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Supplementary Information for

Nitric oxide resets kisspeptin-excited GnRH neurons via PIP2 replenishment

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Other supplementary materials for this manuscript include the following:

n/a

Supplementary Material and Methods.

GnRH neurons maintained in explants. Embryonic day 11.5 embryos (undetermined sex) were obtained from time-mated NIH Swiss mice as previously described (1). One explant is generated from each embryo. Explants were grown in a defined serum-free medium (SFM) and maintained at 37°C in a humidified atmosphere with 5% CO_2. On day 3, SFM was replaced with fresh SFM supplemented with fluorodeoxyuridine (2.3 µM, Sigma) to inhibit proliferation of dividing neurons and nonneuronal explant tissue. The medium was replaced with fresh SFM on culture day 6 and every 2 days afterwards.

Calcium imaging. Briefly, Calcium Green-1 AM (Life Technologies) was first dissolved at 2.7 mM in 80% dimethyl sulfoxide and 20% pluronic F-127 (Life Technologies), then diluted at 13.5 µM in SFM, aliquoted and frozen. Before use, the solution was warmed up and explants were incubated for 20 min at 37°C in a 5% CO2 humidified incubator. After a 20-min wash in fresh SFM, explants were mounted in a perfusion chamber (Warner Instruments, Hamden, CT) and continuously perfused at a rate of approximately ~300 µL/min. The fluorescence emitted by Calcium Green-1 was visualized with an inverted Nikon microscope through a 20X fluorescence objective. Excitation wavelength were provided with a medium-width excitation bandpass filter at 465-495 nm, and emission was monitored through a 40-nm bandpass centered on 535 nm. Images were acquired every 3 seconds with a charge-coupled device camera (QImaging, Surrey, Canada) piloted by iVision-MAC imaging software (BioVision Technologies, Exton, PA). All experiments were terminated with a brief KCl stimulation (40 mM) to ensure the viability of the cells. After the experiments, explants were fixed (0.1 M phosphate buffer saline (PBS) pH7.4 containing 4% formaldehyde, 1 h, room temperature (RT)), and stored in cryoprotectant until immunocytochemically stained against GnRH was done to confirm the phenotype of the recorded cells.

Immunocytochemistry for GnRH. Explants were washed in PBS, blocked in 10% normal horse serum/0.3% Triton X-100 for 1 h, washed several times in PBS, and then incubated in GnRH antibody (1:5000, SW-1; (2)) overnight at 4°C. The next day, explants were washed in PBS, incubated in biotin-conjugated donkey anti-rabbit antibody (1:500, 1 h RT; Jackson ImmunoResearch Laboratories Inc, West Grove, PA). Explants were washed in PBS incubated in Vectastain Elite® ABC kit (1:600, 1 h RT; Vector Laboratories Inc, Burlingame, CA) then washed, reacted in nickel 3,3'-diaminobenzidine (Ni-DAB), washed and mounted. The recorded field was identified in the stained explant and only GnRH-positive cells were further analyzed.

Electrophysiology. GnRH-GFP mice were killed at approximately 1030h by cervical dislocation. The brain was removed from the skull, glued to a specimen disc and submerged with iced-cold 0.5 mM [Ca] / 6 mM [Mg] artificial cerebrospinal fluid (aCSF), bubbled with 95% O2 / 5% CO2. Conventional coronal sections (200 µm) were cut using a vibratome (Leica VT1000S). After sectioning, slices were incubated at 30°C in normal aCSF containing: 118 mM NaCl, 3 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 10 mM HEPES, 25 mM NaHCO3, and 11 mM D-glucose (pH 7.3), bubbled with 95% O2 / 5% CO2. After >1 h recovery, individual slices were transferred into a recording chamber mounted on an upright microscope (Nikon Eclipse FN1, Tokyo, Japan) and continuously superfused with oxygenated normal aCSF maintained at 28–30°C, at a rate of approximately 2 mL/min. Individual GnRH neurons were identified with fluorescence (20-nm narrow bandpass EGFP filter centered at 480 nm) using a 40X water immersion objective (Nikon 40X/0.80 W, WD 2.0). Visualized with a charge-coupled device camera (QImaging Retiga EXi Blue, Surrey, Canada) piloted by the open source software Micro-Manager version 1.4, the neurons were patched under fluorescence and differential interference contrast. The pipettes (3-5 MOhm) were backfilled with aCSF. Electrophysiological recordings were acquired with a Multiclamp 700B amplifier (Molecular Devices, Synnyvale, CA) using a lowpass filter at 10 kHz and digitized by a Digidata (1550) analog-to-digital converter at 10 kHz (Molecular Devices).

Statistical Analysis.

Calcium imaging analysis. An M-file was coded to detect the KP-evoked response in each cell and the time course of [Ca2+]i response (Fig. 1B). Briefly, the mean baseline fluorescence and standard deviation (SD) were determined during the 3 last minutes of TTX application. The response to KP was validated in individual cells when [Ca2+]i levels during the 2nd-3rd and 4th-5th minutes following KP application were greater than mean baseline fluorescence + 6 SD. Calcium traces of responding cells are expressed as $\Delta F/F = (F - F0)/F0$, where F is the fluorescence at a given time frame and F0 is the mean baseline fluorescence. All the responding cells from one explant gave a single average trace per explant (Fig. 1C). For the quantification of the KP recovery, the raw trace of each responding cell was smoothed by averaging data in 1-min bins to reduce the impact of noise. The mean baseline level was determined again, this time using the smoothed trace, and used as the baseline reference. The maximum response reached after the 2-min application of KP, but within the 5-min following KP application, was used as a maximum reference (100%).

To test the repeatability of the KP response, KP was applied on explants between 10-12th min then reapplied between 37-39th min, i.e. after a 25 min washout period. The baseline and the response to KP were determined over 5 min periods for the first and second KP application. To test whether nitric oxide facilitated the repeatability, DEA/NO (100 µM) was applied between KP applications, from 17th to 32th min.

Electrophysiology analysis. APs were detected with Clampfit 10 on continuous recordings and summed into 30 s bins. The response to 2-min KP was determined by averaging 10 bins before KP (baseline) and 2-min after the start of KP perfusion (response). The two KP applications were spaced by 20-25 min. During some recordings, DEA/NO (100 μM) was applied for 5 min, 5-min after the start of the washout period following the first KP application. The baseline firing (Hz) was determined during a 5-min window preceding KP application. The response firing to KP was determined during a 5-min window positioned 2-min after the onset of KP application. Cells that did not reduce firing in the baseline firing window following the first KP application were excluded.

SI References

1. S. Fueshko, S. Wray, LHRH cells migrate on peripherin fibers in embryonic olfactory explant cultures: an in vitro model for neurophilic neuronal migration. *Dev Biol* **166**, 331–48 (1994).

2. S. Wray, A. Nieburgs, S. Elkabes, Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode. *Brain Res Dev Brain Res* **46**, 309–18 (1989).