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Kiss1$^{-/-}$ Mice Exhibit More Variable Hypogonadism than *Gpr54*$^{-/-}$ Mice

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The G protein-coupled receptor Gpr54 and its ligand metastin (derived from the *Kiss1* gene product kisspeptin) are key gatekeepers of sexual maturation. *Gpr54* knockout mice demonstrate hypogonadotropic hypogonadism, but until recently, the phenotype of *Kiss1* knockout mice was unknown. This report describes the reproductive phenotypes of mice carrying targeted deletions of *Kiss1* or *Gpr54* on the same genetic background. Both *Kiss1* and *Gpr54* knockout mice are viable but infertile and have abnormal sexual maturation; the majority of males lack preputial separation, and females have delayed vaginal opening and absence of estrous cycling. *Kiss1* and *Gpr54* knockout males have significantly smaller testes compared with controls. *Gpr54* knockout females have smaller ovaries and uteri than wild-type females. However, *Kiss1* knockout females demonstrate two distinct phenotypes: half have markedly reduced gonadal weights similar to those of *Gpr54* knockout mice, whereas half exhibit persistent vaginal cornification and have gonadal weights comparable with those of wild-type females. FSH levels in both *Kiss1* and *Gpr54* knockout males and females are significantly lower than in controls. When injected with mouse metastin 43–52, a Gpr54 agonist, *Gpr54* knockout mice fail to increase gonadotropins, whereas *Kiss1* knockout mice respond with increased gonadotropin levels. In summary, both *Kiss1* and *Gpr54* knockout mice have abnormal sexual maturation consistent with hypogonadotropic hypogonadism, although *Kiss1* knockout mice appear to be less severely affected than their receptor counterparts. *Kiss1* knockout females demonstrate a bimodal phenotypic variability, with some animals having higher gonadal weight, larger vaginal opening, and persistent vaginal cornification. (*Endocrinology* 148: 4927–4936, 2007)

PROGRESSIVE INCREASE IN the neurosecretory activity of the GnRH system is the defining neuroendocrine and hormonal event for pubertal maturation. GnRH stimulates the gonadotrophs of the pituitary to release LH and FSH, which in turn stimulate gametogenesis and the production of sex steroids. The physiological mechanisms that drive GnRH secretion at the time of sexual maturation have been difficult to identify.

In 2003, the G protein-coupled receptor Gpr54 was discovered as a key regulator of pubertal development through genetic approaches in families with hypogonadotropic hypogonadism, a condition characterized by the absence of spontaneous sexual maturation in the face of inappropriately low gonadotropins (1, 2). Mice carrying targeted deletions for *Gpr54* also demonstrate failure of adult sexual development (2, 3). Gpr54 is a member of the rhodopsin family of G protein-coupled receptors and is activated by endogenous peptides derived from a precursor protein named kisspeptin (4–6). The longest of these peptides is kisspeptin 68–121 (human numbering), also called metastin because of its ability to suppress metastatic potential in melanoma and breast cancer cell lines (7).

Although a handful of patients with hypogonadotropic hypogonadism and mutations in *GPR54* have been described, no inactivating mutations in the kisspeptin gene (*KISS1*) have yet been reported. Until recently, there was no loss-of-function mouse model available for *in vivo* analysis of the role of kisspeptin (16). To determine whether kisspeptin knockout mice have a distinct phenotype from *Gpr54* knockout mice (2, 3), we generated and characterized in parallel mice carrying targeted deletions of *Gpr54* and *Kiss1*.

Materials and Methods

Generation of Kiss1 and Gpr54 knockout mice

Knockout mice were created in collaboration with the Harvard Partners Center for Genetics and Genomics core facility. The strategy for disrupting the *Kiss1* gene was to replace exon 1 (120 bp) with the neomycin/G418 resistance cassette (Neo) (Fig. 1). Similarly, exon 2 (125 bp) was replaced for *Gpr54*. The targeting vectors were electroporated into the 129/S line of mouse embryonic stem (ES) cells. To screen the ES cells for homologous recombination of *Kiss1* and *Gpr54* alleles, DNA was isolated and PCR performed with primers complementary to sequences outside and inside the targeted region using the Expand High Fidelity PCR System (Roche, Indianapolis, IN). Primer sequences are published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. The positive colonies were verified by Southern blot analysis of genomic DNA with probes designed to confirm single recombination events.

ES cells from positive colonies were injected into C57BL/6J blastocysts, which in turn were implanted into pseudopregnant foster mice.

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Abbreviations: ES, Embryonic stem; RACE, rapid amplification of cDNA ends; WT, wild type.

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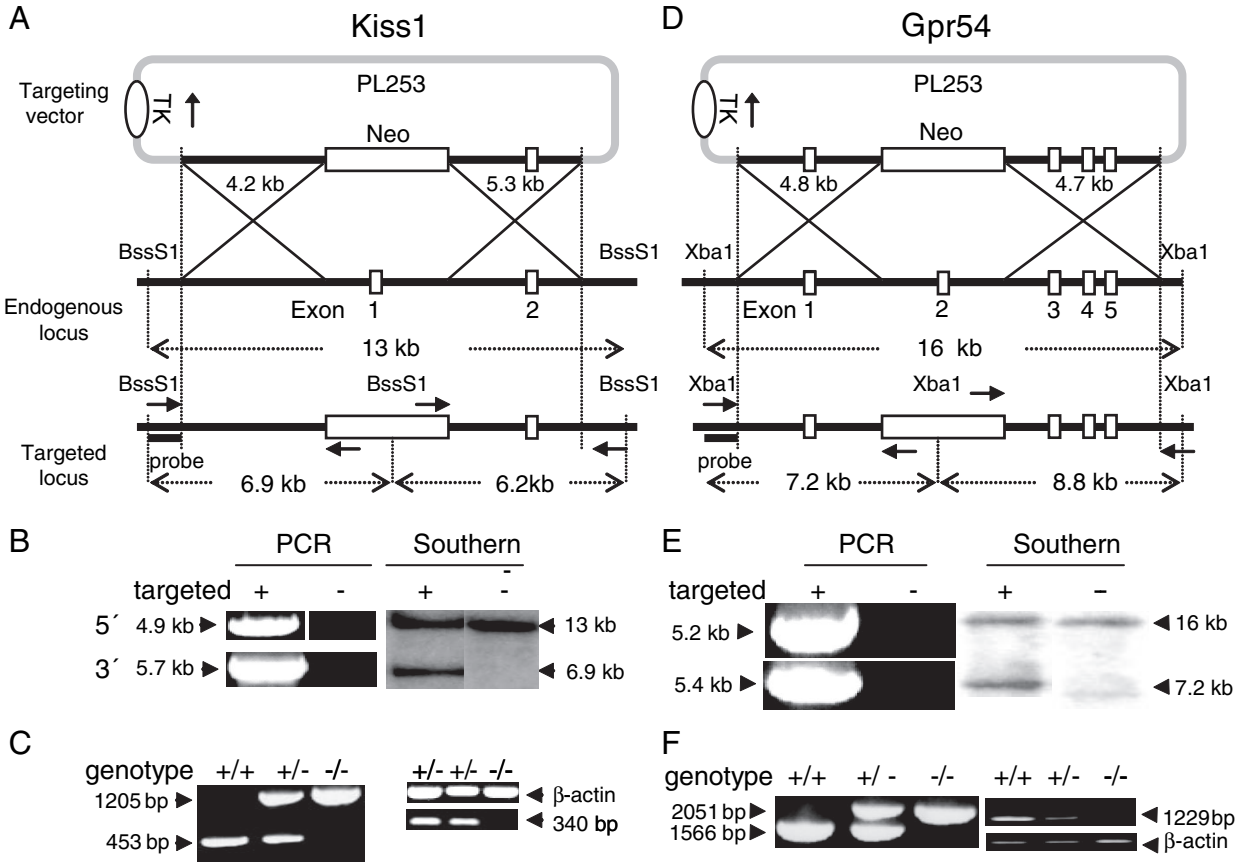

FIG. 1. Targeted disruption of *Kiss1* and *Gpr54* genes. A and D, Schematic diagrams of the targeting vectors, the endogenous loci, and the disrupted loci of *Kiss1* (A) and *Gpr54* (D). B and E, Screening of ES cells by PCR using one primer located outside of the 5′ or the 3′ end of the targeting vector and a second primer in the Neo gene (*arrows*) generates 4.9- and 5.7-kb products in the targeted *Kiss1* ES cells (B) and 5.2- and 5.4-kb products in the targeted *Gpr54* ES cells (E). Southern blot analysis of *Bss*S1- or *Xba*1-digested DNA using a probe outside of the targeting vector detected 6.9- and 13-kb fragments (B) or 7.2- and 16-kb fragments (E) in the targeted locus and in the endogenous locus, respectively. C and F, PCR amplified a fragment of 453 bp in WT (+/+) mice, fragments of 453 and 1205 bp in heterozygous (+/−) mice, and a fragment of 1205 bp in *Kiss1* mutant (−/−) mice (C) and a fragment of 1566 bp in WT (+/+) mice, fragments of 1566 and 2051 bp in heterozygous (+/−) mice, and a fragment of 2051 bp in *Gpr54* mutant (−/−) mice (F). RT-PCR using one primer in exon 1 and a second primer in the last exon of the targeting gene amplified a 340-bp fragment or 1229-bp fragment in both WT (+/+) and heterozygous (+/−) mice but not in *Kiss1* (C) or *Gpr54* (F) mutant (−/−) mice, respectively. β-Actin was used as a control.

Resulting chimeric males were crossed with 129/S1/SvImJ females for germline transmission of the mutated alleles. The resulting male and female heterozygotes were bred to generate wild-type (WT), heterozygous, and knockout animals for each targeted deletion. Genotyping was performed with genomic DNA isolated from a piece of tail using the DNeasy Tissue Kit (QIAGEN, Valencia, CA) and PCR with primers listed in supplemental Fig. 1.

Animal housing

All mice were kept in cages of one to five animals and housed under a 12-h light cycle and controlled temperature in the experimental animal facility of the Massachusetts General Hospital Center for Comparative Medicine. Food and water were available *ad libitum*. All procedures were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital.

Rapid amplification of cDNA ends (5′-RACE) analysis

The DNeasy kit (QIAGEN) was used to isolate cDNA from hypothalami of *Kiss1* knockout and WT mice. The SMART 5′-RACE kit (Clontech, Mountain View, CA) was used to amplify the cDNA of *Kiss1* transcription products using a primer in the last exon of *Kiss1* (primer sequence in supplemental data). The products of RACE were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) for sequence analysis.

Immunohistochemistry for Kiss1 knockout mice

Kiss1 knockout and WT mice were anesthetized with ketamine and xylazine and perfused with 2.5% acrolein plus 4% paraformaldehyde in phosphate buffer. After perfusion, the brain was removed from the skull and sunk in 30% aqueous sucrose solution. Sections were cut at 28 μm with a freezing microtome into cryoprotectant antifreeze solution (8) into 12 serial series and stored at −20 C until they were processed. The kisspeptin antibody was the gift of Dr. Alain Caraty and Dr. Isabella Franceschini-Laurent, Unite de Physiologie de la Reproduction et des Comportementes, University of Tours, France (9), and was used at a dilution of 1:300,000. Staining was performed on a one-in-six series of sections using conventional avidin-biotin complex techniques as described previously (10) and visualized using a nickel sulfate-diaminobenzidine chromogen solution. After reaction with the chromogen solution for 15 min, the sections were rinsed in acetate solution followed by PBS, placed into normal saline, mounted onto glass slides, dried overnight, dehydrated, and coverslipped. Sections were examined with a Nikon E800 microscope, photographed using a Retiga-EX cooled CCD digital camera, and captured onto a Macintosh G4 computer using I Vision-Mac (BioVision Technologies, Exton, PA).

Phenotyping

Details of births, including litter size, abnormalities, and neonatal deaths, were recorded. The mice were weaned and genotyped at 3 wk of age and then weighed and inspected two to three times per week. Sexual maturation was assessed by inspecting the presence or absence of vaginal opening (females) or preputial separation (males) and by measuring the anogenital distance (males) (11). Vaginal smears were obtained using a calcium alginate swab wetted with PBS, applied to a slide, fixed with 95% ethanol, and then stained using the Hema3 system (Fisher Scientific, Pittsburgh, PA). For fertility assessment, a knockout animal of each gender was placed in a cage with a WT mouse of the opposite gender and of proven fertility, and females were observed for evidence of pregnancy.

The 9- to 16-wk-old animals were weighed, inspected, and then killed by asphyxiation with carbon dioxide. Some mice received a single injection of 50 nmol C-terminally amidated mouse kisspeptin 110–119 (corresponding to metastin 43–52 and also known as kisspeptin-10) in PBS sc 30 min before being killed. Some mice were injected with the vehicle only. Blood was obtained by cardiac puncture, and organs were removed, inspected, and weighed. All phenotyping experiments were done without the knowledge of the genotype.

Tissue preparation and analysis

Serum was stored at −80 C. For sperm analysis, a single epididymis was dissected, weighed, and diced in 0.5 ml PBS with 4 mg/ml BSA and then incubated at room temperature. Ten microliters were applied to a hemocytometer for sperm to be counted. A separate sample was examined on a Petri dish for sperm motility.

Tissues for histology were fixed in Bouin's solution (testes and epididymides) or 4% paraformaldehyde in PBS (other tissues) and then stored in 70% ethanol until processing. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Rodent Histopathology Core facility at Harvard Medical School. Samples for RNA analysis were stored in RNAlater (Ambion, Austin, TX) or snap frozen and stored at −80 C.

Hormone assays

All hormone assays were performed by the Ligand Assay Core of the Specialized Cooperative Center for Research in Reproduction at the University of Virginia. The reportable range of the LH assay was 0.04–37.4 ng/ml, and the intra and interassay coefficients of variation were less than 7 and 9%. The reportable range of the FSH assay was 3.4–35.9 ng/ml, and the intra and interassay coefficients of variation were less than 8 and 10%. The reportable range of the testosterone assay was 7.0–801.0 ng/dl, and the intra and interassay coefficients of variation were less than 7 and 9%.

Statistical analysis

All data are reported as mean ± SD unless otherwise stated. Differences between groups of heterozygous and WT animals were analyzed with ANOVA. No significant differences were observed, and no *post hoc* analyses were performed. In contrast, differences between groups of homozygous mutant and WT animals were analyzed with the unpaired Mann-Whitney *U* test because of the nonnormal distribution of data. The χ^2 test was used in the analysis of vaginal opening and in the analysis of sex and genotype ratios. To avoid false positives, Bonferroni correction was applied when multiple testing between groups occurred. The *P* values reported are the corrected *P* values, and *P* values < 0.05 are considered significant. Comparison is with the WT group of the same sex unless otherwise stated. All mice were treated as independent of littermate relationships.

Results

Targeted disruption of Kiss1 and Gpr54

Constructs targeting the first exon of *Kiss1* and the second exon of *Gpr54* for replacement with the Neo cassette were created and used for homologous recombination in ES cells (Fig. 1). PCR and Southern blotting confirmed that recombinant cells carried the targeted deletion with no unintended insertions of the targeting construct. Recombinant cells were injected into blastocysts and the resulting chimeras were used to generate mice heterozygous for the targeted deletion. Heterozygote mice of both lines have litters at the same frequency and of the same size as WT 129 mice.

RT-PCR using primers directed against exon 2 of *Kiss1* did amplify a transcript in *Kiss1* knockout mice. To characterize this transcript, RACE and sequencing of *Kiss1* transcripts were performed on WT and *Kiss1* knockout mice. Transcripts isolated from hypothalami of WT mice revealed a new exon that lies 5′ to the previously known exon 1, with two splice acceptor sites within the previous exon 1 resulting in two transcripts with slightly differing 5′ untranslated regions but identical coding regions (supplemental data). Twenty RACE products isolated from the hypothalami of *Kiss1* knockout mice were the same size. Five were sequenced and found to be identical. This transcript consists of the newly identified 5′-most exon spliced to exon 2. This transcript contains no in-frame translation start site and is therefore predicted to be incapable of producing functional kisspeptin.

To further confirm loss of kisspeptin expression in the *Kiss1* knockout animals, immunohistochemistry was performed on hypothalamic sections with an antibody against kisspeptin. Kisspeptin was clearly expressed in WT hypothalamic slices in the arcuate and anteroventral periventricular nuclei, consistent with previous descriptions (12–14), but no staining was seen in hypothalamic slices of *Kiss1* knockout mice (Fig. 2).

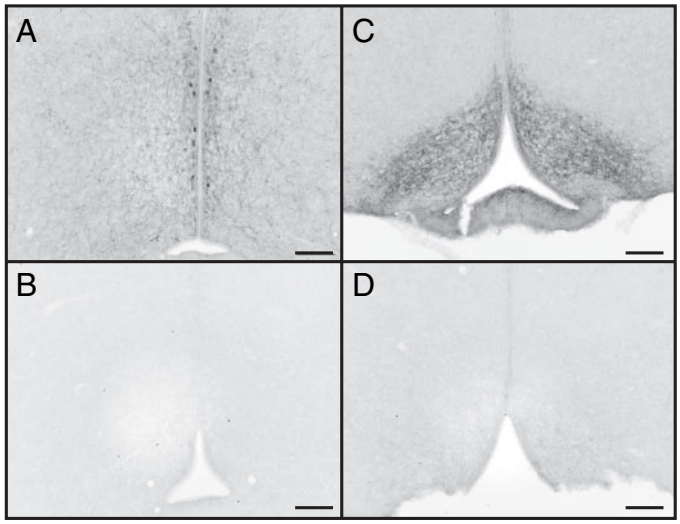

FIG. 2. Immunohistochemistry of kisspeptin in the hypothalamus. A–D, Immunocytochemical localization of kisspeptin in WT (A and B) and knockout (C and D) mice. A micrograph of the anteroventral periventricular nuclei of a female WT mouse shows the cells for kisspeptin as well as abundant axons within the periventricular region (A) (males have a few neurons and fewer axons, data not shown). In the same region of the *Kiss1* knockout mouse, no kisspeptin is detected (B). In the arcuate nucleus, the abundant axons for kisspeptin are easily seen in both female (C) and male WT mouse. *Kiss1* knockout mice show absence of kisspeptin immunoreactivity in the arcuate (D). *Bar*, 100 μm.

Characterization of Kiss1 and Gpr54 null mice

Kiss1 and *Gpr54* null mice are viable. Knockout adult animals display normal feeding and motor activity; mating behavior was not observed in any cages during routine animal husbandry. Sexual assignment was unambiguous for homozygous mutants of both targeted genes, and males and females were born in expected ratios (supplemental data). *Kiss1* heterozygous crosses produced offspring in expected Mendelian ratios, but *Gpr54* heterozygous crosses produced fewer *Gpr54* homozygous offspring than expected, probably due to a slight skewing of gamete ratios (supplemental data).

The body weight of *Gpr54* knockout males, but not *Kiss1* males, was slightly but significantly less than that of WT males at 9–12 wk (*Kiss1*$^{-/-}$ 22.1 ± 2.3 g, nonsignificant; *Gpr54*$^{-/-}$ 20.4 ± 1.8 g, $P < 0.001$; WT 23.4 ± 2.3 g; Table 1). In contrast, the body weights of *Kiss1* and *Gpr54* knockout females were not significantly different from controls (*Kiss1*$^{-/-}$ 20.2 ± 1.8 g, *Gpr54*$^{-/-}$ 21.6 ± 3.5 g, WT 19.4 ± 1.8 g; Table 1).

Underdevelopment of the genitalia was the only gross abnormality noted on external examination of adult knockout mice. Males of both knockout lines largely lacked preputial separation (present in two of nine of 7- to 31-wk-old *Kiss1*$^{-/-}$ males and zero of eleven of 8- to 28-wk-old *Gpr54*$^{-/-}$ males) and had shorter anogenital distances than WT males (*Kiss1*$^{-/-}$ 13 ± 1 mm, $P < 0.001$; *Gpr54*$^{-/-}$ 13 ± 1 mm, $P < 0.001$; WT 17 ± 2 mm; Fig. 3B and Table 1). Heterozygous males were indistinguishable from WT males, and preputial separation always occurred by 5 wk of age. Both showed preputial separation as expected. Anogenital distance, a marker of androgen exposure (11), in both lines of knockout male mice diverged from that of WT animals early in the course of sexual maturation (d 23–25 of life) (Fig. 3B).

In females, vaginal opening was significantly delayed in both lines (*Kiss1*$^{-/-}$ $P < 0.005$; *Gpr54*$^{-/-}$ $P < 0.001$, Fig. 3D). The delayed vaginal opening of the knockout female mice was not attributable to differences in body weight, because both WT and knockout animals followed the same weight trajectory (Fig. 3C). Some animals not included in the study shown in Fig. 3D had no vaginal opening even by 9–13 wk of age, but most animals ultimately achieved this milestone (Fig. 3D). The vaginal orifices of the adult knockout animals were often small and round compared with those of WT or heterozygous animals. Dye injection confirmed normal anatomic connectivity to the uterus. Vaginal smears of 9- to 11-wk-old *Kiss1* and *Gpr54* knockout female mice showed absence of estrous cycling. Nearly all *Gpr54* null mice and almost half of *Kiss1* knockout mice had little cellularity on vaginal smears. However, more than half of the *Kiss1* knockout mice and one *Gpr54* knockout mouse exhibited persistent vaginal cornification; these animals had vaginal openings that resembled those of WT females.

At necropsy, internal organs of adult mice (heart, lungs, liver, kidney, spleen, stomach, pancreas, and gut) had similar weights and appearance irrespective of genotype. Knockout males had small testes in comparison with WT mice (*Kiss1*$^{-/-}$ testis 39.0 ± 14.8 mg, $P < 0.001$; *Gpr54*$^{-/-}$ testis 24.2 ± 10.8 mg, $P < 0.001$; WT testis 141.0 ± 17.8 mg; Fig. 4A and Table 1). Furthermore, the reduction was significantly more severe in *Gpr54* males than in *Kiss1* males ($P < 0.05$). Sex-hormone-dependent organs such as seminal vesicles and preputial glands were small and sometimes difficult to detect. Heterozygous animals were not significantly different from WT animals. The female reproductive hemi-block (half of the uterus, one oviduct, and one ovary) was significantly smaller in *Gpr54* knockout females compared with WT females but not in *Kiss1* knockout females when compared with WT females as a group (*Kiss1*$^{-/-}$ 39.1 ± 27.0 mg, nonsignificant; *Gpr54*$^{-/-}$ 20.1 ± 23.4 mg, $P < 0.001$; WT 48.5 ±

TABLE 1. Data for all mice grouped by genotype

	Gpr54$^{-/-}$		*Gpr54*$^{+/-}$		WT		*Kiss1*$^{+/-}$		*Kiss1*$^{-/-}$	
	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	n
Females										
Body weight (g)	21.6 ± 0.9	17	19.8 ± 0.5	16	19.4 ± 0.3	27	18.8 ± 0.5	18	20.2 ± 0.5	14
Gonadal weight (mg)	20.1 ± 6.0[a]	15	47.1 ± 3.7	16	48.5 ± 3.6	26	54.1 ± 6.3	18	39.1 ± 7.2	14
VO age (d)	36.6 ± 5.4[a]	9	28.7 ± 0.5	15	28.1 ± 0.7	16	28.5 ± 0.5	17	35.5 ± 2.0[a]	8
LH (ng/ml)	0.4 ± 0.2	9	0.3 ± 0.3	9	0.2 ± 0.0	18	1.2 ± 1.0	9	0.2 ± 0.1	8
Stim. LH (ng/ml)	0.1 ± 0.0[a,b]	6	5.5 ± 1.0	7	4.3 ± 1.0	6	7.1 ± 1.0	9	6.9 ± 2.2[b]	6
FSH (ng/ml)	3.3 ± 0.6[a]	8	9.7 ± 3.0	8	6.0 ± 0.6	16	5.7 ± 0.9	7	3.8 ± 0.9	8
Stim. FSH (ng/ml)	1.4 ± 0.3[a,b]	5	12.7 ± 2.4	6	13.6 ± 4.9	5	8.0 ± 1.6	9	19.2 ± 5.5[b]	6
Males										
Body weight (g)	20.4 ± 0.4[a]	22	23.4 ± 0.6	17	23.4 ± 0.6	16	22.8 ± 0.5	17	22.1 ± 0.6	17
Gonadal weight (mg)	24.2 ± 2.3[a,b]	22	143.8 ± 2.9	17	141.0 ± 4.4	16	153.5 ± 4.5	17	39.0 ± 3.6[a,b]	17
AGD (mm)	12.9 ± 0.2[a]	22	16.9 ± 0.4	17	17.3 ± 0.4	16	17.1 ± 0.2	17	12.8 ± 0.3[a]	17
LH (ng/ml)	0.1 ± 0.0[a]	10	0.1 ± 0.0	7	0.2 ± 0.1	9	0.2 ± 0.1	8	0.1 ± 0.0	8
Stim. LH (ng/ml)	0.1 ± 0.1[a,b]	11	7.0 ± 0.4	7	6.2 ± 0.6	6	6.7 ± 0.7	8	8.1 ± 1.0[b]	7
FSH (ng/ml)	1.5 ± 0.2[a]	9	17.0 ± 2.4	7	20.5 ± 3.3	7	19.4 ± 2.3	8	1.9 ± 0.4[a]	8
Stim. FSH (ng/ml)	1.5 ± 0.1[a,b]	11	29.5 ± 4.4	7	29.7 ± 4.6	6	32.1 ± 2.5	8	9.1 ± 0.7[a,b]	7
T (ng/dl)	19.4 ± 7.3[a,b]	9	130.1 ± 76.7	7	155.1 ± 62.2	8	130.0 ± 30.4	8	90.9 ± 21.5[b]	8
Stim. T (ng/dl)	35.2 ± 7.4[a,b]	10	4027 ± 285	7	3848 ± 369	6	4530 ± 241	5	437 ± 107[a,b]	7

Gonadal weight is the average of testis weights for males and average of weights for genital hemiblock (ovary, oviduct, and half of uterus) for females. AGD, Anogenital distance; Stim., animals stimulated 30 min before being killed with 50 nmol metastin C-terminal decapeptide sc; T, testosterone; VO, vaginal opening.

[a] Statistically significant difference (corrected $P < 0.05$) when compared with the WT group.

[b] Statistically significant difference (corrected $P < 0.05$) between *Gpr54* and *Kiss1* knockout mice.

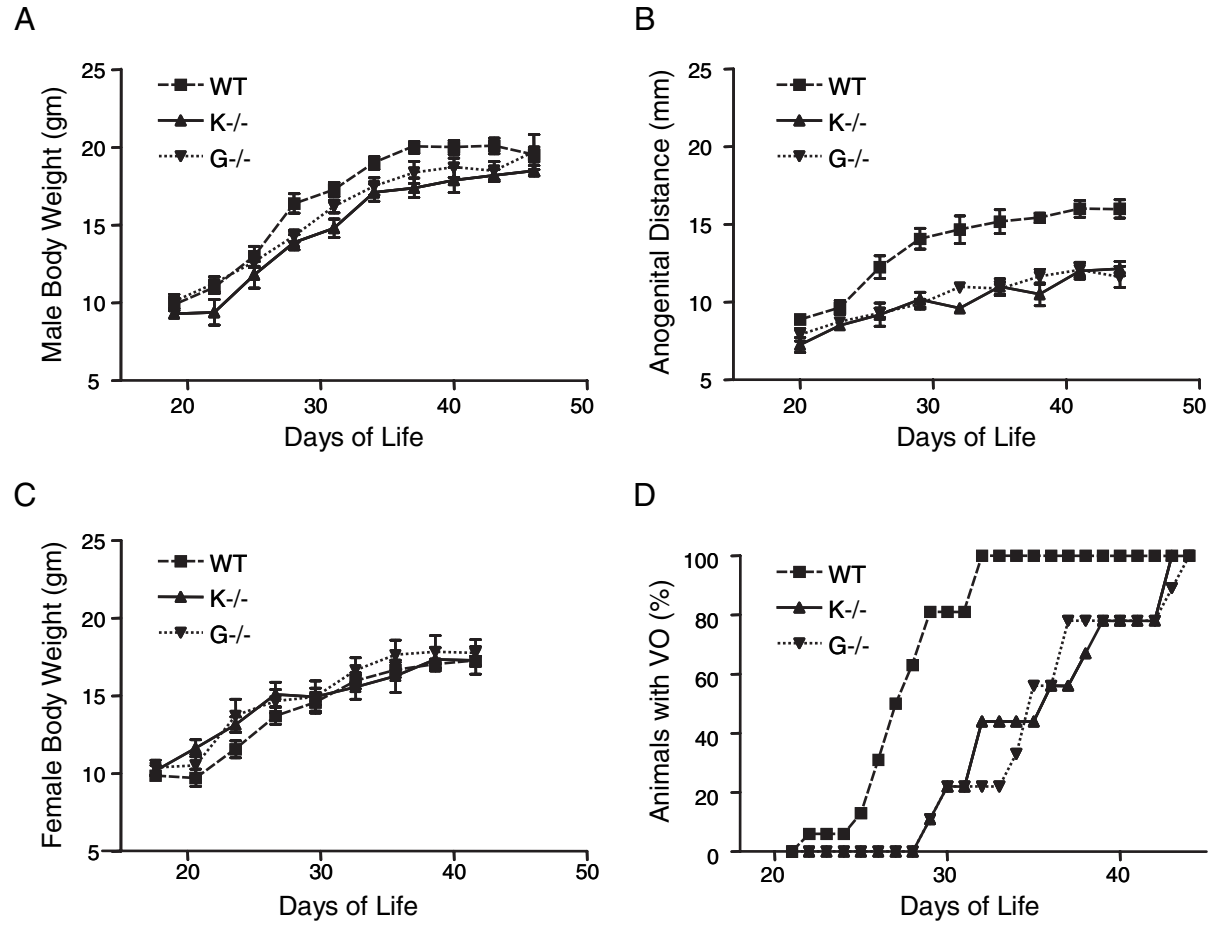

FIG. 3. Maturation in the *Kiss1* and *Gpr54* mutant mice. A, Body weight development in male mice; B, anogenital distance development in male mice; C, body weight development in adult female mice; D, time course of vaginal opening (VO) in female mice. G−/−, *Gpr54* knockout; K−/−, *Kiss1* knockout. Values are mean ± SD.

18.3 mg; Fig. 4B and Table 1). The gonadal weight of *Kiss1* knockout females followed a bimodal distribution. One group had a mean gonadal weight of 11.6 ± 2.3 mg, similar to *Gpr54* knockout females and significantly different from WT females ($P < 0.001$). The other group had a mean gonadal weight of 59.8 ± 14.8 mg, similar to that of WT females. *Kiss1* and *Gpr54* heterozygotes were not significantly different from WT animals.

Gonadal histology

The seminiferous tubules and interstitial cells of null males of both lines had normal histological architecture. However, although early stages of spermatogenesis appeared normal, mature spermatozoa were either absent or present in reduced numbers in mutant mice of both lines (Fig. 5, A–F). Leydig cell density was not overtly different between knockout and WT lines. Because the testes of knockout animals were significantly smaller, this suggests that total Leydig cell number was reduced in the null animals. Epididymides of knockout males contained less sperm and more nonsperm cells than epididymides of WT or heterozygous males (Fig. 5, A–F).

Ovaries from knockout females of both lines displayed follicles up to the antral stage of development, but no preovulatory follicles or corpora lutea were seen. In addition, many atretic follicles were observed in mutant ovaries (Fig. 5, G–L). *Kiss1* knockout females with persistent vaginal cornification had larger ovaries that contained multiple large cysts, but no sign of ovulation was seen (Fig. 5, G–L).

Serum gonadotropin levels with and without kisspeptin stimulation

Baseline levels of LH and FSH were obtained at dissection of adult mice. In both sexes and both lines, FSH was reduced in knockout animals compared with WT mice (males: *Kiss1*$^{-/-}$ 1.9 ± 1.2 ng/ml, $P < 0.001$; *Gpr54*$^{-/-}$ 1.5 ± 0.5 ng/ml, $P < 0.001$; WT 20.5 ± 8.8 ng/ml; females: *Kiss1*$^{-/-}$ 3.8 ± 2.5 ng/ml, nonsignificant; *Gpr54*$^{-/-}$ 3.3 ± 1.8 ng/ml, $P < 0.05$; WT 6.0 ± 2.3 ng/ml; Fig. 6, C and D, and Table 1). However, only *Gpr54* knockout males had significantly reduced LH levels (*Gpr54*$^{-/-}$ 0.05 ± 0.02 ng/ml, $P < 0.001$; WT 0.20 ± 0.16 ng/ml). *Kiss1* knockout males and knockout females of both lines had LH levels that were not statistically significantly different from WT (Fig. 6, A and B, and Table 1). Gonadotropin levels did not differ significantly between WT and heterozygous mice.

Administration of a peptide that consists of the C-terminal 10 amino acids of metastin is a powerful stimulus for GnRH-

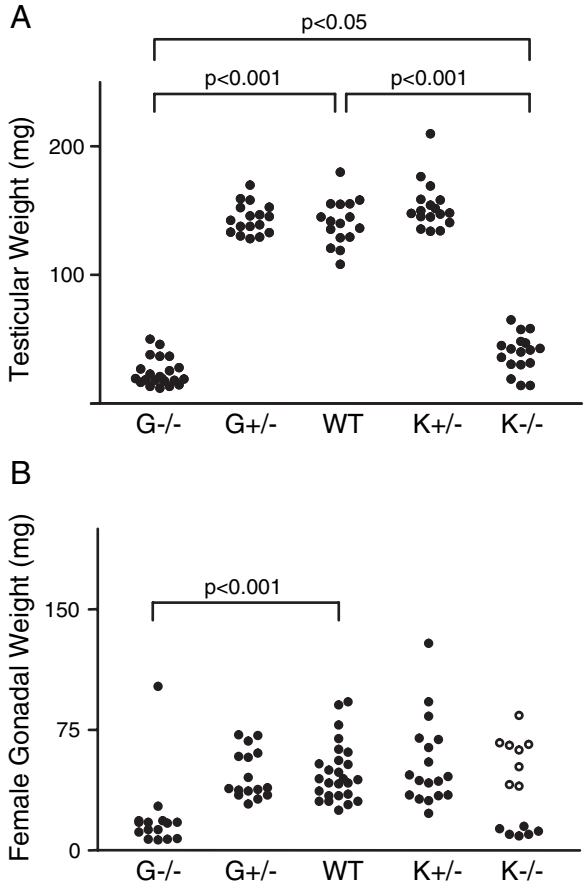

FIG. 4. Gonadal weight. A, Testicular weights (average of left and right testis weight was used for each animal). B, Weight of female reproductive hemi-block (ovary, oviduct, and half of uterus) (average of left and right used for each animal). G−/−, *Gpr54* knockout; G+/−, *Gpr54* heterozygote; K+/−, *Kiss1* heterozygote; K−/−, *Kiss1* knockout. *Open circles* indicate *Kiss1* knockout females with large vaginal openings.

induced gonadotropin secretion in many species (as reviewed in Ref. 15). Animals were injected sc with 50 nmol C-terminally amidated mouse kisspeptin 110–119 (metastin 43–52) and killed 30 min after injection. As expected, gonadotropin levels did not rise in male or female *Gpr54* knockout animals. The gonadotropin responses in *Kiss1* knockout animals were comparable to those of WT and heterozygous animals (Fig. 6, A–D, and Table 1). The subgroup of *Kiss1* knockout females with higher gonadal weights (*open circles* in Fig. 6) had less robust gonadotropin responses to exogenous kisspeptin 110–119 than *Kiss1* knockout females with lower gonadal weights.

Testosterone levels and sperm production

Baseline serum testosterone levels were significantly lower in *Gpr54* knockout male mice but not in *Kiss1* knockout males in comparison with WT (*Kiss1*$^{-/-}$ 91 ± 61 ng/dl, nonsignificant; *Gpr54*$^{-/-}$ 19 ± 22 ng/dl, $P < 0.05$; WT 155 ± 176; Fig. 7A and Table 1). The sperm count of both *Kiss1* and *Gpr54* knockout mice was reduced compared with WT (Fig. 7B). However, considerable phenotypic variation was observed because some knockout animals of both lines had no sperm, whereas others had sperm counts approaching those of WT animals.

Fertility

Five *Kiss1* and five *Gpr54* knockout males were mated with WT females of proven fertility for 11–36 wk, but no pregnancies were observed. Similarly, no pregnancies have been observed when *Kiss1* and *Gpr54* knockout females (five each) were mated with WT males of proven fertility.

Discussion

In this report, we demonstrate abnormal sexual development in mice with a targeted deletion of *Kiss1*, whose protein product is proteolytically processed to generate ligands for Gpr54, and contrast these findings with those of *Gpr54* knockout mice generated in the same genetic context. Both *Kiss1* and *Gpr54* knockout mice have impaired sexual maturation, low gonadotropin levels, and abnormal gametogenesis as documented by these studies and others (16).

The endocrinological phenotypes of the *Kiss1* and *Gpr54* knockout mice were consistent with hypogonadotropism. When mouse metastin 43–52 was injected sc, all genotypes and genders (except *Gpr54* null mice) responded with a significant increase in LH and FSH. In several previous studies, kisspeptin/metastin has been found to be a robust stimulus for GnRH release from the hypothalamus (reviewed in Ref. 15). In the absence of any other data, we assume that the robust response to exogenous metastin in *Kiss1* knockout mice is due to the same mechanism of triggering GnRH and, by extension, LH release. Additional studies will determine whether a direct effect of metastin at the pituitary, where *Gpr54* is expressed, can be uncovered using this model.

Pregnancies were not observed when either *Kiss1* or *Gpr54* knockout mice were housed with WT mice of proven fertility. The infertility of these mice could be multifactorial including 1) lack of preputial separation in males impeding intercourse, 2) lack of estrous cycling in females, 3) changes in mating behavior in both genders, although this was not formally assessed in this study, and 4) the proposed role of kisspeptin/Gpr54 system in placentation (17, 18). *In vitro* fertilization using sperm from null males would determine whether sperm, when present, are capable of fertilization.

Males of both knockout lines exhibited a slightly decreased anogenital distance at the time of weaning, although this difference did not reach statistical significance. This suggests that kisspeptin/Gpr54 may function during fetal and/or juvenile development, a possibility raised by a male patient carrying mutations in *GPR54* who exhibited microphallus and cryptorchidism (19). After weaning, the difference in anogenital distance between knockout and WT males became marked; similar patterns of anogenital distance have been observed in LH receptor knockout mice (11). Anogenital distance at this age primarily reflects the action of testosterone (11), although influences from the fetal and juvenile time windows may contribute to a total cumulative effect. Along with the failure of preputial separation to develop in most of the knockout males, the postweaning difference in anogenital distance further suggests a failure of sexual maturation.

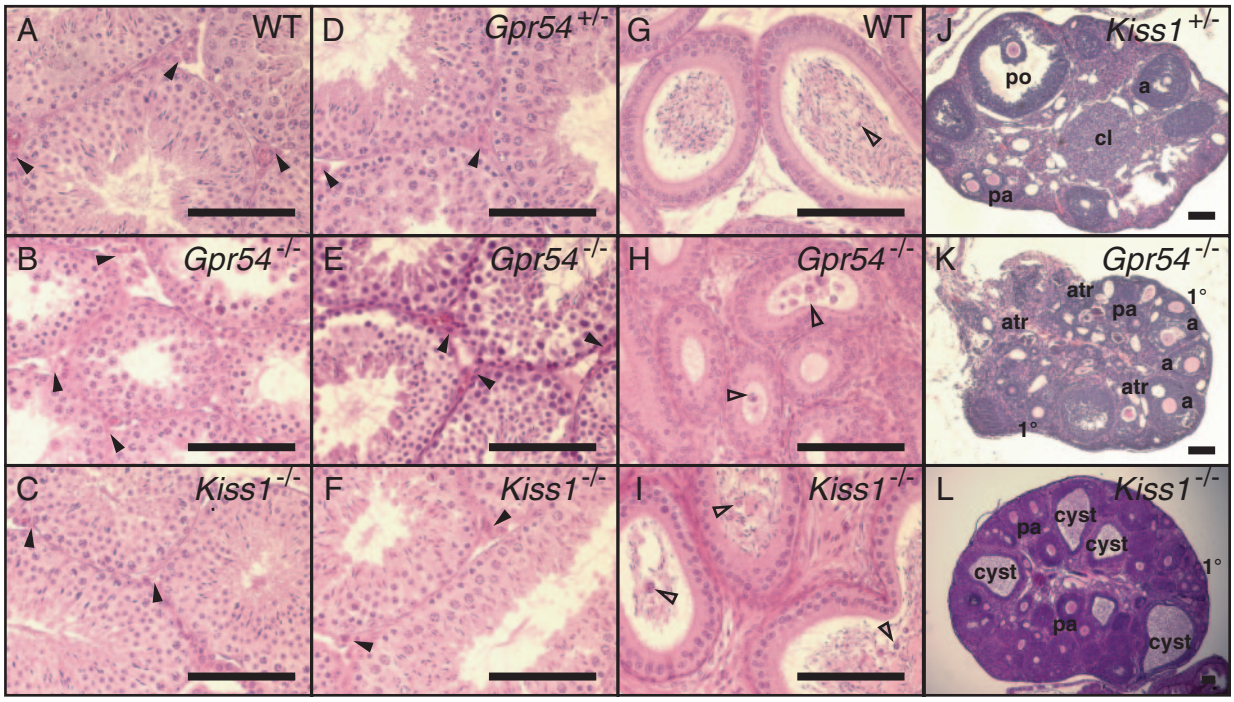

FIG. 5. Gonadal histology. A–F, Representative sections of testes. A and D, Seminiferous tubules from a WT mouse (A) and from a *Gpr54* +/− mouse (D) show all stages of spermatogenesis with numerous spermatozoa (identifiable by their condensed heads) and sperm tails present. Interstitial Leydig cells are indicated with *arrowheads*. The seminiferous tubules of *Gpr54*$^{-/-}$ (B and E) and *Kiss1*$^{-/-}$ (C and F) mice have severely (B) to moderately (C and E) to mildly (F) reduced numbers of spermatozoa and sperm tails. The density of interstitial cells (*arrowheads*) is comparable with that of testes from WT or heterozygous mice. G–I, Representative sections of epididymides. G, The lumen of the epididymis from a WT mouse is filled with sperm. H and I, The epididymides of *Gpr54*$^{-/-}$ (H) and *Kiss1*$^{-/-}$ (I) mice have fewer sperm and have numerous nonsperm cells (*open arrowheads*) in the lumen. J–L, Representative sections of ovaries. An ovary from a *Kiss1*$^{+/-}$ mouse (J) shows follicles at all stages of development, including primary (1°), preantral (pa), antral (a), and preovulatory (po) follicles as well as a corpus luteum (cl) and some atretic follicles (atr). Ovaries from nearly all *Gpr54*$^{-/-}$ (K) and many *Kiss1*$^{-/-}$ mice, in contrast, do not contain follicles past the antral stage and contain numerous atretic follicles. The ovaries of over half of *Kiss1* mice and one *Gpr54* mouse, however, exhibit multiple large cysts (L). Sections are not always through the maximum width of the ovary. *Scale bars*, 100 μm.

Importantly, considerable phenotypic variability was apparent in the *Kiss1* knockout females. One subgroup had a more severe phenotype closely resembling that of *Gpr54* knockout female mice, with small vaginal openings, small ovaries, and scant folliculogenesis. When given kisspeptin, this subgroup of mice responded with the largest increments in LH and FSH, possibly due to the lack of negative feedback by estrogen.

The other subgroup of *Kiss1* knockout mice had larger gonadal weights, larger vaginal openings, and vaginal smears showing persistent vaginal cornification. This pattern has been observed in senescent mice and can also be induced by administration of high-dose estradiol in the neonatal period (20, 21). Although blood volume requirements precluded direct assay of estradiol in these animals, the large uterine weight, the persistent vaginal cornification, and the blunted response to kisspeptin suggested the presence of higher estrogen levels. Although we first thought that this bimodal pattern belonged only to the *Kiss1* group, we did find one *Gpr54* knockout female with persistent vaginal cornification and a large uterus. Although this is only one observation, we feel it may reflect specific mechanisms or thresholds required for pubertal development.

Kiss1 knockout males have a more modest phenotype than *Gpr54* knockout males. The testes of *Kiss1* null males are significantly larger than those of *Gpr54* null males probably explaining the differences in serum testosterone, body weight, and sperm count. Basal gonadotropins are also slightly higher in *Kiss1* knockout males than in *Gpr54* knockout males, although this is not statistically significant. Although the *Kiss1* null male data are not obviously bimodal, unlike the *Kiss1* null female data, additional studies are needed to determine whether real heterogeneity exists within *Kiss1* and *Gpr54* knockout male mice.

Although the hypogonadism of *Kiss1* knockout mice is consistent with the absence of a major compensatory ligand or constitutive activity of Gpr54, the phenotypic variability observed in *Kiss1* knockout female mice does raise several important physiological possibilities: 1) another currently unknown ligand that can weakly stimulate Gpr54, 2) a previously unappreciated inhibitory role for kisspeptin, 3) modest constitutive activity of the Gpr54 receptor not previously detected but known to exist for many G protein-coupled receptors (22), 4) minute amounts of functional *Kiss1* transcript and kisspeptin below the limits of detection of the assays employed in this study, 5) intrauterine position and possible steroid or kisspeptin transfer via amniotic fluid (23) or from the mother, and 6) genetic polymorphisms, although we failed to identify any founder or clear inheritance pattern in our colony of *Kiss1* null mice. Although there are clear

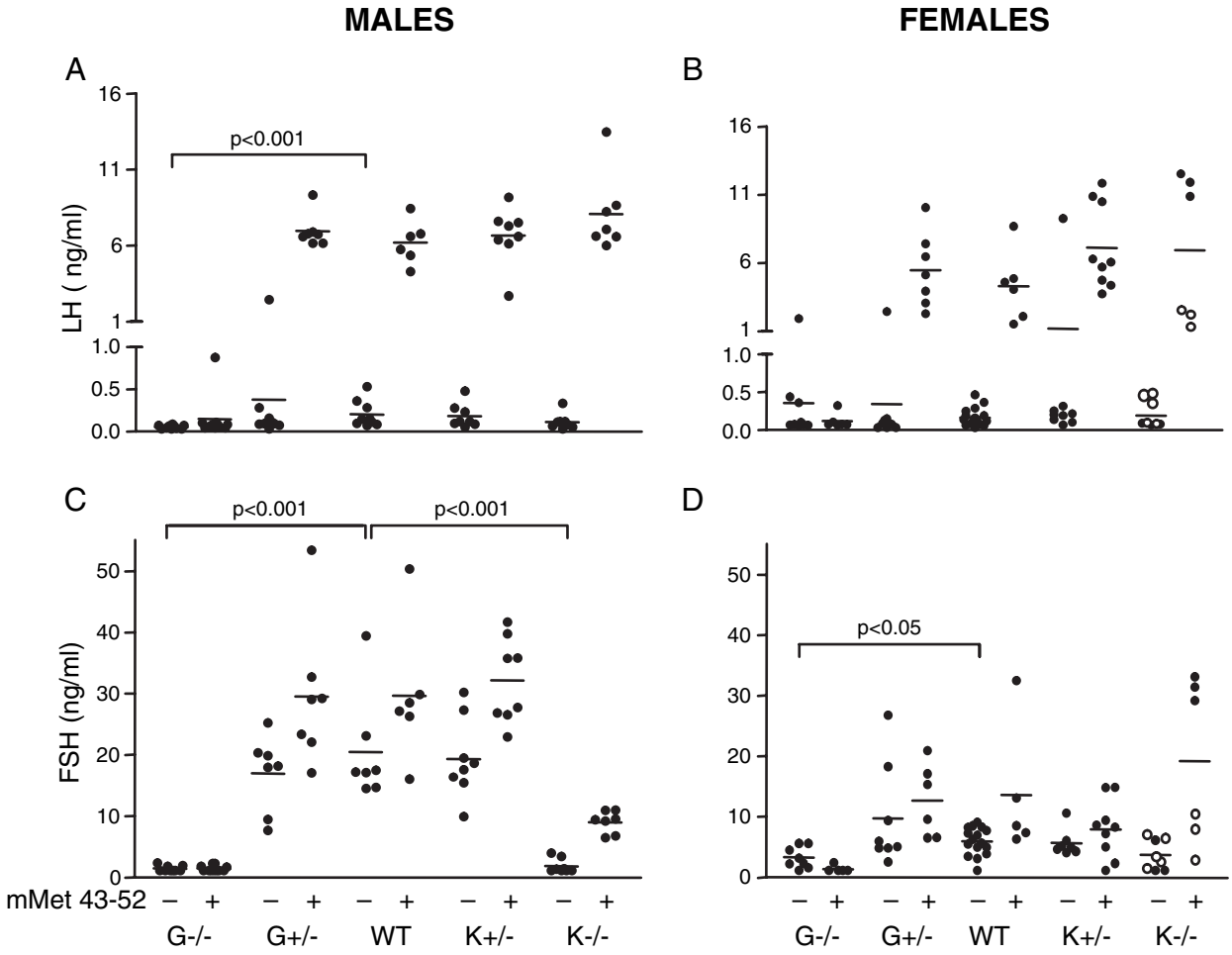

FIG. 6. Gonadotropin levels at baseline (−) and 30 min after sc injection (+) of 50 nmol mouse metastin 43–52. mMet 43–52 −/+, without or with stimulation with 50 nmol sc mouse metastin 43–52 30 min before mice were killed. A, LH—male; B, LH—female; C, FSH—male; D, FSH—female. G−/−, *Gpr54* knockout; G+/−, *Gpr54* heterozygote; K+/−, *Kiss1* heterozygote; K−/−, *Kiss1* knockout. ●, Low gonadal weight; ○, high gonadal weight. Different animals were used for baseline and poststimulation groups. Statistics are pertinent to baseline groups only.

differences between rodent and human reproduction, it is interesting to note that some patients harboring mutations in *GPR54* do not have complete hypogonadotropic hypogonadism but rather exhibit partial pubertal development, low-amplitude LH pulsations, and increased (as opposed to decreased) responsiveness to exogenous GnRH (2, 24). *Gpr54*$^{-/-}$ female mice, when stimulated with exogenous estrogen, can produce an LH surge. However, this surge is blocked when the animals are pretreated with acyline, suggesting the presence of a Gpr54-independent pathway capable of stimulating GnRH secretion (25). Collectively, these observations suggest that the kisspeptin/Gpr54 system is a key regulator of GnRH release but is not strictly required for GnRH secretion.

In general, analogous phenotypes are seen between ligand and receptor knockouts across the hypothalamic-pituitary-gonadal axis. For example, the LH receptor knockout mouse (26, 27) is remarkably comparable to the LHβ null model (28), indicating that cross-talk with other structurally comparable ligand-receptor pairs is unlikely. The FSH receptor knockout mouse (29, 30) and the FSHβ knockout mouse (31) both exhibit sterility in females and oligospermia and reduced fertility in males. Because kisspeptin modulates GnRH secretion, our targeted deletions of *Kiss1* and *Gpr54* are best compared with *hpg* and *Gnrhr* mutant mice. The *hpg* mice harbor a 33.5-kb deletion that results in a null mutation of the *Gnrh* gene. Spermatogenesis in *hpg* mice is arrested at the diplotene stage, and folliculogenesis rarely proceeds beyond the preantral stage (32). GnRH receptor mutant mice, recently created by *N*-ethyl-*N*-nitrosourea mutagenesis, also demonstrate a meiotic block of spermatogenesis, but folliculogenesis proceeds until the early antral stage (33). Although precise comparison between the *hpg* and *Gnrhr* mutant mice and our models is not possible due to strain differences, *hpg* mice have several features suggesting a more severe phenotype, including smaller ovarian size, earlier arrest of folliculogenesis, atretic interstitial testicular tissue, and undetectable FSH levels, and thus providing further support to the concept that the kisspeptin/Gpr54 system is not strictly required for GnRH secretion.

In summary, the phenotypes of *Kiss1* and *Gpr54* knockout mice are directionally parallel, with both sets of mice demonstrating hypogonadotropism. However, the degree of severity of the two sets of knockout mice are different with

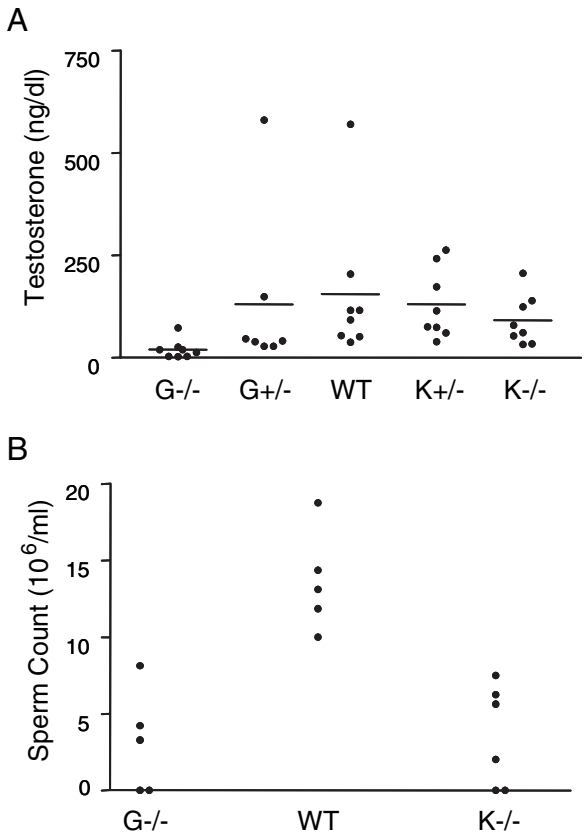

FIG. 7. Serum testosterone values and sperm production. A, Testosterone values in males; B, sperm production. G−/−, *Gpr54* knockout; G+/−, *Gpr54* heterozygote; K+/−, *Kiss1* heterozygote; K−/−, *Kiss1* knockout.

Kiss1 knockout mice being less severely affected than their receptor counterparts. In particular, the *Kiss1* knockout females have a bimodal phenotype, with about 50% of the females having larger gonadal weight and persistent vaginal cornification. Although the reason(s) underlying this variability in phenotype are not known, these observations suggest the presence of novel biological thresholds for the initiation of sexual maturation, acting either alone or in concert with modest GnRH secretion.

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