Supporting Information

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SI Materials and Methods

Development of the Mouse Mutant. *Gnrhr*^{E90Kneo} mice were generated by introducing a point mutation (G to A) into the first exon of the *Gnrhr* gene at position 313 by homologous recombination as described (1). For this study, the floxed *PGK-neo-pA* (*neo*) resistance cassette was not removed from the locus. All mice were bred on a C57BL/6; 129 mixed genetic background and fed ad libitum a sterilized standard rodent diet and sterilized water.

Genotyping. *Gnrhr*^{E90Kneo} mice were genotyped by two PCRs. PCR A uses primers L1 and R1 and produces a band size of 169 bp for the wild-type allele. PCR B uses primers L2 and R1 and produces a band size of 550 bp for the *E*^{90}*Kneo* allele. The primer sequences were L1, 5′-TCA GCA GTA GCC TTT AAC CCT GAC; L2, 5′-CGT GCT ACT TCC ATT TGT CAC G; R1, 5′-GGG GAA GAG GAT AGA GTC AGT TGT G.

Tissue Collection and Preservation. Tissues were collected at 90 d of age. Testes were weighed after trimming away the epididymis and fat. Depending on use, the testes and pituitary were either fixed in Bouin's fluid and stained by standard techniques or frozen in liquid N_2 and stored at −80 °C.

Breeding. *E*^{90}*Kneo*/*E*^{90}*Kneo* mice were maintained by breeding heterozygotes inter se.

Endocrine Responses and Endocrine Histology. Testosterone and luteinizing hormone (LH) values were means of individual determinations. Circulating levels of testosterone were determined by RIA on serum collected from individual animals (at least five determinations were averaged). Serum levels in mice were measured by ether extraction and RIA at the Endocrine Technology and Support Core Lab at the Oregon National Primate Research Center/Oregon Health and Science University (2). Briefly, samples for testosterone were extracted in 5 mL of ether in heated-treated 13 × 100 glass tubes. Hormonal values were corrected for extraction losses determined by radioactive tracer recovery at the same time with sample extraction; recovery is typically >90%. The sensitivity was 5 pg per tube for the testosterone RIA. The intra-assay and interassay variations were less than 10% and 15%, respectively. Cross-reaction of the antibody was testosterone, 100%; dihydrotestosterone, 59%; 5β-androstane-17β-ol-3-one, 4%; and 5α-androstane-3α-17β-diol, 3%. Six other related steroids were tested, and all had <1% cross-reactivity.

The LH RIA used a highly purified rat LH for iodination (obtained from National Institute of Diabetes and Digestive and Kidney Diseases) and a mouse reference preparation obtained as a kind gift from Al Parlow (Harbor-University of California, Los Angeles Hospital, Torrance, CA). LH antisera (C102) was prepared, characterized, and used as described (3).

Surgery and Infusion. For insertion of the catheter into 60-d-old *E*^{90}*Kneo*/*E*^{90}*Kneo* male mice, the midscapular region (MR), cranial ventral thorax, and neck were shaved, and a small skin incision was made in the MR. Mice were maintained in a surgical plane of anesthesia with isoflurane (1.5–2%) throughout the procedure. They were placed in dorsal recumbency, and a small skin incision was made in the midline of the neck. A loop of sterile polypropylene suture was tunneled s.c. from the MR to the incision on the neck. The osmohyoid muscle was separated longitudinally and retracted laterally to expose the left carotid artery. A portion of the artery, close to the thyroid gland, and below where it branches to internal and external carotid vessels, was isolated. Two 6-0 silk ligatures were placed caudally and cranially. A small vascular clamp was placed as far cranially as possible, below the branching. Once the vessel expanded with blood, the caudal ligature was tied, ligating the vessel. A 25-gauge needle, bent ∼45° so that the effective angle is ∼135°, was used to make an incision in the vessel between the ligatures into which the catheter was inserted. The vascular clamp was removed and the catheter advanced. The catheter was secured by tying the cranial ligature around the catheterized vessel, and patency was confirmed. The caudal ligating suture was also secured around the catheter. A drop of tissue adhesive (VetBond) was placed on the catheterization site. The catheter was run through the previously placed polypropylene suture loop, and the loop was then pulled from the midscapular incision site, pulling the catheter with it, tunneling the catheter from the entry site to the exit site. The position of the catheter was checked before closing the ventral incision site. A single suture was also used to close the midscapular incision if needed, and a drop of tissue adhesive was placed there as well. Patency was tested, and the catheter was filled with a locking solution and sealed with a stainless steel plug. Postoperative Carprofen was delivered s.c. before surgery and again 24 h later as needed for analgesia. The animal was attached to an infusion system (Instech) and placed in a cage designed for the equipment. The 60-d-old male animal was first infused for 45–48 h with heparin (1 U/mL) saline at a rate of 25 μL/h (which is constant for all studies) before the start of the experimental period (infusion with drugs) by using two Harvard infusion pumps. IN3 was infused for 30 d with the indicated frequency (pulses per day) and duration of dose (h).

Morphology. Pituitaries and testes were fixed by immersion in Bouin's fluid, dehydrated through an ethanol gradient, and embedded in paraffin wax by standard methods. Hematoxylin/eosin (H&E) staining was performed by standard methods on 5-μm sections. A single large image comprising most, if not all, of each tissue section was captured at 200× magnification with a Nikon Ti-E microscope by using the large image feature of NIS Elements software (Nikon). Spermatogenic activity was assayed by seminiferous tubule diameter and the presence of elongated spermatids in testis cross-sections. Seminiferous tubule diameters were measured by using the length measurement feature in the NIS Elements. Only internal tubules were selected for measurement. The shortest diameter of each tubule was chosen for measurement because the tubules are rarely perfect circles in tissue sections. Depending on the availability of tissue, 6–41 measurements were made per animal and averaged to generate a mean seminiferous tubule diameter for each animal, which was treated as $n = 1$. Six to 12 animals were assayed per group (wild type, $n = 6$; *E*^{90}*Kneo*/+, $n = 12$; *E*^{90}*Kneo*/*E*^{90}*Kneo*, $n = 10$; IN3, $n = 13$). Elongated spermatid production was classified into three categories. "None/extremely few" were those with less than five elongated spermatids in a single 200× image. "Some" were those with a clear reduction in the number of elongated spermatids compared with wild type. "Abundant" were those that appeared indistinguishable from wild type.

Real-Time PCR. Total RNA was extracted from whole pituitaries or testes by using TRIzol reagent according to the manufacturer's protocol (Invitrogen). mRNA levels were measured by using TaqMan gene expression assays (Mm00439143_m1 for *Gnrhr*, Mm00515131_m1 for Hsd17b3) from Applied Biosystems (Life

Technologies). The level of *Gapdh* mRNA was used for normalization. The PCR was run by using an ABI Prism 7900HT thermocycler and SDS2.1 software (Applied Biosystems). Data were analyzed by the comparative ΔΔCT method.

Immunoblotting. Immunoblotting studies were carried out by using total cellular protein. After removing the tunica albuginea, each testis was sonicated in 50 mM Tris at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitors, then centrifuged at 10,000 × g for 10 min. Fifty micrograms of supernatant protein was solubilized in sample buffer and loaded onto 10% (mass/vol) SDS/PAGE (Mini Protean II System, Bio-Rad Laboratories). The proteins were transferred onto Immuno-Blot PVDF membranes (Bio-Rad). Membranes were probed with primary antisera that recognize StAR [1:20,000 (4), CYP11A1 (1:4,000, Chemicon) (5)]; and β-actin (1:3,000, Applied Biosystems/Ambion). Following overnight incubation at 4 °C with primary antisera, membranes were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antisera for 1 h at room temperature. Membranes were washed again, and immunodetection of different proteins was determined with a Chemiluminescence Imaging Western Lightning Kit (Perkin-Elmer).

Sperm Assessment and IVF. Pregnant mare serum gonadotropin (PMSG) and human CG (hCG) were purchased from the National Hormone and Peptide Program. Mouse sperm recovery, capacitation, and in vitro fertilization (IVF) were carried out as described (6). Briefly, cauda epididymides were dissected from adult male mice, and sperm was isolated into a 0.2-mL drop of human tubal fluid (HTF; catalog no. MR-070-D, Millipore) and preincubated at 37 °C, 5% CO_2 for 2 h to allow for capacitation. A portion of sperm was analyzed for each: total count, viability, motility, forward progression, and general morphology.

Mature oocytes were flushed from B6D2F1 females (C57BL/6×DBA/2) following standard superovulation with PMSG and hCG. Cumulus cells were dispersed with 0.1% hyaluronidase treatment, and oocytes were placed into a 0.25-mL HTF fertilization drop. Sperm was then added to the drop at the concentration 10^6 sperm per mL, and sperm with oocytes were incubated for 5 h. Next, 15–20 oocytes were placed into a 20-μL drop of KSOM medium (catalog no. MR-020P-5F; Millipore) and cultured at 37 °C, 5% CO_2 overnight. Pronuclear formation and cleavage was recorded to determine fertilization the next day after IVF. Embryos were cultured to blastocysts and then surgically transferred into uteri of pseudopregnant random breeding stock females following standard protocols.

Statistical Analyses. For experiments with more than two experimental groups, quantitative data were subjected to one-way analysis of variance (ANOVA) by using MedCalc v12.4.0 software (MedCalc Software). Before ANOVA, Levene's Test for Equality of Variances was performed. If the Levene test was positive ($P < 0.05$), a logarithmic transformation was applied to the data. If the Levene test was still positive by using log transformed data, the nonparametric Kruskal–Wallis test was performed. If the ANOVA was positive ($P < 0.05$), a post hoc Student–Newman–Keuls test was performed for pairwise comparison of subgroups. For experiments with two experimental groups, a *t* test was performed. A Pearson's χ^2 test was used to compare differences in categorical data (e.g., presence of elongated spermatids). $P < 0.05$ was considered significant for all tests.

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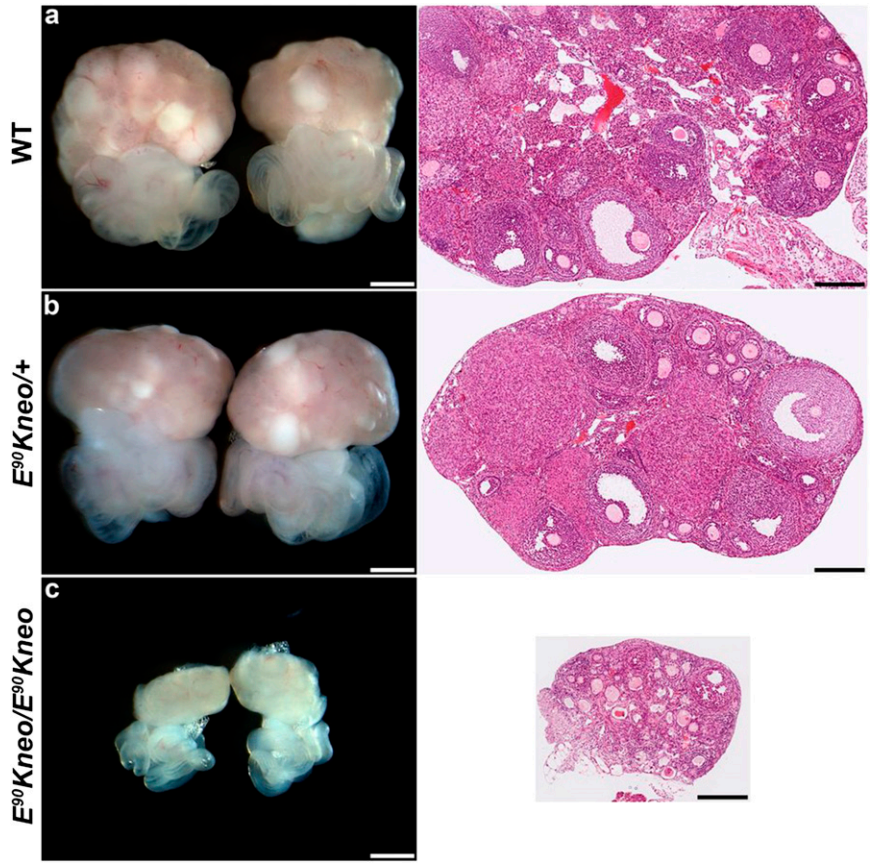

Fig. S1. Phenotype of E^{90}Kneo heterozygous and homozygous female mice. Ovaries from mice of the indicated genotypes were collected at 90 d of age, imaged with a stereomicroscope, and processed for histology (H&E staining). Wild-type (*A*) and E^{90}Kneo/+ (*B*) ovaries were indistinguishable. Both exhibited similar size, presence of follicles at all stages of development, and presence of corpora lutea. The ovaries of E^{90}Kneo/E^{90}Kneo females (*C*) were smaller and exhibited no follicular development past the secondary follicle stage. The stereomicroscope image is shown on *Left*, and the H&E-stained sections are on *Right*. (Scale bars: *A–C Left*, 0.5 mm; *A–C Right*, 0.2 mm.)

Table S1. Evaluation of spermatids

Presence of elongated spermatids in H&E sections	WT	K^{90}neo/+	K^{90}neo/K^{90}neo	K^{90}neo/K^{90}neo + IN3
None/very few, %	0	0	30.8*	0
Some, %	0	0	15.4*	0
Abundant, %	100	100	53.8*	100
No. of animals	7	15	13	13

The frequency of elongated spermatids was determined in WT, homozygous and heterozygous *E^{90}Kneo* male mice, and in IN3-rescued *E^{90}Kneo* homozygotes. Elongated spermatid production was classified into three categories. "None/very few" were those with less than five elongated spermatids in a single 200x image. "Some" were those with a clear reduction in the number of elongated spermatids compared with wild type. "Abundant" were those that appeared quantitatively indistinguishable from wild type. Forty-six percent of *E^{90}Kneo/E^{90}Kneo* males exhibited reduced numbers of elongated spermatids. In contrast, 100% of *E^{90}Kneo/E^{90}Kneo* males treated with IN3 exhibited normal numbers of elongated spermatids. Pearson's χ^2 test was used to test for significant differences between the frequencies of the different classification levels. Categorical frequencies observed for K^{90}neo/K^{90}neo were significantly different from that of the other groups (*$P = 0.005$).