Supporting Information

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SI Materials and Methods

Immunohistochemistry. Animals were anesthetized with ketamine/xylazine i.p. and perfused intracardially with 0.1 M PBS followed by 4% paraformaldehyde in 0.1 M PBS. Brains were removed and postfixed in 4% paraformaldehyde for 24 h at 4 °C, followed by immersion in 30% sucrose cryoprotectant. Brains were cut into three sets of 30-μm-thick coronal sections, and two of these sets were used for both single- and double-immunocytochemistry staining as previously described (1, 2). Sections were treated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. After rinses in Tris-buffered saline (TBS), the tissue was incubated for 48 h at 4 °C in TBS Tween 20 (TBST; 0.3% Triton X-100, 0.25% BSA, and 2% normal goat serum) containing a polyclonal rabbit anti–kisspeptin-10 antiserum (1:10,000; a gift from A. Caraty, Institute National de la Recherche Agronomique, Paris). After washes in TBS, sections were incubated in TBST containing biotinyilated goat anti-rabbit IgG (1:500; Vector Laboratories), and this was followed by incubation in Vectastain avidin-biotin complex reagent (Vector Laboratories) for 90 min at room temperature. Sections were washed and the immunoreactivity was visualized using glucose oxidase, nickel-enhanced diaminobenzidine hydrochloride. Sections were washed, mounted on slides, dried overnight, and coverslipped.

Kisspeptin neurons were counted under a Nikon Eclipse TE2000-S microscope. Scoring of kisspeptin neurons was carried out by visualizing kisspeptin neurons located between the A-P coordinates of the mouse stereotaxic atlas (3), extending from 0.62 mm anterior to bregma to 0.10 posterior to bregma. Only cells with darkly stained cytoplasm were included in tallies. Kisspeptin cell numbers were quantified in the AVPe only; as previously reported (2), kisspeptin fiber immunoreactivity precluded such analysis in the ARC.

Dual-label chromogen immunocytochemical analysis of ERα and kisspeptin coexpression was performed according to methods previously reported by Clarkson et al. (2). Briefly, sections were treated with 3% hydrogen peroxide for 10 min and then washed in TBS. Sections were incubated for 48 h at 4 °C in TBST containing rabbit polyclonal primary antisera directed against ERα (1:10,000; Millipore). Sections were then washed and incubated in biotinylated anti-rabbit immunoglobulins (Vector Laboratories) at 1:500 for 90 min at room temperature. After subsequent washing in TBS, the sections were incubated in Vector Elite avidin–peroxidase (Vector Laboratories) for 90 min at room temperature. Immunoreactivity was revealed using glucose oxidase, nickel-enhanced diaminobenzidine hydrochloride that resulted in a black precipitate within the nucleus of the labeled cell. For kisspeptin immunolabeling, sections were washed in TBST containing a polyclonal rabbit anti-kisspeptin antiserum for 48 h at 4 °C. Sections were then washed and incubated in biotynilated anti-rabbit immunoglobulins (1:500; Vector Laboratories) for 2 h at room temperature. Immunoreactivity was then revealed using glucose oxidase, diaminobenzidine hydrochloride without nickel to generate a brown precipitate within the cytoplasm. Double-labeled neurons were indentified as clearly exhibiting a brown cytoplasm and a black nucleus. Singly labeled kisspeptin neurons exhibited a brown cytoplasm only.

Corpora Lutea and Cyst Counts. One H&E-stained section was selected randomly for each animal. Counts were performed in a blinded fashion.

Estrous Cyclicity. Vaginal lavages from female mice (3 wk to 3 mo old) were obtained and viewed under a microscope daily (0900–1,000 h) for at least 31 consecutive days. To determine the percent of days in each stage, lavages from 21 consecutive days, starting from at least 10 d after vaginal opening, were analyzed.

Statistical Analysis. Data are presented as the mean ± SEM. Two-tailed, unpaired *t* tests with Welch's correction were used to determine statistical significance for corpora lutea and cyst counts, P35 male LH data, and testes weights. Two-way ANOVA followed by Bonferroni post hoc tests were used to determine statistical significance in the remaining experiments. Differences were considered significant when $P < 0.05$.

1. Clarkson J, Herbison AE (2006) Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology* 147:5817–5825.
2. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE (2008) Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci* 28: 8691–8697.
3. Paxinos GAF (2001) *The Mouse Brain in Stereotaxic Coordinates* (Academic, San Diego), 2nd Ed.

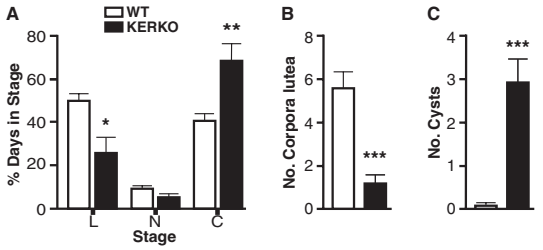

Fig. S1. (*A*) KERKO females spend significantly more days in estrus and fewer days in metestrus or diestrus than WT females (n = 15–18). C, cornified (estrus); N, nucleated (proestrus); L, leukocytic (metestrus and diestrus). (*B*) Ovaries of KERKO mice contain few or no corpora lutea (CLs), whereas WT ovaries contain numerous CLs (n =12–14). (*C*) KERKO ovaries contain numerous follicular cysts, which are absent from WT ovaries (n = 12–14). Data are presented as mean ± SEM. *$P < 0.01$; **$P < 0.001$.

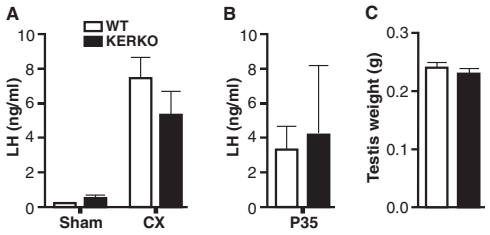

Fig. S2. (*A*) Serum LH levels were not different in adult male WT and KERKO mice after sham surgery (Sham) or castration (CX) (n = 6–9). (*B*) Serum LH levels were not different in P35 male WT and KERKO mice (n = 3–8). (*C*) Testes weights did not differ among adult male WT and KERKO mice (n = 32–35). Data are presented as mean ± SEM.