Supporting Information

Clasadonte et al. 10.1073/pnas.1107533108

SI Materials and Methods

Animals. Electrophysiological recordings were performed on brain slices from adult gonadotropin-releasing hormone (GnRH)-GFP and GFAP–DN-erbB4/GnRH-GFP transgenic mice. The generation of animals expressing GFP in GnRH neurons and DN-erbB4 in astrocytes have been previously reported (1, 2). Mice were bred and housed in a room with controlled photoperiod (12 h of light and 12 h of darkness) and temperature (21–23 °C), with food and water ad libitum. Both males and females were used for experiments. Vaginal smears were carried out at the time of death. Smears were transferred to microscopic slides and dried, fixed in absolute ethanol for 30 s, and stained with hematoxylin–eosin and the Papanicolaou method (Merck) to identify phases of the estrous cycle (3). All experiments were carried out in accordance with the European Communities Council Directive of November 24th, 1986 (86/609/EEC) regarding mammalian research, and experimental protocols were approved by the institutional animal research committee.

Electrophysiological Experiments. For electrophysiological recordings, animals were deeply anesthetized with a mixture of ketamine hydrochloride and xylazine hydrochloride, according to institutional guidelines. They were perfused via the left ventricle with 15 mL of chilled artificial cerebrospinal fluid (ACSF) with the following composition (in mM): NaCl, 117; KCl, 4.7; NaH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; $MgCl_2$, 1.2; and glucose, 10, bubbled with 95% O_2–5% CO_2. (pH 7.4; osmolarity, 304 mOsm). The brains were removed and 150-μm thick frontal brain slices were made throughout the septum and preoptic area using a Vibratome. The slices were stored for 35 min at 32 °C in ACSF. They were then transferred to a recording chamber continuously perfused with 2 mL/min ACSF, at 25 °C, on the stage of an upright microscope (Leica DL MFSA). GnRH-GFP neurons were visualized thanks to their green fluorescence under a GFP filter and the chosen cell body observed under infrared differential interference contrast. Patch pipettes were produced from borosilicate capillary tubes. Two types of pipette solution (ps) were used. Pipette solution 1 (ps1) contained (in mM): K-gluconate, 125; Hepes, 10; $CaCl_2$, 1; $MgCl_2$, 1; ATP-Mg, 2; EGTA, 11; GTP, 0.3; and NaCl, 15 (pH 7.3 with KOH; osmolarity, 270–280 mOsm). This was the normal internal solution used for most recordings, unless otherwise stated. Pipette solution 2 (ps2) contained (in mM): K-gluconate, 140; Hepes, 10; ATP-Mg, 2; EGTA, 1; and KCl, 10 (pH 7.3 with KOH; osmolarity, 270–290 mOsm). This ps was only used when the spontaneous activity of a neuron needed to be recorded. Previous data have shown that almost all GnRH-GFP neurons (74/75) fire action potentials when they are recorded in the whole-cell configuration with this internal solution (4).

Recordings were carried out with an Axoclamp 2A amplifier (Axon Instruments) in the bridge mode for current-clamp and voltage-clamp recordings and displayed on a pen recorder (Gould Windograph). Acquisition and analysis of potentials were performed with pClamp8 software (Axon Instruments). During whole-cell recordings, the liquid junction potential of −12 mV, calculated for ps1 and ps2, was corrected. A series of constant current pulses (from −70 to +70 pA for 300 ms with a 10 pA increment, at 1-s intervals) was used to characterize the passive membrane properties of recorded neurons using ps1. Drugs were applied to the perfusing system (bath applications) to obtain the final concentrations indicated. The drugs used were: PGE_2, butaprost, sulprostone, 17-phenyl trinor PGE_2 (Cayman Chemical); CNQX, DL-AP5, tetrodotoxin citrate (TTX), thapsigargin (Tocris); sodium fluoroacetate, indomethacin, AH 23848, H89, KT 5720, Rp-cAMPs, and bicuculline methiodide (Sigma).

Statistical analysis was performed using Sigma-Stat software (Jandel). Differences between several groups were analyzed by one-way ANOVA, followed by a Student-Newman-Keuls multiple comparison test. The Student *t* test was used to compare two groups. The data points of the dose–response curve shown in Fig. 1*D* were fitted using Sigma Plot 2001 (SPSS) applying the four-parameter logistic equation: $y = \min + (\max - \min)/(1 + (x/EC_{50})^{Hillslope})$, in which y is the magnitude of the effect, min is the minimum effect, max is the maximum effect, x is the drug concentration, EC_{50} is the half-maximal effective concentration, and Hillslope is a constant, characterizing the slope of the curve at its midpoint. A P value of <0.05 was considered to indicate a statistically significant difference. Values are reported as the mean ± SEM.

SR101 Staining. Brain slices (150 μm thick) were incubated in the presence or absence of fluoroacetate (FA) (5 mM) for 1 h at 32 °C in ACSF, bubbled with 95% O2–5% CO_2. They were then transferred to a 1-μM solution of sulforhodamine 101 (SR101) (Sigma) in bubbled ACSF and maintained at 32 °C for an additional 20 min. Next, they were rinsed in ACSF and fixed in 4% formaldehyde overnight. Finally, they were washed twice in PBS and coverslipped with buffered glycerin mounting medium. Sections were analyzed using an Axio Imager.Z1 ApoTome microscope (Zeiss), equipped with a motorized stage and an AxioCam MRm camera (Zeiss). Specific filter cubes were used for the visualization of green (EX, 475/40 nm; DM, 500 nm; and BA, 530/50 nm) and red fluorescence (EX, 550/25 nm; DM, 570 nm; and BA, 605/70 nm).

Immunohistochemistry. Under deep anesthesia, four wild-type mice were perfused through the ascending aorta with 20 mL of chilled ACSF followed by 20 mL of cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) pH 7.4. Brains were removed immediately after perfusion and postfixed at 4 °C in the same solution for 24 h. After fixation, they were cryoprotected overnight in 20% sucrose in PB at 4 °C and imbedded in Tissue-Tek before being frozen in chilled isopentane. Frontal sections of the brain (14 μm thick) were cut on a cryostat and mounted on gelatin-coated slides. Sections were washed twice with 0.1 M PB − 0.9% NaCl (PBS), treated overnight at 4 °C with a blocking solution (0.1 M PBS containing 0.3% Triton X-100 and 10% normal goat serum), and then incubated for 24 h at 4 °C with the primary antibody (EP2 receptor polyclonal antibody, Cayman Chemical) diluted 1:400 in the blocking solution. Sections were subsequently washed in PBS and incubated for 1 h at room temperature with the secondary antibody (biotinylated-goat antirabbit IgG; Vector) diluted 1:5,000 in PBS containing 0.3% Triton X-100, and then incubated in Elite AB reagent (Vector) for 30 min at room temperature. Signal intensification was obtained by using biotinylated tyramide as follows. Sections were rinsed in PBS and incubated for 20 min at room temperature in 5 μL biotinylated tyramide per milliliter of PBS with 0.005% H_2O_2. After rinsing in PBS, sections were incubated in streptavidin-conjugated Alexa Fluor 568 (Molecular Probes) diluted 1:1,000 in PBS containing 0.4% Triton-X for 1 h and rinsed in PBS. Finally sections were washed in PBS and coverslipped with buffered glycerin mounting medium. Controls consisted of omit-

ting the primary antibody: no staining could be detected under this condition.

SI Results

The electrical activity of 213 GnRH neurons was studied in brain slices from transgenic mice expressing a green fluorescent protein (GFP) under the GnRH promoter, using whole-cell patch-clamp recordings. Most GnRH neurons (193) were recorded using a pipette solution (ps1) that endowed cells with an average resting potential of −75.54 ± 0.38 mV (n = 151) and an input resistance of 1417.76 ± 43.07 MΩ (n = 97). In this configuration, 87% of the neurons remain silent at the resting potential. The remaining neurons discharged in an irregular manner without bursting (n = 2, Fig. S1*A*), in an irregular pattern with sporadic bursts of several action potentials (n = 14, Fig. S1*B*) or with repetitive bursts of action potentials (n = 10, Fig. S1*C*). The latter were driven by depolarizing plateau potentials with an amplitude ranging from 2 to 11 mV and a duration of 1–12 s (Fig. S1*C*).

1. Prevot V, et al. (2003) Normal female sexual development requires neuregulin-erbB receptor signaling in hypothalamic astrocytes. *J Neurosci* 23:230–239.
2. Spergel DJ, Krüth U, Hanley DF, Sprengel R, Seeburg PH (1999) GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. *J Neurosci* 19:2037–2050.
3. Oksjoki S, Sallinen S, Vuorio E, Anttila L (1999) Cyclic expression of mRNA transcripts for connective tissue components in the mouse ovary. *Mol Hum Reprod* 5:803–808.
4. Clasadonte J, Poulain P, Beauvillain JC, Prevot V (2008) Activation of neuronal nitric oxide release inhibits spontaneous firing in adult gonadotropin-releasing hormone neurons: a possible local synchronizing signal. *Endocrinology* 149:587–596.

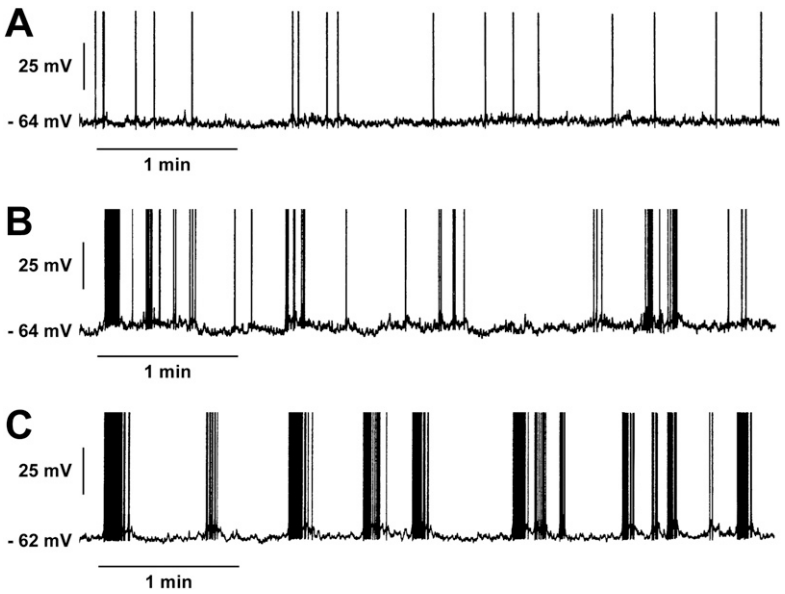

Fig. S1. GnRH neurons display different patterns of spontaneous activity. Recordings of GnRH neurons were performed under whole-cell current-clamp using pipette solution 1 (ps1) (*Materials and Methods*). (*A*) Irregularly firing neuron. (*B*) Neuron showing irregular firing with bursts of action potentials. (*C*) Neuron showing periodic bursts of activity.

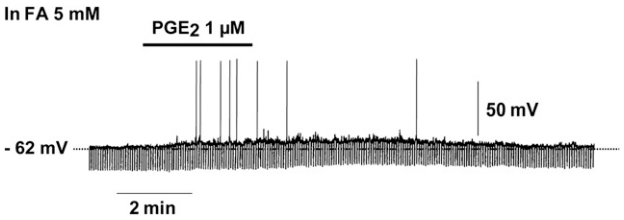

Fig. S2. PGE_2 retains its ability to trigger action potential firing in GnRH neurons recorded from brain slices treated with the glial toxin fluoroacetate (5 mM, 120 min).

Table S1. Membrane depolarization induced by PGE_2 in GnRH neurons in male and female mice and in mice at different stages of the estrous cycle

Sex	
Male	9.24 ± 0.72 mV (*n* = 24)
Female	8.77 ± 0.50 mV (*n* = 34)
Stage of the estrous cycle	
Diestrus	8.84 ± 1.04 mV (*n* = 12)
Proestrus/estrus	9.62 ± 0.61 mV (*n* = 11)
Proestrus	7.50 ± 1.16 mV (*n* = 8)
Estrus	8.75 ± 0.72 mV (*n* = 3)

No statistical difference was noted between sexes or between stages of the estrous cycle. PGE_2 was used at a concentration of 1 μM.