Supporting Information

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SI Materials and Methods

Animal Care and Handling. C57BL/6J mice were housed in temperature-controlled (22–23 °C) quarters under a 12-h light and 12-h dark photoperiod (light on at 8:00 AM); standard mouse chow and water were available for ad libitum consumption. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University.

Construction of GnRHp-dsLuc Plasmid. Luciferase gene fragments were amplified from pGL3-basic (Promega) by PCR with a forward primer containing the HindIII restriction site and a reverse primer with the XbaI and EcoRI restriction sites. The luciferase gene of pGL3-basic was replaced with the PCR product. The PEST sequence was derived from mouse ornithine decarboxylase ubiquitination fragments. The PCR product containing the PEST sequence was tagged to the luciferase gene by cloning into the EcoRI/XbaI sites to construct destabilized luciferase. The rat gonadotropin-releasing hormone (GnRH) promoter (3.0 kb, −3,002 to +88 from the transcription start site) was constructed from the plasmid pGnRH3.0-Luc (1). The promoter was cloned into the XhoI/HindIII sites of the modified pGL3-basic vector. The primer sequences for cloning were as follows: Luciferase forward, 5′-AAG CTT AAG CCA CCA TGG AAG ACG CCA-3′; Luciferase reverse, 5′-TCT AGA GGG GAA TTC CAC GGC GAC TTT CCG CCC TT-3′; PEST forward, 5′-GAA TTC AGC CAT GGC TTC CCG CCG GCG GTG GCG GCG CAG GAT GAT-3′; and PEST reverse, 5′-TCT AGA CTA AAG CAG GTC CTC CTC TGA GAT CAG CTT CTG CAT TGA TGC CAT CAC ATT GAT CCT AGC A-3′.

GnRHp-dsLuc Transgenic Mice. GnRHp-dsLuc transgenic (TG) mice were generated as described previously (2). Briefly, the inserted fragment containing the GnRH promoter, destabilized luciferase, and SV40 poly-A signal was cut out by XhoI/XbaI digestion and purified by agarose gel electrophoresis. TG mice were generated by microinjection of the purified DNA into the pronuclei of the fertilized eggs of C57BL/6J mice. The animal genotypes were identified by PCR amplification of tail DNA. PCR genotyping was carried out with the following two primers: GnRHp-dsLuc forward for genotyping, 5′-GTG GCT TCA GCT GTG AAA GT-3′; GnRHp-dsLuc reverse for genotyping, 5′-CAC CTC GAT ATG TGC ATC T-3′. The tissue distribution of luciferase activities was determined with a commercial enzyme assay kit (Promega) and normalized with a protein assay kit (BioRad) according to the manufacturer's instructions. The Bmal1-knockout mouse (3) was a generous gift from Marina Antoch (Roswell Park Cancer Institute, Buffalo, NY) and Karyn Esser (University of Kentucky, Lexington, KY). GnRHp-dsLuc mice were crossbred with $Bmal1^{+/-}$ mice to obtain GnRHp-dsLuc;$Bmal1^{+/-}$ mice. Male GnRHp-dsLuc;$Bmal1^{+/-}$ mice were bred with $Bmal1^{+/-}$ females to produce GnRHp-dsLuc;$Bmal1^{+/+}$ or GnRHp-dsLuc;$Bmal1^{-/-}$ mice.

Immunohistochemistry. Male GnRHp-dsLuc mice (15 wk of age) were perfused with 4% (wt/vol) paraformaldehyde in PBS, and brains were postfixed in the same solution for 24 h. Brains were cryoprotected in 30% (wt/vol) sucrose in PBS and sectioned (20 μm). Subsequent immunostaining was performed with a free-floating method. Brain sections containing POA were washed with PBS and blocked with 3% (wt/vol) BSA and 0.1% Triton X-100 in PBS for 30 min. The following primary antibodies were applied overnight at 4 °C: anti-GnRH (Millipore), 1:500 and anti-luciferase (Sigma), 1:500. After three washes with PBS, the appropriate secondary antibodies conjugated with fluorescent dye were applied for 30 min. Subsequently, the sections were washed, mounted, and observed under fluorescence microscopy (Carl Zeiss).

Organotypic Slice Culture. The materials for slice culture were obtained from Invitrogen. The slice culture was prepared as previously reported with minor modifications (4). Briefly, the neonate genotype was determined on postnatal days 3–5 by PCR from tail genomic DNA. Transgenic mice (postnatal days 5–7) were anesthetized with ether, and brains were immediately transferred to ice-cold Gey's balanced salt solution (with 10 mM Hepes and 30 mM glucose) bubbled with 5% CO_2 and 95% O_2. The coronal or sagittal brain slices (400 μm thick) were made with a vibratome (Campden Instruments). The brain slices were carefully dissected under a stereomicroscope to minimize the outside regions of the preoptic area (POA), which were located at 3.10–3.50 mm according to the Paxinos coordinate description (postnatal day 6) (5). The POA region was clearly distinguished from the suprachiasmatic nucleus, located at 4.10–4.60 mm, and the arcuate nucleus, which was located at 4.80–6.00 mm. For the adult slices, 200-μm brain slices containing the POA were sectioned with a vibratome. The POA explants (~1 mm long and 1 mm wide) were maintained on a membrane (Millicell-CM; Millipore) dipped into culture medium (50% minimum essential medium, 25% HBSS, 25% horse serum, 36 mM glucose, and 100 U/mL penicillin–streptomycin) at 36 °C. One POA explant was obtained per mouse, and four explants were maintained on a membrane. The medium was changed every 3 d by lifting the Millicell culture membrane and placing it into a new culture dish containing fresh medium.

Drugs. The drug concentrations were as follows: kisspeptin (Anygen) at 10 nM (a single bolus or intermittent administration) or 2.5 nM (continuous exposure);15a (6) (4Chem) at 30 μM; Gö 6983 (Tocris) at 10 μM; cycloheximide (Sigma) at 100 μM; brefeldin A (Sigma) at 10 μg/mL; tetrodotoxin (Tocris) at 1 μM; nimodipine (Tocris) at 10 μM; senktide (Tocris) at 100 nM; dynorphin A (Phoenix Pharmaceuticals) at 200 nM; norepinephrine (Sigma) at 50 μM, glutamate (Sigma) at 5 mM; and γ-aminobutyric acid (Tocris) at 100 μM.

Single-Cell Real-Time Bioluminescence Monitoring. The bioluminescence from the POA cultures was monitored as previously reported with slight modifications (4). Bioluminescence was monitored with Cellgraph (AB-1000; ATTO), a specialized microscope for bioluminescence observation, equipped with a Nikon S Fluor 10× objective lens (N.A., 0.75). The POA slice cultures were maintained in a customized chamber (Live Cell Instruments) and kept in an incubating unit inside the Cellgraph [36 °C, 5% (vol/vol) CO_2 balanced with air and humidified]. One millimolar D-luciferin (Promega) was included in the recording medium (DMEM:Ham's F12 supplemented with 1× N2 supplement, 36 mM glucose, and 100 U/mL penicillin–streptomycin). Images of 4-min exposure duration were acquired at 5-min intervals with the Cellgraph Controller (ATTO). The image acquisition conditions were as follows: cooling temperature, −80 °C; binning, 1 × 1; readout rate, 1 MHz at 16 bits; output amplifier, electron multiplying; electron multiplier gain, 200; and preamplifier gain, 1.0. The acquired bioluminescence images were analyzed with MetaVue (Molecular Devices). All of the analyses were restricted to cells that were clearly distinguished from adjacent cells. Luminescence intensity was measured within a region of interest defined manually for each cell and filtered through a median filter to eliminate

cosmic ray-induced noise. Average value of the region of interest was presented in arbitrary units after background correction. The detrended value was obtained by subtracting the 25-min average from the background-eliminated luminescence intensity.

Pulsatile Kisspeptin Stimulation and Measurement of the Hormone Level. For the simultaneous determination of GnRH secretion with bioluminescence monitoring, the POA cultures were maintained in a customized chamber with two input ports and one output port made of stainless steel. Two NE-1000 syringe pumps controlled by a Syringe Pump Pro program (New Era Pump Systems) were connected with each of the input ports (void volume, ~200 μL). The media in the chamber was withdrawn with a Minipuls Evolution peristaltic pump (Gilson) connected with the output port (void volume, ~300 μL). Kisspeptin was administered by altering the input from the normal media to kisspeptin-containing media. For pulsatile kisspeptin administration, six consecutive kisspeptin pulses (15min of kisspeptin at 10 nM followed by 45 min of media washout) were administered to the slice cultures. For continuous administration, cultures were perifused with kisspeptin-containing media (2.5 nM) for 6 h. The recording media was perfused at a flow rate of 2.4 mL/h and collected every 15 min. The collected media was centrifuged at 1,200 × *g* for 3 min and stored at −80 °C until the RIA was performed. GnRH RIA was performed as described previously (1) with Chen–Ramirez GnRH antiserum, CRR13B73 (generously provided by V. D. Ramirez, University of Illinois, Urbana, IL). Cumulative GnRH secretion was calculated by adding the amount collected for 6 h after the intermittent kisspeptin stimulation was started. The sensitivity at 90% binding was ~5 pg/tube. The intra- and interassay coefficients of variation were 4–5% and 5–10%, respectively, for a 10-pg dose of synthetic GnRH.

Single-Cell Real-Time Monitoring Data Analysis. The pulse peak and nadir of the background-subtracted bioluminescence profile were identified with the Cluster-8 program (7). Cluster sizes for peaks and nadir were defined as 3, and the *t* statistic to identify a significant increase or decrease was 2.0. The pulse period of an individual cell was defined as the duration between the first and last peak divided by the number of interpulse intervals. The pulse amplitude was normalized by the average bioluminescence activity. The synchronization of a GnRH neuronal population was calculated as previously described (8) with minor modifications. Briefly, the time points of the peaks in each cell were compiled into a single file for the entire neuronal population within the imaged field. The Excel program assigned either a 0 (no significant peak) or 1 (significant peak detected) for a given point. This series of 0s and 1s was summed and normalized by the number of cells detected in the culture. For the raster plots, detrended bioluminescence intensity data were normalized by SD and color-coded in red for a SD higher than 1.5 and green for a SD lower than 1.5. Plots were constructed with HCE3.5 (9).

Statistical Analysis. Statistical analysis was carried out with GraphPad Prism 4 (GraphPad Software). Statistical significance was assessed by unpaired two-tailed Student *t* test or two-way ANOVA with repeated measures. The Bonferroni posttest was used for post hoc comparison of ANOVA. Statistical significance was set as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$.

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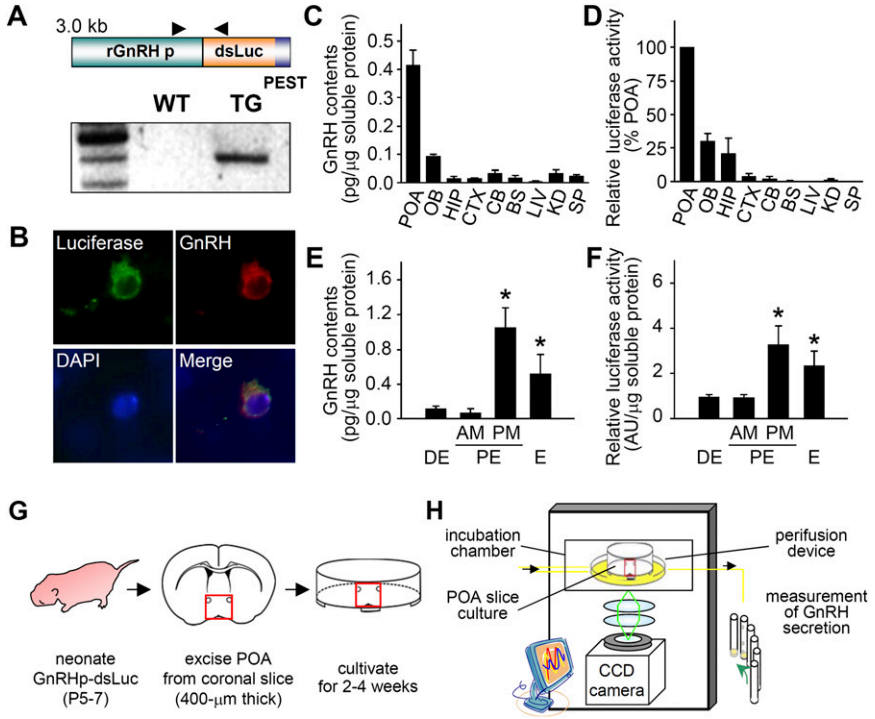

Fig. S1. Validation of GnRHp-dsLuc transgenic mice. (*A*) The structure of the GnRHp-dsLuc fragment and the position of the genotyping primers. The arrowheads indicate the genotyping primers. PCR products were resolved on 2% agarose gel. (*B*) Immunohistochemical analysis of the preoptic area (POA) region of an adult transgenic mouse. Luciferase (green) was simultaneously labeled with GnRH (red). The nucleus was counterstained with 4′,6-diamino-2-phenylindole (DAPI). More than 90% of 36 luciferase-immunoreactive cell bodies from randomly selected POA slices (20-μm thickness) of three different mice were GnRH-immunoreactive. (*C* and *D*) Tissue distribution of endogenous GnRH peptide (*C*) and luciferase reporter activity (*D*). (*E* and *F*) Regulation of endogenous GnRH content (*E*) and luciferase reporter activity (*F*) across the estrous cycle ($n = 4$–6 for each group). DE, diestrous; PE, proestrous; E, estrous. Data are presented as the mean ± SEM, $*P < 0.05$ vs. DE by two-tailed *t* test. (*G*) Preparation of POA slice culture. On postnatal days 5–7, 400-μm brain slices were prepared from the GnRHp-dsLuc transgenic mice. The POA region was excised under a microscope and cultivated for 2–4 wk before experiments. (*H*) Real-time bioluminescence monitoring system. POA slice culture was maintained in an incubation chamber attached to a perifusion device. The emitted photon from a luciferase-positive cell was imaged with a specialized microscope (Cellgraph) equipped with a highly sensitive CCD camera with a perifusion device. GnRH secretion in the perifused media was determined by RIA.

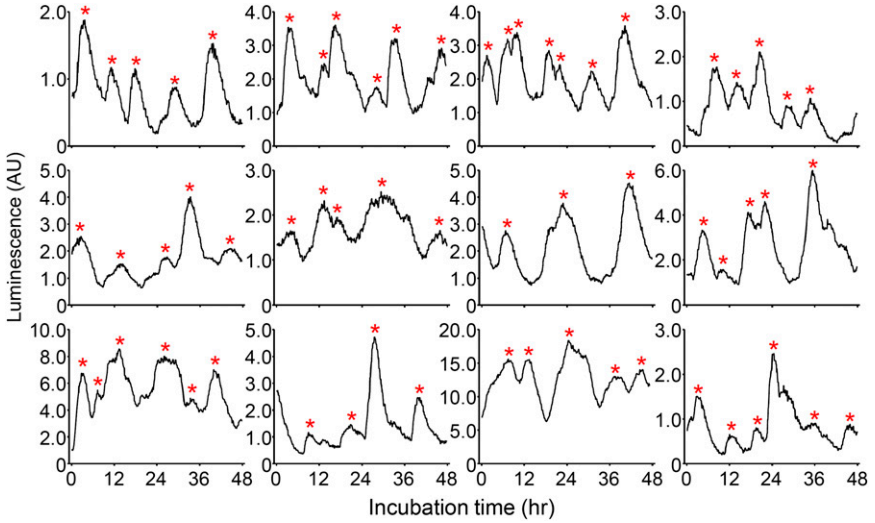

Fig. S2. Representative bioluminescence profiles of individual GnRH neurons. Bioluminescence images of individual GnRH neurons in the POA culture were acquired at 5-min intervals with Cellgraph in the presence of 1 mM D-luciferin. Luminescence intensity was measured within a region of interest (ROI) and filtered through a median filter to eliminate cosmic ray-induced noise. The average value of the ROI was presented in arbitrary units (AU) after background correction. Asterisks represent peaks identified by the Cluster-8 program.

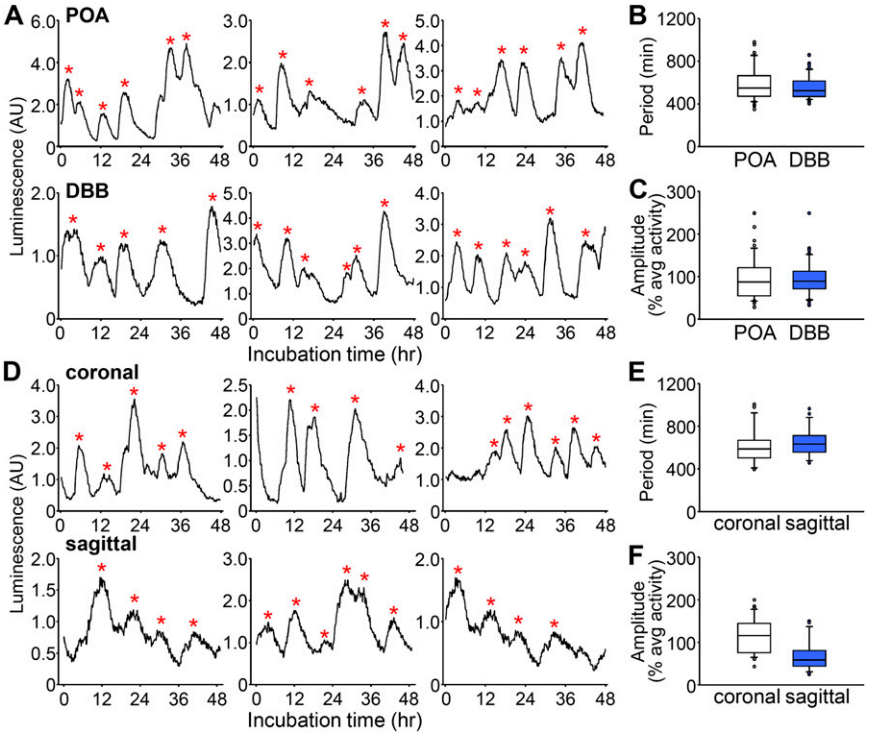

Fig. S3. Ultradian rhythm of GnRH promoter activities in the various slice preparations. (*A*) Representative bioluminescence profiles of GnRH promoter activity in the cultures of POA (*Upper*) and the diagonal band of Broca (DBB, *Lower*). (*B* and *C*) Peak period (*B*) and peak amplitude (*C*) of the GnRH promoter activity observed in GnRH neurons in the POA and DBB were compared (n = 52–55 cells from two batches). Similar ultradian rhythms were observed in the cultured POA and DBB slices (period, $P = 0.7114$; amplitude, $P = 0.9571$ by two-tailed t test). (*D*) Representative bioluminescence profiles of GnRH promoter activity in the cultures of coronally cut (*Upper*) and sagittally cut (*Lower*) POA slices. (*E* and *F*) Peak period (*E*) and peak amplitude (*F*) of the GnRH promoter activity observed in GnRH neurons from coronal and sagittal slice cultures were compared (n = 27–46 cells from two batches). The period of ultradian GnRH promoter activity obtained from the coronal slices was similar to that from sagittal slices ($P = 0.1488$ by two-tailed t test), whereas the amplitude was greater in the GnRH neurons obtained from coronal slices than those from sagittal slices ($P < 0.01$ by t test). Asterisks represent peaks identified by the Cluster-8 program. In the box plots (*B*, *C*, *E*, and *F*), data are expressed as median (line), interquartile (box), 10%, and 90% (bar).

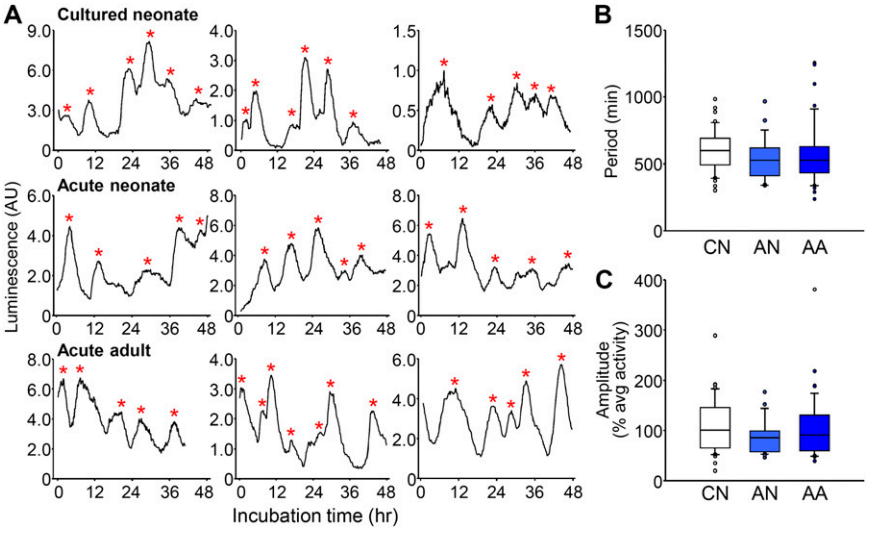

Fig. S4. Effects of slice culture and the preparation age on ultradian GnRH promoter activities. (*A*) Representative bioluminescence profiles of GnRH promoter activity monitored in the POA region from cultured neonatal slices, acutely prepared neonatal slices, and acutely prepared adult slices. Asterisks represent peaks identified by the Cluster-8 program. (*B* and *C*) Peak period (*B*) and peak amplitude (*C*) of GnRH promoter activity observed in GnRH neurons from cultured neonatal slices (CN), acute neonatal slices (AN), and acute adult slices (AA) were used for comparison (n = 46 cells from two batches of CN, 23 cells from two batches of AN, and 54 cells from five batches of AA). Note that the ultradian rhythm of the GnRH promoter activity in GnRH neurons was similar for CN, AN, and AA [one-way ANOVA, $P = 0.3135$ (period) and $P = 0.2199$ (amplitude)]. Owing to thinning of the slices during cultivation, more GnRH neurons were available for simultaneous monitoring in cultured slices than in acutely prepared slices. The viability of the GnRH neurons determined by the luminescence signal intensity was higher in the slices derived from neonatal rather than adult brains. Therefore, we focused on the cultured neonatal slice rather than the acute neonatal or acute adult slice. In the box plots (*B* and *C*), data are expressed as median (line), interquartile (box), 10%, and 90% (bar).

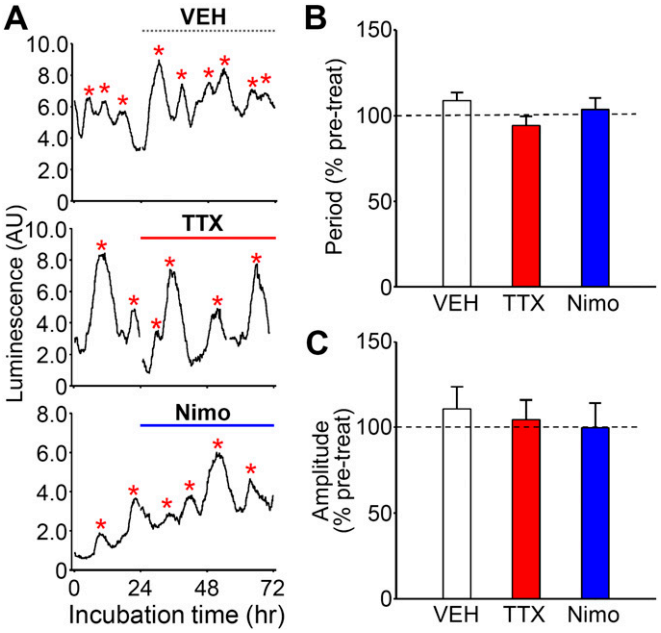

Fig. S5. Effects of tetrodotoxin and nimodipine on the ultradian rhythm of GnRH transcriptional activity. (*A*) Representative profiles showing GnRH promoter activities in the presence of vehicle (VEH, 0.1% dimethyl sulfoxide), 1 μM tetrodotoxin (TTX), and 10 μM nimodipine (Nimo). POA slice cultures were monitored under basal conditions for 24 h and monitored for an additional 48 h after application of the designated drugs. Asterisks represent peaks identified by the Cluster-8 program. (*B* and *C*) Effects of TTX and Nimo on the period (*B*) and amplitude (*C*) of the ultradian rhythm expressed as a percentage of the pretreatment average. TTX and Nimo barely affected the ultradian rhythm of spontaneous GnRH promoter activities [one-way ANOVA, $P = 0.2967$ (period); $P = 0.7110$ (amplitude), $n = 32–86$ cells from two to three slices per group]. Data are shown as the mean ± SEM.

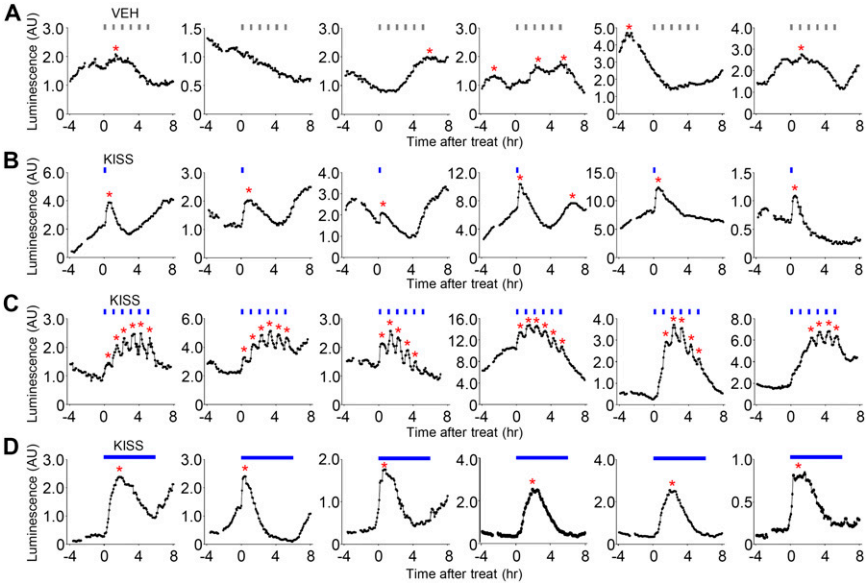

Fig. S6. Representative bioluminescence profiles of GnRH promoter activity in response to the different modes of kisspeptin administration. (*A*) Vehicle (0.1% distilled water) was applied in a pulsatile manner (15-min administration per 1-h cycle for six cycles). (*B*) A single bolus of kisspeptin (10 nM) was administered for 15 min and followed by washout. (*C*) Kisspeptin (10 nM) was administered in an intermittent manner (15 min on, 45 min off). Four luciferase-positive cells that rapidly responded to kisspeptin pulses and reached their peaks are presented at the left, whereas another two cells showing an increase in response to the first one or two boluses of episodic stimulation and exerting pulsatile responses are displayed on the right. (*D*) Chronic exposure of POA slice cultures to kisspeptin (2.5 nM). The concentration used in the different modes of administration was selected to match the total amount of kisspeptin in the intermittent and chronic administrations. Asterisks represent peaks identified by the Cluster-8 program.

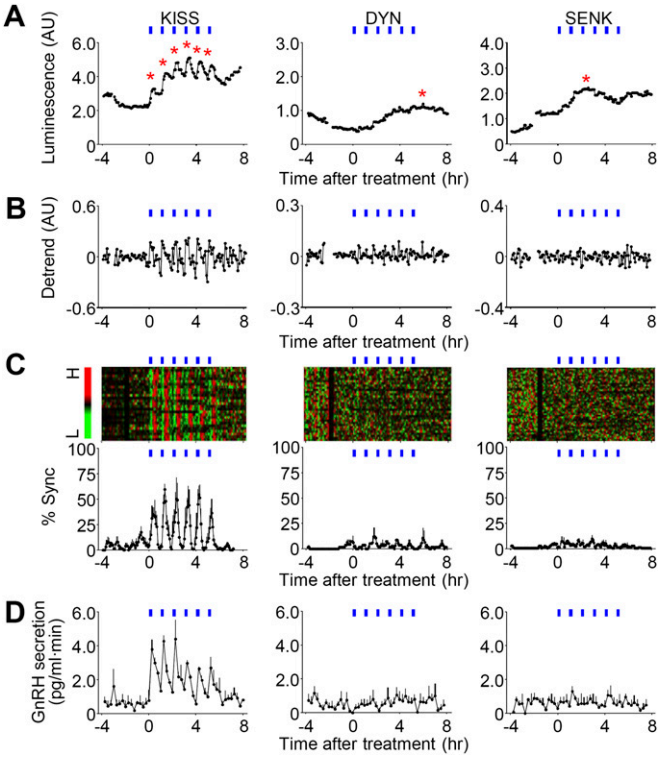

Fig. S7. Effects of neuropeptide signaling related to kisspeptin. (*A* and *B*) Representative profiles of an individual GnRH neuron with intermittent application of indicated neuropeptides (DYN, dynorphin A at 200 nM; KISS, kisspeptin at 10 nM; SENK, senktide, a neurokinin B receptor (NK3R) agonist at 100 nM). Intermittent administration was composed of six cycles (15 min of treatment and 45 min of washout per cycle) at a flow rate of 2.4 mL/h. Data are shown as raw (*A*) or the detrended profile (*B*). (*C*) Synchronization of GnRH neuronal population. Raster plot of normalized detrended luminescence of a representative batch is color-coded according to the scale shown on the left (H, high; L, low). Each row represents an individual GnRH neuron. Synchronization index (% Sync) represents the percentage of luciferase-positive cells simultaneously reaching their peaks. (*D*) GnRH secretion in perifused media was determined by RIA. For *C* and *D*, data are shown as the mean ± SEM (n = 3–4 batches for each treatment). Asterisks represent peaks identified by the Cluster-8 program.

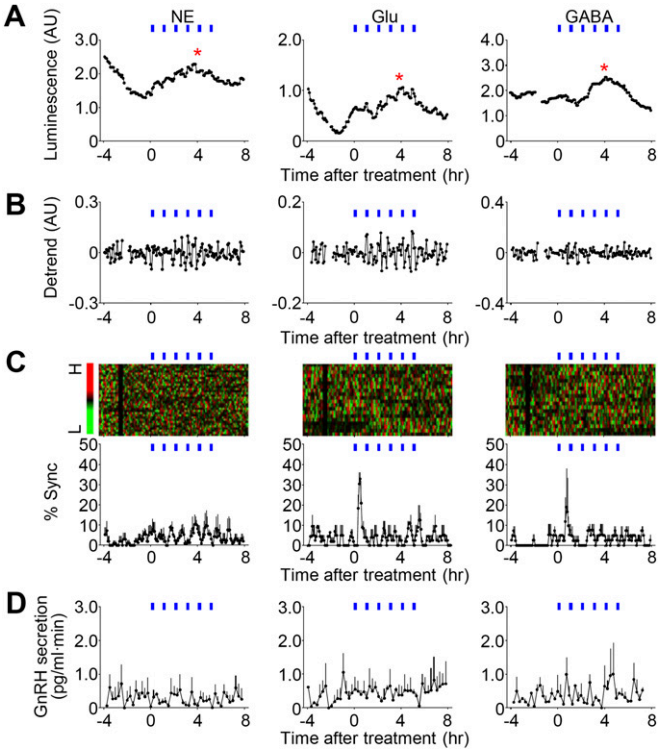

Fig. S8. Effects of pharmacological agents. (*A* and *B*) Representative profiles of an individual GnRH neuron with intermittent application of the indicated neurotransmitters (GABA, γ-aminobutyric acid at 100 μM; Glu, glutamate at 5 mM; NE, norepinephrine at 50 μM). Intermittent administration was composed of six cycles (15 min of treatment and 45 min of washout per cycle) at a flow rate of 2.4 mL/h. Data are shown as raw (*A*) or the detrended profile (*B*). (*C*) Synchronization of GnRH neuronal population. Raster plot of normalized detrended luminescence of a representative batch is color-coded according to the scale shown on the left (H, high; L, low). Each row represents an individual GnRH neuron. Synchronization index (% Sync) represents the percentage of luciferase-positive cells simultaneously reaching their peaks. (*D*) GnRH secretion in perifused media. For *C* and *D*, data are shown as the mean ± SEM (n = 3–4 batches for each treatment). Asterisks represent peaks identified by the Cluster-8 program.

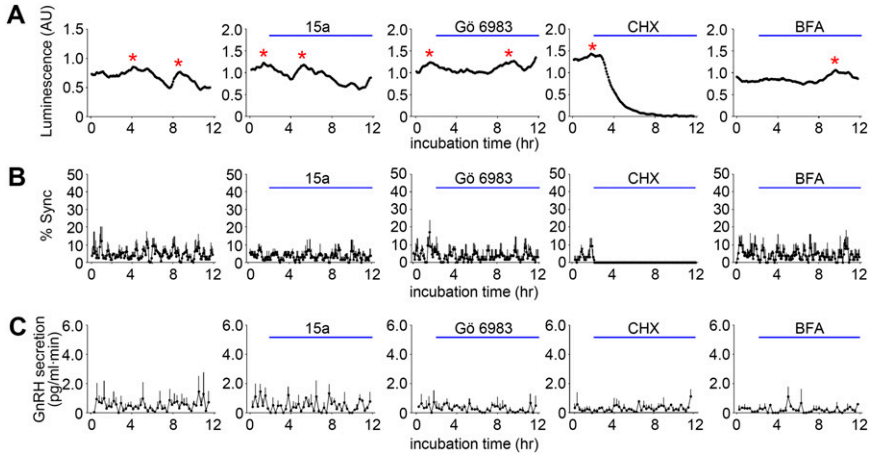

Fig. S9. Pharmacological agents on synchronous GnRH transcription and secretion under basal conditions. (*A*) Representative luminescence profiles of a single GnRH neuron with treatment of 15a (30 μM), Gö 6983 (10 μM), cycloheximide (CHX, 100 μM), or brefeldin A (BFA, 10 μg/mL). The line indicates the incubation period for each pharmacological agent. Asterisks represent peaks identified by the Cluster-8 program. (*B*) Synchronization index. (*C*) GnRH secretion in perifused media. For *B* and *C*, data are shown as the mean ± SEM (n = 3 batches for each agent).