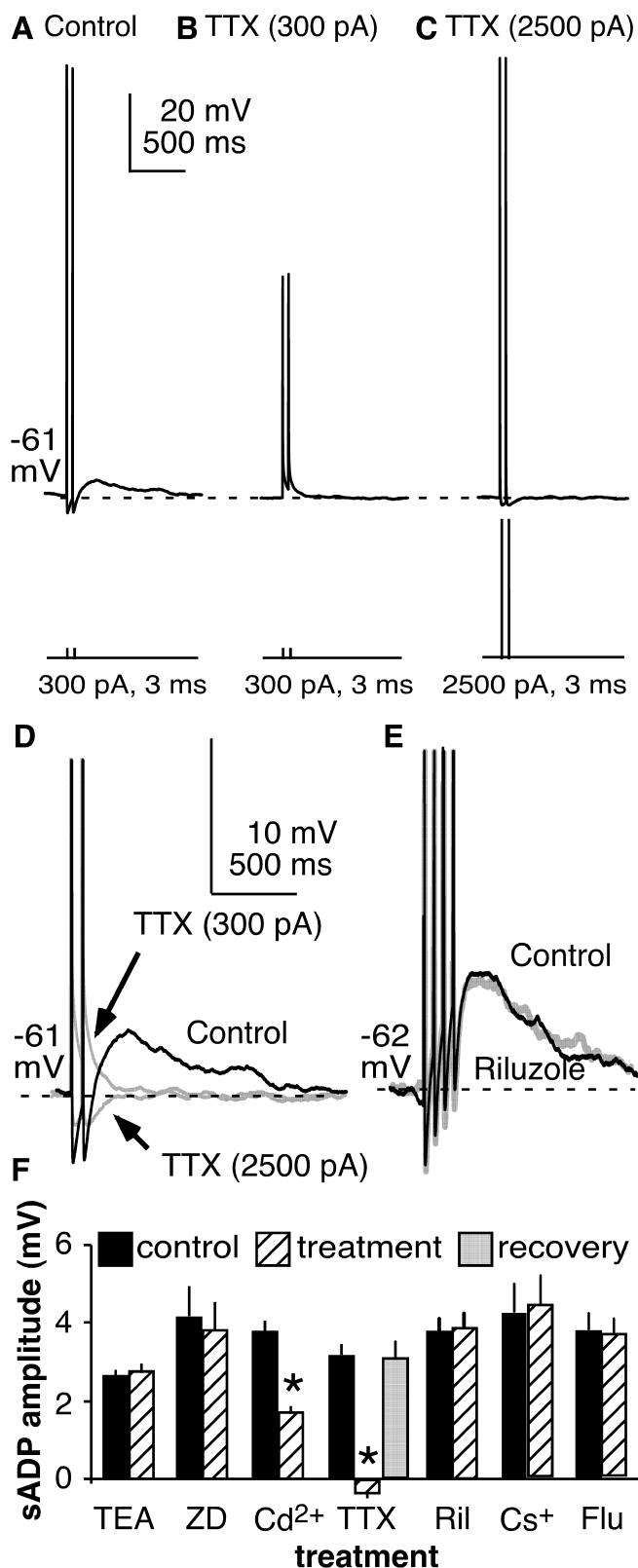


Figure 5. Blocking specific sodium conductances eliminates sADP. **A**, Two brief depolarizing current pulses (300 pA, 3 ms) evokes two spikes followed by an sADP. **B**, TTX (0.5 μ M) eliminates the sADP. **C**, Higher-amplitude current pulses (2500 pA, 3 ms) restore membrane potential changes but not sADP. **D**, Overlap of expanded and truncated traces from **A–C**. **E**, The persistent sodium current blocker riluzole (5–10 μ M) had no effect on sADP amplitude. **F**, Summary of pretreatment (black) and posttreatment (hatched) sADP amplitudes by treatment. Dashed lines show prespike baseline. ZD, ZD7288; Ril, riluzole; Flu, flufenamic acid (200 μ M). * $p < 0.001$.

tance during the I_{ADP} by measuring input resistance before and during the inward current ($n = 17$). A small but consistent reduction in input resistance was observed during the inward current (Fig. 6D–F), suggesting that it is mediated by activation rather than inhibition of a conductance.

Specific blockers were next used to explore the ionic nature of I_{ADP} . Consistent with the observed increase in conductance during I_{ADP} , TEA had no effect on amplitude of the inward current ($n = 10$) (Fig. 7A, G, H). Likewise, Cs⁺ and flufenamic acid had no effect on I_{ADP} (Fig. 7H). Cadmium did not alter the amplitude of the I_{ADP} ($n = 15$) (Fig. 7B, C, G, H). Cadmium did, however, reduce the latency to peak of I_{ADP} ($p < 0.01$, $n = 14$) (Fig. 7D), similar to the reduction it induced in latency to peak of the sADP (Fig. 4). This raised the question of whether or not the latency reduction may represent a secondary effect of cadmium on calcium-sensitive potassium channels. Treatment with blockers (iberiotoxin, 100 nM; apamin, 200 nM; $n = 11$) of calcium-activated potassium currents (I_{KCa}) reduced latency to peak of the I_{ADP} ($p < 0.02$) and, furthermore, blocked subsequent reductions in latency by cadmium (Fig. 7G, H). These data are consistent with the hypothesis that cadmium reduces latency by blocking I_{KCa} . Interestingly, blocking I_{KCa} tended to increase current amplitude (single spike, 3.0 ± 0.3 vs 3.2 ± 0.4 pA, $p < 0.02$; double spike, 3.7 ± 0.4 vs 3.9 ± 0.4 pA, $p < 0.08$; quadruple spike, 4.4 ± 0.5 vs 4.6 ± 0.5 pA, $p < 0.05$; $n = 11$) (double spike data shown in Fig. 7H; because this effect varied in significance with spike number, no significance marker is shown in Fig. 7). This suggests that I_{KCa} activated during action potentials may oppose I_{ADP} and limit the magnitude of the sADP. In contrast to the lack of effect of other ion channel blockers on I_{ADP} , TTX eliminated I_{ADP} as it had the sADP ($n = 12$) (Fig. 7E, F, H). Together, these data indicate the sADP is mediated by a slow inward TTX-sensitive sodium current.

Function of sADPs in GnRH neuron excitability and regulation by estradiol

The amplitude and duration of the afterpotential, including hyperpolarizing and depolarizing components, can influence neuronal excitability and spike firing patterns. To examine the role of sADPs in controlling GnRH neuron activity, current-clamp recordings were made of GnRH neurons, and the effect on pattern of firing was examined. Strong sADPs were suprathreshold, with spontaneous action potentials riding on the depolarizing envelope (Fig. 8A). This suggests that sADPs can alter the pattern of GnRH neuron firing.

To test whether the sADP is subject to physiological regulation, we chose an established model of estradiol feedback, treating some animals *in vivo* with estradiol implants (Christian et al., 2005). Recordings from the latter animals were made in both the morning and evening because estradiol feedback is regulated by the circadian clock (Chappell, 2005). Specifically, estradiol exerts negative feedback in the morning and positive feedback in the evening; in ovary-intact nocturnal rodents, this times the ovulatory signal to the onset of night. The mechanisms for this switch in feedback action are unknown but likely involve both synaptic and intrinsic mechanisms. Estradiol-induced feedback persists in GnRH neurons in brain slices, with reduced GnRH neuron activity in the morning and increased activity in the evening; in contrast, there are no diurnal changes in GnRH neuron activity in OVX mice (Christian et al., 2005).

To facilitate comparison between cells, DC (≤ 10 pA) was injected to maintain membrane potential between -58 and -62 mV. There was no difference in either resting membrane poten-

tial before this injection (OVX, $n = 20$, -64.3 ± 1.1 mV; OVX plus estradiol in the morning, $n = 5$, 61.6 ± 2.4 mV; OVX plus estradiol in the evening, $n = 14$, 62.7 ± 2.2 mV) or in input resistance (OVX, 794 ± 57 M Ω ; OVX plus estradiol in the morning, 849 ± 69 M Ω ; OVX plus estradiol in the evening, 820 ± 61 M Ω) among the cells studied. Two brief current injections were used to trigger action potentials as described above. Estradiol increased sADP duration and amplitude (Fig. 8B,E). Estradiol increased the frequency of spontaneous action potentials during the depolarizing envelope of the sADP by an average of fivefold (Fig. 8C,D). This observation is consistent with a recent report showing that estradiol acutely increases firing activity when applied in the bath solution (Abe and Terasawa, 2005) (note that all estradiol treatments in the present study were *in vivo*). Estradiol also increased I_{ADP} amplitude (Fig. 8E). Most estradiol effects on the sADP were independent of time of day. This suggests that effects on the sADP and resulting changes in GnRH neuron excitability are a response of this system to estradiol alone and that time-of-day information is conveyed via alternative mechanisms such as changes in synaptic transmission. This estradiol-induced increase in sADP and excitability may be an important component of the increased firing rate of GnRH neurons that occurs at the time of the GnRH surge.

Discussion

GnRH neurons express a variety of intrinsic conductances that shape their firing patterns in an incompletely understood manner (Bosma, 1993; Kusano et al., 1995; DeFazio and Moenter, 2002; Kelly and Wagner, 2002; Moenter et al., 2003) (Chu, Takagi, and Moenter, unpublished observation). Here, we show that a TTX-sensitive sodium conductance mediates an intrinsically generated sADP in GnRH neurons. This sADP is modulated by estradiol and can contribute to repetitive firing in these cells and potentially to hormone release. Although these studies were conducted on sADPs after induced spikes, spontaneous spikes are also followed by sADPs, indicating that sADPs are a component of nonstimulated GnRH neuron function.

ADPs in neuroendocrine and non-neuroendocrine neurons can facilitate burst firing. The ADP in GnRH neurons more closely resembles that in other neuroendocrine neurons in amplitude, delayed onset after an AHP, and longer duration (Andrew and Dudek, 1984; Li and Hatton, 1997a,b; Kuehl-Kovarik et al., 2005) than that observed in neurons of the hippocampus and cortex (Metz et al., 2005; Yue et al., 2005). The different characteristics of ADPs may relate to different functions of these cell types. The long duration of neuroendocrine ADPs provides an

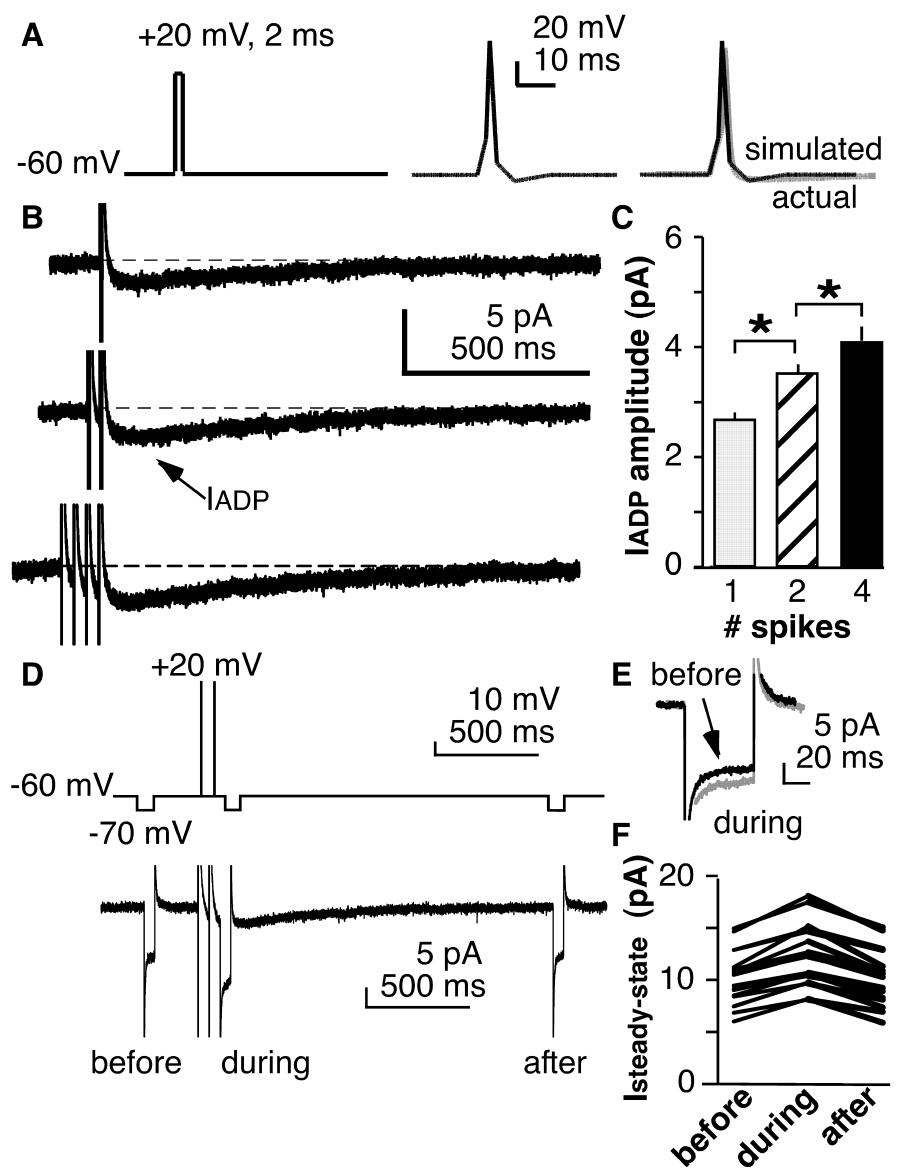


Figure 6. An inward current underlies the sADP. *A*, Square pulses (left) or simulated action potentials (middle) were used as voltage commands to elicit the current underlying the sADP. Right shows comparison of simulated action potential (black) versus actual action potential (gray). *B*, Inward current (I_{ADP}) is elicited by voltage commands shown in *A*. *B*, *C*, Increasing number of simulated spikes increases the current. *D*, Top, Voltage protocol to estimate input resistance before, during, and after induced I_{ADP} . Bottom, Corresponding current trace. *E*, Overlap of current trace from before and during I_{ADP} showing increased steady-state conductance during I_{ADP} . *F*, Changes in $I_{steady-state}$ in all cells tested illustrating the increase in current and return to precommand levels. $*p < 0.001$.

extended period of increased excitability. Neuroendocrine neurons studied thus far are spontaneously active but tend to have low firing rates compared with other neurons. The activity of a network of neuroendocrine neurons often must be synchronized to produce the discrete pulses of hormone release required for function of downstream targets. The extended period of increased excitability after a GnRH neuron fires an action potential may help increase response to excitatory feedforward and feedback mechanisms required to recruit the network to activity.

Similar to magnocellular neurons (Andrew and Dudek, 1984; Li and Hatton, 1997a), GnRH neuron sADP amplitude was dependent on the number of preceding spikes, but only to a point. sADPs in these cells appear to be self-limiting. By producing a finite increase in excitability, sADPs may dictate a pattern of pha-

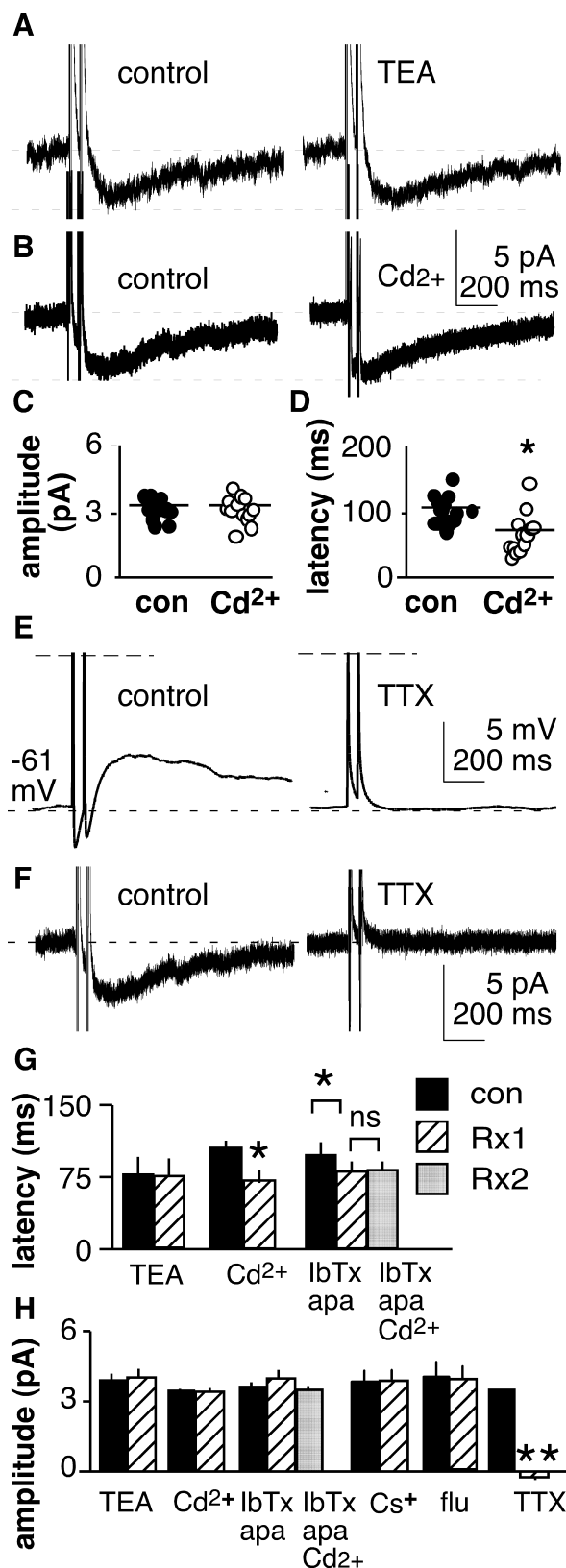


Figure 7. Effect of channel blockers on I_{ADP} . **A**, TEA (10 μ M) does not alter I_{ADP} . **B–D**, Cadmium (200 μ M) does not affect amplitude of I_{ADP} (**B, C**) but does reduce latency. **C** and **D** show each individual cell with horizontal line indicating the mean. * $p < 0.05$. sADP and I_{ADP} are TTX sensitive. con, Control. **E, F**, Current-clamp (**E**) and voltage-clamp (**F**) recordings of the same cell before (left) and after (right) application of TTX. **G, H**, Summary of effect of channel blockers on I_{ADP} latency (**G**) and amplitude (**H**). lbtTx, 100 μ M ibertoxin; apa, 200 μ M apamin; Rx, treatment. * $p < 0.05$; ** $p < 0.001$.

sic burst firing, with groupings of action potentials separated by quiescence, rather than a tonic increase in firing rate. Burst firing causes increased hormone release (Dutton and Dyball, 1979), suggesting that sADPs may play a role in this primary function of neuroendocrine neurons. Interestingly, qualitative examination of GnRH neuron firing patterns indicates that many fire groups of action potentials the same size as the number of spikes needed to maximize the sADP.

Mechanisms that underlie ADPs can be broadly classified as intrinsic and network. Blockade of selected transmitter and neuromodulator receptors failed to alter the sADP in GnRH neurons, suggesting that it is intrinsically generated. Consistent with this, sADPs persist in isolated GnRH neurons (Kuehl-Kovarik et al., 2005). Blockade of specific ion channels suggests that calcium, calcium-activated potassium, and sodium conductances play roles in shaping the postspike response in GnRH neurons. Cadmium blockade of calcium channels reduced the absolute amplitude of the sADP, but this appears secondary to reducing latency and increasing the amplitude of the AHP because the trough-to-peak amplitude was similar. This is different from magnocellular neuroendocrine neurons, in which the sADP was completely eliminated by the calcium removal or blockade of release from intracellular stores. In contrast to the altered waveform with cadmium, TTX eliminated the sADP even when the current injection was increased to produce excursions of membrane potential similar to those during an action potential. The voltage dependence of the sADP of GnRH neurons also differs from other neuroendocrine neurons. Specifically, the sADP of GnRH neurons was a bell-shaped curve peaking at approximately -75 mV, whereas that in magnocellular neurons was minimal at -70 mV and not evident at more hyperpolarized potentials. Together, these data suggest different mechanisms.

Voltage clamp was used to explore the mechanism under controlled conditions. In response to a voltage command mimicking an action potential or a square depolarizing pulse of similar duration, GnRH neurons generate an inward current. Several characteristics of this current suggest that it may underlie the sADP. First, it is inward and would thus be depolarizing. Second, its peak is advanced relative to the change in membrane potential of the sADP after a spike; this is expected because of the time required to charge the membrane capacitor. Third, increasing the number of spikes in the voltage command increased the amplitude of the current, just as increasing the number of spikes in current clamp increased the amplitude of the sADP. Finally, the product of this current amplitude and input resistance is similar to the change in membrane potential during the sADP.

Membrane conductance was increased during the inward current of the sADP, suggesting that it is conducted via sodium, calcium, or mixed cation channels rather than reduced outward current. Consistent with this, blocking delayed-rectifier potassium channels did not affect the current. Rather, it was abolished by TTX, suggesting that it is conducted via sodium channels. Cadmium, which reduced the sADP by half, had no effect on the amplitude of the inward current but did reduce the latency to current peak. We hypothesized that this was secondary to blockade of calcium-activated potassium currents in GnRH neurons (Bosch et al., 2002). In support of this, I_{KCa} blockade reduced latency and prevented a subsequent reduction in latency by cadmium. I_{KCa} blockade had a strong tendency to increase amplitude of the inward current, suggesting that I_{KCa} opposes and/or times generation of the sADP.

Two of the remaining questions are the nature of the sodium conductance and the subcellular location of the channels. With

regard to the former, two possibilities are persistent sodium current (I_{NaP}) and resurgent sodium currents (I_{NaR}). I_{NaP} can generate the progressive depolarization of a pacemaker that brings neurons to the threshold for firing (Alonso and Llinas, 1989; Pape et al., 1998; Bennett et al., 2000; Taddese and Bean, 2002; Del Negro et al., 2005), as well as amplify dendritic depolarizations and facilitate repetitive firing (Schwindt and Crill, 1995; Stuart and Sakmann, 1995; Parri and Crunelli, 1998). I_{NaR} facilitates conglomerate firing in cerebellar Purkinje neurons (Raman and Bean, 1997). The onset of I_{ADP} in GnRH neurons appears slower than reported for I_{NaR} , and the time course is prolonged. Although I_{NaR} flows through the same pore as the transient sodium current of the action potential spike (I_{NaT}) (Grieco et al., 2005), there is ongoing debate about whether I_{NaT} and I_{NaP} are conducted through the same pore. In the present study, TTX but not riluzole, which blocks I_{NaP} , abolished the sADP. These data suggest either that different pores conduct these currents or that the pharmacological sensitivity of a single pore is state dependent, and that the state which conducts I_{ADP} is sensitive to TTX but not riluzole.

With regard to subcellular location, dendritic sodium channels can promote retrograde conduction of a spike from soma to dendrite or amplify the weight of synaptic transmission by initiating a spike at the dendritic level (Benardo et al., 1982; Huguenard et al., 1989; Kim and Connors, 1993; Stuart and Sakmann, 1994; Hausser et al., 2000, 2004). Although dendritic sodium currents can modulate the excitability of soma (Fernandez et al., 2005), the persistence of the sADP in isolated GnRH neurons lacking visible dendrites argues that the operative sodium channels are proximal to the soma (Kuehl-Kovarik et al., 2005).

Regardless of the precise mechanism generating sADPs, they clearly modulate the firing properties of GnRH neurons. Spontaneous excitatory discharges rode on top of induced sADPs, changing the firing pattern of GnRH neurons. This response is modulated by an important physiological regulator. Specifically, magnitude of the sADP, I_{ADP} , and frequency of spontaneous discharges were all increased by estradiol. The apparent discrepancy between a lack of time of day effect for estradiol enhancement of the sADP and previous work in this animal model indicating firing rate of GnRH neurons is elevated only during positive feedback (in the evening) (Christian et al., 2005) can be resolved by invoking different mechanisms estradiol may use to induce the positive feedback response: changes in synaptic transmission to GnRH neurons and changes in the intrinsic properties of GnRH neurons. The present work indicates that sADPs are intrinsically generated and that estradiol enhancement of sADPs

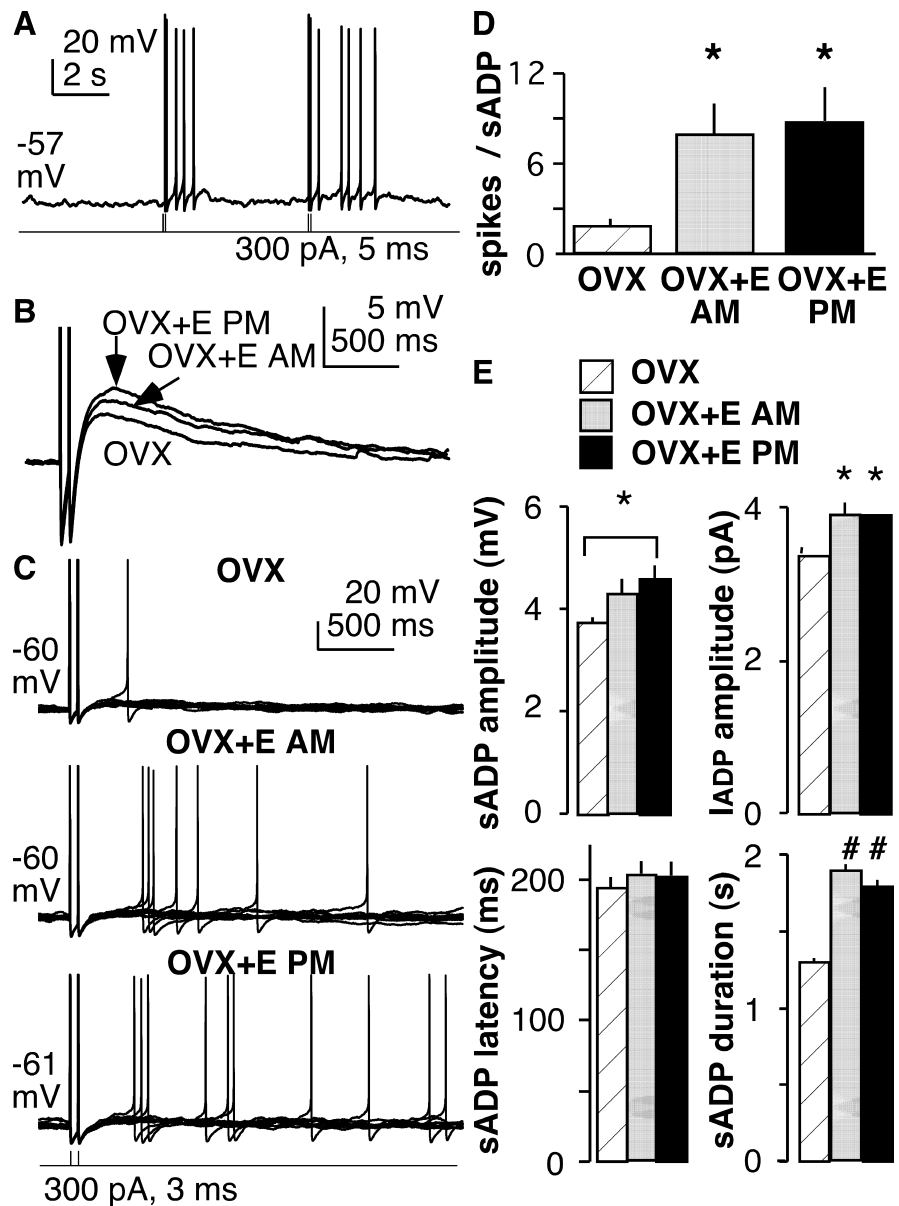


Figure 8. sADPs contribute to repeat firing in GnRH neurons, and both sADPs and the resulting firing are enhanced by estradiol. *A*, Spontaneous action potentials can occur during the sADP. *B*, Superimposition of sADP in representative cells (V_m of -58 to -62 mV) from OVX and OVX plus estradiol mice showing the increased magnitude induced by estradiol (measured at 200 ms). *C*, Current-clamp recordings showing that induction of a pair of spikes by brief current injection elicits spontaneous spikes during the subsequent sADP and that this is enhanced by estradiol treatment in both the morning and evening. Ten traces from each cell are superimposed. *D*, Effect of estradiol on spikes generated during the sADP. *E*, Summary of estradiol effects on sADP amplitude, latency, and duration and I_{ADP} amplitude. * $p < 0.05$; # $p < 0.01$, unpaired analyses. E, Estradiol; AM, morning; PM, evening.

increases firing after initiation of an action potential. Although GnRH neurons fire spontaneously, firing rate is likely modulated by neurotransmission. The response of GnRH neurons to excitatory inputs would be amplified during the sADP, and this amplification would be increased by estradiol. Ongoing studies indicate that estradiol increases depolarizing transmission during positive feedback but decreases transmission during negative feedback (C. A. Christian and S. M. Moenter, unpublished observation). Estradiol enhancement of the intrinsic sADP and perhaps changes in other conductances (DeFazio and Moenter, 2002) may facilitate firing in response to increased transmission in the evening. In ovary-intact cycling animals, such estradiol-induced changes combine to produce the surge mode of GnRH release and ovulation.

We hypothesize that intrinsic generation of sADPs in GnRH neurons not only integrates with other intrinsic conductances to generate burst firing but also provides, through prolonged depolarization, an altered responsiveness to network influences. This may help increase the robustness of GnRH neuron rhythms, alter the frequency of GnRH pulses, help synchronize the network to produce the overall pattern of hormone release, and participate in the switch.

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