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Kisspeptin/Gpr54-independent GnRH activity in *Kiss1* and *Gpr54* mutant mice

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Abstract

The kisspeptin/Gpr54 signaling pathway plays a critical role in reproduction by stimulating the secretion of GnRH, yet mice carrying mutations in *Kiss1* (which encodes kisspeptin) or *Gpr54* exhibit partial sexual maturation. For instance, a proportion of female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice exhibit vaginal oestrus, and some male *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice exhibit spermatogenesis.

To characterise this partial sexual maturation, we examined the vaginal cytology of female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice over time. Nearly all mutant mice eventually enter oestrus, then spontaneously transition from oestrus to dioestrus and back to oestrus again. These transitions are not associated with ovulation, and the frequency of these transitions increases with age. The oestrus exhibited by female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice was disrupted by administration of the competitive GnRH antagonist acyline, which also resulted in lower uterine weights and, in *Kiss1*$^{-/-}$ mice, lower serum FSH and LH concentrations. Similarly, male *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice treated with acyline had smaller testicular sizes and absence of mature sperm.

In addition to examining intact *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice, we also assessed the effects of acyline on gonadotrophin concentrations in gonadectomised mice. Gonadectomy resulted in a significant increase in serum FSH concentrations in male *Gpr54*$^{-/-}$ and *Kiss1*$^{-/-}$ mice. Acyline administration to gonadectomised *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ male mice lowered serum FSH and LH concentrations significantly. In contrast to males, gonadectomy did not result in significant gonadotrophin changes in female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice, but acyline administration was followed by a decrease in LH concentrations.

These results demonstrate that, while kisspeptin signaling is critical for the high levels of GnRH activity required for normal sexual maturation and for ovulation, *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice retain some degree of GnRH activity. This GnRH activity is sufficient to produce significant effects on vaginal cytology and uterine weights in female mice and on spermatogenesis and testicular weights in male mice.

Keywords

metastin; Kiss1r; LHRH

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Introduction

Signaling by the neuropeptide kisspeptin through its receptor Gpr54 (also called Kiss1r) is the most potent stimulus for GnRH-induced gonadotrophin release identified to date (reviewed in refs. 1–2). Both humans and mice with mutations in *GPR54*/*Gpr54* exhibit failure of sexual maturation, impaired gametogenesis, and infertility (3–6). Three groups, including our own, have characterised mice with targeted deletions of *Gpr54* and have determined that their reproductive phenotypes are due to low levels of sex steroids and of the pituitary gonadotrophins FSH and LH, that is, hypogonadotropic hypogonadism (4–6). Recently, mice carrying mutations in *Kiss1* have been reported to have similar defects (7–8), emphasizing the importance of this receptor-ligand pair for reproduction.

However, the hypogonadism of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice is not complete. While all animals are infertile, we and others have observed that testes from male *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice sometimes contain mature sperm (7–8). In addition, we have observed that a subset of female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice exhibit normal uterine weights, vaginal cornification, and folliculogenesis but no evidence of ovulation (8). Vaginal cornification is a characteristic of the oestrus phase of the female oestrous cycle and reflects the action of oestrogen (9). Therefore, despite their failure of sexual maturation and infertility, some *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ animals have findings consistent with weak activity of the reproductive endocrine axis.

Our previous finding that only some female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice exhibit partial sexual maturation could be due to a subset of mice never achieving sexual maturation, or alternatively could be due to cross-sectional observations of a dynamic phenotype that changes over time. To distinguish between these possibilities, we examined the vaginal cytology of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice longitudinally. We also sought to determine whether the differences in vaginal cytology in *Gpr54*$^{-/-}$ mice were due to differences in hypothalamic *Gnrh1* mRNA expression.

We then sought to demonstrate and quantify GnRH activity in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice. To demonstrate that the partial sexual maturation of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice is due to GnRH activity, we administered the competitive GnRH antagonist acyline and observed its effect on vaginal cytology, uterine weight, and reproductive hormone levels in female mice, and on testicular size, spermatogenesis, and hormone levels in male mice. We also assessed the effects of acyline administration on gonadotrophin levels in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice that had been gonadectomised to remove negative feedback inhibition of gonadotrophin secretion.

Materials and Methods

Animals and animal care

Kiss1$^{-/-}$ and *Gpr54*$^{-/-}$ mice were generated as previously described (8). Animals were housed in the Massachusetts General Hospital (MGH) Center for Comparative Medicine at controlled temperature on a 12-hour light-dark cycle. All protocols were approved by the MGH Subcommittee on Research Animal Care.

Vaginal smears

To examine vaginal cytology, calcium alginate swabs were wetted with PBS and inserted in the vaginal opening. The swab was smeared on a glass slide, which was then stained using the Hema 3 system (Fisher). The cellular appearance of the smears was used to assign an oestrous cycle phase: dioestrus (predominantly neutrophils), prooestrus (predominantly

nucleated epithelial cells), oestrus (abundant cornified epithelial cells), or metoestrus (mixture of cell types, predominantly neutrophils).

Histology

Dissected tissues were fixed in Bouin's solution. Fixed tissues were embedded in paraffin, sectioned at 4 μm, mounted on slides, and stained with hematoxylin and eosin by the Harvard Medical School Rodent Histopathology Core.

Hormone assays

FSH concentrations for Experiments 2, 3, and 4 and LH and testosterone concentrations for all experiments were measured by RIA performed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. The measurable range for FSH was 2.6–21.7 ng/mL, for LH 0.04–37.4 ng/mL, and for testosterone 6.2–695.8 ng/dL. Intraassay and interassay coefficients of variation were 6.7% and 8.4% for FSH, 3.9% and 6.7% for LH, and 3.4% and 8.1% for testosterone, respectively. FSH concentrations for Experiment 5 were measured using the FSH (Rat) EIA kit (Alpco Diagnostics). The measurable range was 0.89–57.0 ng/mL, and intraassay and interassay coefficients of variation were 5.5% and 7.7%, respectively. There was not sufficient serum for all hormones to be measured in all animals.

Experiment 1: Longitudinal assessment of vaginal smears

Daily vaginal smears were obtained for two cohorts of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice. A younger cohort of 7 *Kiss1*$^{-/-}$ and 11 *Gpr54*$^{-/-}$ mice was followed from weaning on postnatal day 21 for up to 9 months; an older cohort of 5 *Kiss1*$^{-/-}$ and 6 *Gpr54*$^{-/-}$ mice was followed for 9 months starting from 2–12 months of age. Male bedding was added weekly to cages to prevent entry into anoestrus. Four *Kiss1*$^{-/-}$ mice that had undergone an oestrus-to-dioestrus transition were sacrificed for ovarian histology, with sections every 100 μm mounted and examined for the presence of corpora lutea.

Experiment 2: *Gnrh1* mRNA levels in *Gpr54*$^{-/-}$ females in oestrus and dioestrus

Gpr54$^{-/-}$ female mice 24–58 weeks of age were sacrificed, brains rapidly removed, and the basal forebrain dissected with cuts at the posterior edges of the olfactory bulbs, at the anterior edge of the optic chiasm, extended from the lateral hypothalamic sulci, and at a depth of 2 mm. The dissected tissue was immediately frozen and stored at −80°C. RNA was extracted using the RNeasy Lipid Kit (Qiagen), treated with DNase I (Invitrogen), and used for cDNA synthesis with the Superscript III First-Strand Synthesis kit (Invitrogen). Quantitative PCR was performed with the TaqMan 2X Universal PCR Mix (Applied Biosystems) on an ABI 7000 Real-Time PCR system using the following primers at 200 nM concentration: for *Gnrh1*, forward primer 5′-GAACCCCAGCACTTCGAATGTA-3′, reverse primer 5′-TGGCTTCCTCTTCAATCAGACTTT-3′, probe 5′-6-FAM-TCCACTGGCCCCGTTCACCCCTC-BHQ-1-3′; for *Gadph*, forward primer 5′-CAATGTGTCCGTCGTGGATCT-3′, reverse primer 5′-AGAGTGGGAGTTGCTGTTGAAG-3′, probe 5′-HEX-CGTGCCGCCTGGAGAAACCTGCC-BHQ-1-3′. The accompanying software was used to calculate C_t's, and relative expression levels calculated by normalizing C_t's to standard curves generated from serial dilutions of a reference sample. Expression levels were analyzed unadjusted, normalized to *Gapdh* expression, and normalized to the weight of the dissected tissue. No amplification was seen with samples from which reverse transcriptase was omitted (data not shown).

Experiment 3: Effects of acyline on female reproductive phenotypes in intact mice

Acyline was a generous gift of R. Blye, Eunice Kennedy Shriver National Institute of Child Health and Human Development. Female mice (17–59 weeks of age) exhibiting vaginal estrus were given two daily subcutaneous injections of acyline 50 μg (1 mg/mL in PBS; 13 *Kiss1*$^{-/-}$, 8 *Gpr54*$^{-/-}$) or PBS alone (17 *Kiss1*$^{-/-}$, 7 *Gpr54*$^{-/-}$), and vaginal smears were obtained for five days. After five days, a subset of these animals as well as WT animals treated with acyline or PBS were sacrificed, blood was collected by cardiac puncture, and ovaries and uteri were dissected, weighed, and fixed.

Experiment 4: Effects of acyline on male reproductive phenotypes in intact mice

Male mice 14–24 weeks in age received subcutaneous injections of acyline 50 μg or PBS alone on days 1, 2, 8, 15, 22, and 23 of the study. After the final injection, mice were sacrificed, blood was collected by cardiac puncture, and testes were dissected, weighed, and fixed.

Experiment 5: Effects of acyline on gonadotrophin levels in gonadectomised mice

Paired subcutaneous injections of acyline or PBS were given 24 h and 1 h before each blood collection, which was performed by submandibular puncture using Goldenrod lancets (Medipoint, Inc.). Male mice 9–24 weeks and female mice 10–39 weeks were used. For most animals, pre-gonadectomy gonadotrophin levels were obtained after injection with PBS. Animals were then anaesthetised with an intraperitoneal injection of ketamine 118 mg/kg and xylazine 11.8 mg/kg. The abdomen was shaved and opened with midline incisions through the skin and peritoneum. The gonads were identified, ligated, and resected (this step was omitted for animals undergoing sham surgery). The peritoneum was closed with a running chromic gut suture (Ethilon) and the skin closed with simple interrupted nylon sutures (Ethilon). After at least one week for recovery, paired PBS injections were given and post-gonadectomy blood samples obtained. After another week for recovery, paired injections of acyline 50 μg were given, followed by blood collection and sacrifice.

Statistics

Changes in vaginal smears after acyline or PBS injections were compared using Fisher's two-tailed exact test. Length of oestrous phases, RT-PCR results, and levels of hormones were compared with unpaired, two-tailed t-tests, except for comparisons of FSH and LH levels pre-and post-acyline, for which paired t-tests were used. Because most LH levels were close to the detection limits of the assay, the sign test (binomial probability) was also used to analyze directions of change in LH levels. For quantitative calculations, assay results that were below the measurable range were set at the lower limit of the measurable range. Data are presented as mean ± SEM.

Results

Longitudinal examination of vaginal smears in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice

We examined daily vaginal smears of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice over time to characterise the oestrous cyclicity of mice lacking kisspeptin/Gpr54 signaling (Figure 1). *Kiss1*$^{-/-}$ mice first exhibited oestrus at a median of 130 days of life (n=7), whereas *Gpr54*$^{-/-}$ mice first exhibited oestrus at a median of 265 days (n=11). Nearly all *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice of both genotypes eventually exhibited oestrus, though some *Gpr54*$^{-/-}$ females were never seen to enter oestrus. Indeed, rare mice in our *Gpr54*$^{-/-}$ colony failed to exhibit even vaginal opening by 2 years of age (data not shown).

Mice that had entered oestrus were observed to spontaneously exit oestrus and enter a state of prolonged dioestrus, then enter oestrus once again (Figure 1). The frequency of these transitions increased with age. In *Kiss1*$^{-/-}$ mice 2–12 months of age, oestrus lasted 17.9 ± 2.2 days (n=12) and dioestrus 28.1 ± 6.2 days (n=13), whereas in *Kiss1*$^{-/-}$ mice 12–24 months of age oestrus lasted 7.7 ± 1.0 days (n=43, $P < 0.001$ compared to younger mice) and dioestrus lasted 7.6 ± 1.2 days (n=34, $P = 0.007$). In *Gpr54*$^{-/-}$ mice 2–12 months of age, oestrus lasted 23.1 ± 4.7 days (n= 14) and dioestrus 13.6 ± 4.5 days (n=11), and in *Gpr54*$^{-/-}$ mice 12–24 months of age oestrus lasted 15.3 ± 3.4 days (n=13, $P = 0.19$ compared to younger mice) and dioestrus 11.6 ± 3.1 days (n=19, $P = 0.71$).

To determine whether these oestrus-to-dioestrus transitions were the result of ovulation, four *Kiss1*$^{-/-}$ mice were sacrificed and their ovaries examined histologically. Follicles at all stages of development and large numbers of atretic follicles were present in *Kiss1*$^{-/-}$ mice, as we had observed previously (8), but no corpora lutea were seen on serial sections through the entirety of these ovaries (data not shown). Thus, even though older *Kiss1*$^{-/-}$ mice transitioned from dioestrus to oestrus and back again at a frequency approaching that of wild-type animals, these “cycles” were in fact an ovulatory.

Gnrh1 mRNA expression in female *Gpr54*$^{-/-}$ mice in oestrus and dioestrus

To determine whether the changes in vaginal cytology were due to changes in *Gnrh1* expression, *Gnrh1* mRNA levels in the basal forebrain of *Gpr54*$^{-/-}$ mice were measured by quantitative RT-PCR. No difference was seen in *Gnrh1* expression in mice in oestrus compared to mice not in oestrus (Supplemental Table 1).

Effects of acyline on reproductive phenotypes of female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice

To determine whether the persistent vaginal oestrus seen in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice is due to GnRH activity, mice exhibiting vaginal oestrus were given two daily injections of the competitive GnRH antagonist acyline or vehicle (PBS). Of the 13 *Kiss1*$^{-/-}$ mice that received acyline, 12 left oestrus within 4 days, whereas only 2 of 17 mice that received vehicle left oestrus (Figure 2A, $P < 0.001$). Similarly, 7 of 8 *Gpr54*$^{-/-}$ mice that received acyline left oestrus compared to 1 of 7 that received vehicle (Figure 2A, $P = 0.01$).

Five days after acyline administration, a subset of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ females was sacrificed to examine internal sex organs. Uterine weights of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ females treated with acyline were significantly lower than those of females treated with vehicle (Figure 2; *Kiss1*$^{-/-}$ after acyline 35.8 ± 3.9 mg, after vehicle 72.1 ± 6.7 mg, $P = 0.0003$; *Gpr54*$^{-/-}$ after acyline 29.2 ± 1.2 mg, after vehicle 66.7 ± 9.8 mg, $P = 0.007$). No differences in ovarian histology were seen after this relatively short exposure to acyline (data not shown).

Serum LH concentrations were significantly lower in *Kiss1*$^{-/-}$ females treated with acyline (Table 1, acyline 0.05 ± 0.01 ng/mL, vehicle 0.61 ± 0.17 ng/mL, $P = 0.01$), with a similar pattern in *Gpr54*$^{-/-}$ mice (acyline 0.04 ± 0 ng/mL, vehicle 0.96 ± 0.06 ng/mL, $P = 0.26$). Similarly, FSH concentrations were lower in *Kiss1*$^{-/-}$ females treated with acyline (acyline 2.6 ± 0 ng/mL, vehicle 3.4 ± 0.2 ng/mL, $P = 0.007$). Thus, treatment with a GnRH antagonist resulted in disruption of vaginal oestrus and lower uterine weights in female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice and lower FSH and LH concentrations in female *Kiss1*$^{-/-}$ mice.

Effects of acyline on reproductive phenotypes of male *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice

To determine whether the spermatogenesis seen in male *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice is also due to GnRH activity, male mice were treated with acyline or vehicle for three weeks. As

expected, wild-type male mice treated with acyline had testicular weights lower than those of mice treated with vehicle (Figure 3; acyline 59.7 ± 5.4 mg, vehicle 95.3 ± 2.8 mg, P = 0.0007). Similarly, male *Kiss1*$^{-/-}$ mice treated with acyline had significantly smaller testes than *Kiss1*$^{-/-}$ mice treated with vehicle (acyline 8.2 ± 0.8 mg, vehicle 40.5 ± 4.6 mg, P = 0.004), and the same was seen for male *Gpr54*$^{-/-}$ mice (acyline 4.3 ± 0.3 mg, vehicle 16.5 ± 3.9 mg, P = 0.02).

Histological examination of testes from *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice treated with vehicle showed varying degrees of spermatogenesis (Figure 3), as we and others have previously observed (7–8), ranging from arrest at meiosis II to appearance of mature sperm. In contrast, no mature sperm were seen in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ males treated with acyline (Figure 3), with arrest of spermatogenesis at meiosis II. No significant changes in FSH, LH, or testosterone concentrations were seen with acyline treatment of male *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice; many of these measurements were near or below the measurable range of the assays employed (Table 1).

Effects of gonadectomy on gonadotrophin concentrations in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice

Because changes in hormone levels with acyline treatment were difficult to demonstrate consistently in intact *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice, we examined gonadotrophin levels in animals that were gonadectomised to remove feedback inhibition of GnRH and gonadotrophin secretion. Gonadectomy of wild-type mice resulted in marked increases in serum FSH and LH concentrations, as expected (Figure 4).

FSH concentrations rose in male mice of both lines after gonadectomy. After gonadectomy, FSH concentrations in *Kiss1*$^{-/-}$ males (Figure 4A, 1.51 ± 0.13 ng/mL) were significantly higher than at baseline (0.93 ± 0.02 ng/mL, P = 0.002). Similarly, FSH concentrations in *Gpr54*$^{-/-}$ mice after gonadectomy were significantly higher (Figure 4A, mean ± SD 2.87 ± 0.36 ng/mL) than FSH concentrations before gonadectomy (1.46 ± 0.30 ng/mL, P = 0.008) and were also higher than FSH concentrations of mice that underwent sham surgery (1.25 ± 0.26 ng/mL, P = 0.003). In contrast to male mice, no significant changes in FSH concentrations were seen after gonadectomy in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ female mice (Figure 4B), and no significant changes in LH concentrations were seen after gonadectomy in *Kiss1*$^{-/-}$ or *Gpr54*$^{-/-}$ male or female mice (Figure 4C and D).

Effects of acyline on gonadotrophin concentrations in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice

Acyline administration resulted in significant reductions in gonadotrophin concentrations in gonadectomised wild-type, *Kiss1*$^{-/-}$, and *Gpr54*$^{-/-}$ mice of both sexes. Specifically, administration of acyline to gonadectomised *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ male mice resulted in significant decreases in FSH concentrations (Figure 4A; *Kiss1*$^{-/-}$ pre-acyline 1.51 ± 0.13 ng/mL, post-acyline 1.27 ± 0.09 ng/mL, P = 0.002; *Gpr54*$^{-/-}$ pre-acyline 2.87 ± 0.36 ng/mL, post-acyline 1.95 ± 0.42 ng/mL, P = 0.007). In female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice, there was no detectable change in FSH concentrations after acyline administration (Figure 4B).

Mean LH concentrations were not significantly different before and after acyline in gonadectomized *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ males, with most LH concentrations near the lower end of the measurable range of the assay (Figure 4C; *Kiss1*$^{-/-}$ pre-acyline 0.090 ± 0.040 ng/mL, post-acyline 0.04 ± 0 ng/mL, P = 0.23; *Gpr54*$^{-/-}$ pre-acyline 0.057 ± 0.008 ng/mL, post-acyline 0.040 ± 0.0004 ng/mL, P = 0.08). However, in the 8 *Kiss1*$^{-/-}$ males in which a change in LH concentration could be determined, LH concentrations decreased in all 8 cases, more frequently than would be expected by chance alone (P = 0.002 by sign test); a similar trend was seen in *Gpr54*$^{-/-}$ males (5 of 5 mice with decreases in LH concentrations,

P = 0.06). Mean LH concentrations decreased significantly in *Gpr54*$^{-/-}$ females after acyline administration (Figure 4D, pre-acyline 0.065 ± 0.010 ng/mL, post-acyline 0.041 ± 0.001 ng/mL, P = 0.04 by paired t-test, P = 0.008 by sign test), with a similar trend in *Kiss1*$^{-/-}$ females (pre-acyline 0.053 ± 0.007 ng/mL, post-acyline 0.044 ± 0.004 ng/mL, P = 0.12 by sign test).

Discussion

Multiple groups have shown that *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice have abnormal sexual maturation, with gonadal underdevelopment, impaired gametogenesis, low sex steroids and gonadotrophins, and infertility (4–8). Despite these impairments in reproductive maturation and function, we have now shown persistent GnRH activity in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice of both sexes. *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ females alternate between periods of prolonged dioestrus and prolonged oestrus. These transitions increase in frequency with increasing age, are not due to ovulation, and are not associated with changes in hypothalamic *Gnrh1* mRNA expression. We further demonstrated that this oestrus is disrupted by administration of the competitive GnRH antagonist acyline, which also resulted in lower uterine weights. Similarly, acyline administration to male *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice results in lower testicular weights and blockade of spermatogenesis. Finally, we found that acyline lowers gonadotrophins in gonadectomised *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice. These results demonstrate the presence of low-level GnRH activity in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice and suggest that this activity is sufficient to cause partial sexual maturation.

The persistence of GnRH activity in the absence of kisspeptin/Gpr54 signaling has been suggested by several previous reports, although never formally explored. First, as noted above, *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice exhibit partial sexual maturation (7–8). Second, human patients with severe loss-of-function mutations in *GPR54* exhibit low-amplitude LH pulses (4,10). Third, administration of an oestrogen regimen to *Gpr54*$^{-/-}$ mice can induce a GnRH-dependent LH surge (11), although this effect has not been observed by all investigators (12). Fourth, rhesus macaques undergoing pharmacologic desensitization of Gpr54 exhibit low-amplitude LH pulses at night, though these pulses did not a meet statistically significant threshold (13). Fifth, administration of kisspeptin to rats does not alter the rhythmic electrophysiological activity of the arcuate nucleus, which is associated with the pulsatile release of GnRH (14). Lastly, physiologically distinct subpopulations of GnRH neurones have recently been identified: one subpopulation responds to kisspeptin but not to 3,5-dihydroxyphenylglycine (DHPG), an agonist of group 1 metabotropic glutamate receptors, while the other subpopulation responds to DHPG but not to kisspeptin (15). Our findings add to these studies to provide the most direct evidence to date that physiologically significant GnRH activity persists in the absence of kisspeptin/Gpr54 signaling.

Illustrating the known importance of kisspeptin signaling for GnRH secretion, gonadotrophin levels were markedly lower in gonadectomised *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice compared to gonadectomised wild-type mice. Our findings are consistent with those of Dungan et al. (11), who observed low LH concentrations in gonadectomised *Gpr54*$^{-/-}$ mice that were not higher than those of intact mice, evidence that kisspeptin signaling is an essential mediator of the negative feedback effects of sex steroids. We similarly did not see an effect of gonadectomy on LH, but we did find that FSH increased in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ males after gonadectomy; this can be attributed to loss of gonadal inhibins, which restrain FSH but not LH secretion (16). It is unclear why FSH concentrations in female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice did not change after gonadectomy or acyline treatment, particularly since LH levels were lower with acyline treatment. This may reflect a greater contribution of signals other than GnRH (e.g., activins) to FSH secretion in female than in

male mice, or may be due to lack of specificity of the FSH assay at low FSH concentrations in female mice.

Our results provide an indirect measure of kisspeptin-dependent versus kisspeptin-independent GnRH activity. For example, mean FSH levels of gonadectomised wild-type male mice were 54 ng/mL, whereas those of gonadectomised *Gpr54*$^{-/-}$ male mice were 2.9 ng/mL. In contrast, administration of acyline to gonadectomised *Gpr54*$^{-/-}$ male mice resulted in a decrease in FSH levels from 2.9 to 2.0 ng/mL. A rough calculation shows that kisspeptin-dependent GnRH activity is ~50-fold greater than kisspeptin-independent GnRH activity in the context of gonadectomised mice. Nevertheless, even this modest amount of GnRH and gonadotrophin activity is sufficiently potent to induce spermatogenesis and uterine hypertrophy.

Kisspeptin-independent GnRH activity could be due to low-level constitutive activity of GnRH neurones. Indeed, cultured GnRH neurones spontaneously produce synchronous pulses of GnRH secretion in the absence of external inputs (17). However, it is unclear how this could explain the increasing frequency of oestrus-to-dioestrus transitions exhibited by female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice with advancing age. Alternatively or additionally, the GnRH activity in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice could be induced by one or more of the many neuroendocrine pathways that modulate GnRH neuronal activity and secretion (18,19).

Indeed, decreases in glutamate and increases in GABA inputs have been proposed to produce the neuroendocrine changes with reproductive senescence in female rodents (19,20). The effects of these inputs may superimpose on the residual GnRH activity in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice to accelerate entry into oestrus, thereby causing the increasing frequency of dioestrus-to-oestrus transitions we observed in these mutant mice. The milder phenotypes of *Kiss1*$^{-/-}$ mice compared to *Gpr54*$^{-/-}$ mice suggests a higher basal GnRH "tone" that results in a lower threshold for entry into oestrus, which could account for the more dynamic pattern of vaginal smears seen in female *Kiss1*$^{-/-}$ mice.

A potential alternative, technical explanation for our findings is that the mutations in our *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice are not null. We had previously demonstrated that no *Gpr54* transcript could be detected by RT-PCR in the *Gpr54*$^{-/-}$ mice and no kisspeptin peptide could be detected by immunohistochemistry in the *Kiss1*$^{-/-}$ mice (8), and so this possibility appears unlikely. We are further reassured that low-level GnRH activity was observedin both *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice.

Because we used traditional, global targeted deletions, *Kiss1* and *Gpr54* activity was absent from our mutant mice throughout development. It is therefore possible that alternative pathways emerged to compensate for the absence of *Kiss1*/*Gpr54* activity and that our results underestimate the contribution of kisspeptin signaling to the stimulation of GnRH activity in normal animals. It is also possible that kisspeptin/Gpr54 signaling is required for the proper development of the reproductive endocrine system, independent of the need for *Kiss1*/*Gpr54* activity during sexual maturation and in adulthood. The identification and use of specific kisspeptin/Gpr54 antagonists (21) would allow acute blockade of kisspeptin/Gpr54 signaling and more direct determination of the extent to which kisspeptin-dependent and kisspeptin-independent pathways contribute to GnRH activity in wild-type mice.

To date, most studies of the reproductive role of kisspeptin/Gpr54 signaling have emphasized the importance and potency of this pathway for GnRH secretion. Our results demonstrate the presence of low-level GnRH activity in *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice and emphasize the importance of the contribution of other pathways to the regulation of GnRH secretion. Further study of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice will determine which of these pathways are responsible for inducing sexual maturation and oestrous changes, how these

pathways change with increasing age, and whether they can be manipulated to either enhance or overcome the hypogonadism caused by deficits in kisspeptin/Gpr54 signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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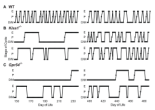

Figure 1.
Representative patterns of vaginal cytology of *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ female mice. A, wild-type mice; B, *Kiss1*$^{-/-}$ mice; C, *Gpr54*$^{-/-}$ mice. D/M, dioestrus/metoestrus; P, prooestrus; E, oestrus.

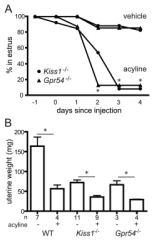

Figure 2.

Effects of acyline administration on oestrus and uterine weights in female *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice. A, female mice remaining in oestrus after administration of two doses of acyline 50 μg or vehicle (PBS) on days 0 and 1. B, uterine weights on day 4 after administration of acyline or vehicle. Asterisks, $P < 0.05$ compared to vehicle-treated mice.

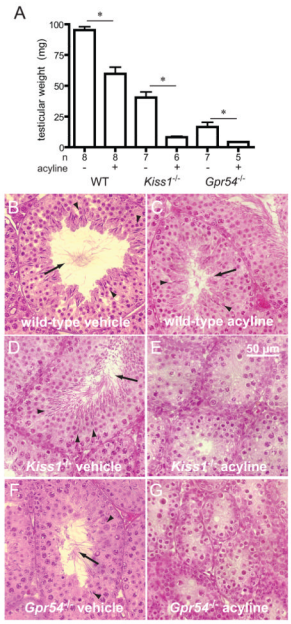

Figure 3.

Effects of acyline administration on male wild-type, *Kiss1*$^{-/-}$ and *Gpr54*$^{-/-}$ mice. A, testicular weights, and B–G, testicular histology after three weeks of weekly doses of acyline 50 μg or vehicle. Asterisks, $P < 0.05$; arrowheads, sperm heads; arrows, sperm tails.

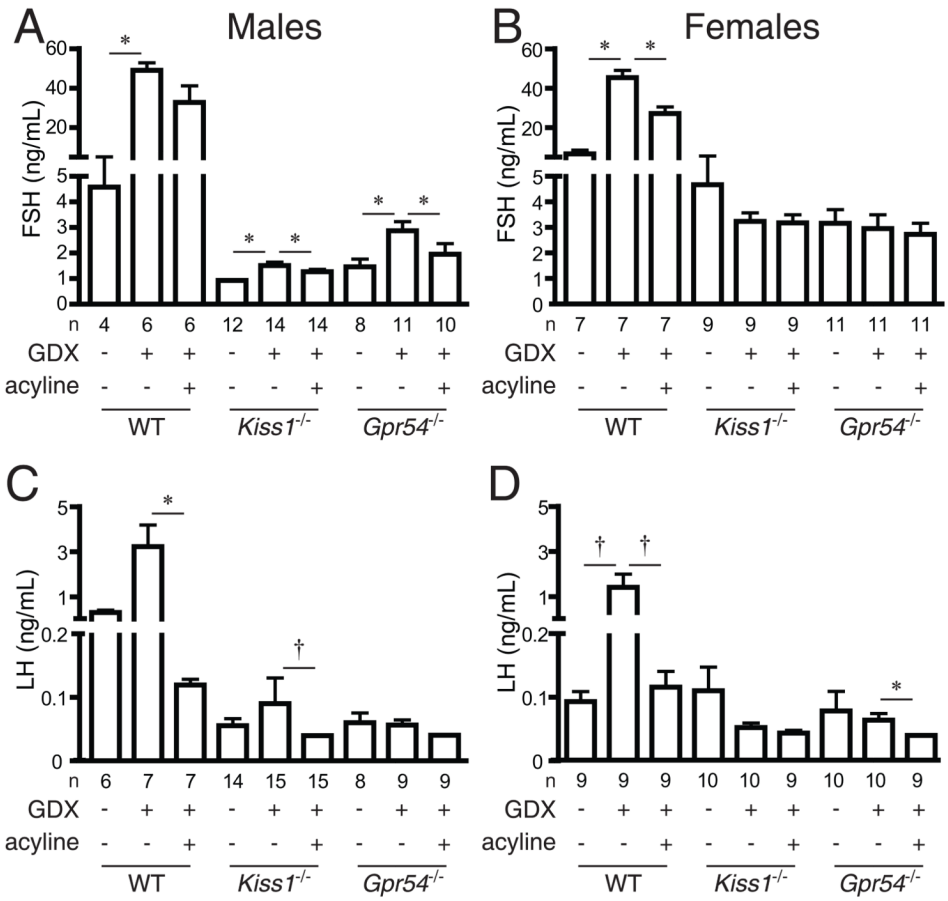

Figure 4.

Gonadotrophin levels after gonadectomy and acyline administration. A, FSH levels in male mice; B, FSH levels in female mice; C, LH levels in male mice; D, LH levels in female mice. WT, wild-type; GDX, gonadectomy. Asterisks, $P < 0.05$ by paired t-test; crosses, $P < 0.05$ by sign test.

Table 1

Hormone levels (mean ± SEM) in intact wild-type, *Kiss1*$^{-/-}$, and *Gpr54*$^{-/-}$ mice treated with acyline or vehicle (PBS). P values are for differences between acyline and vehicle treatment. The lower end of the measurable range was 2.6 ng/mL for FSH, 0.04 ng/mL for LH, and 6.2 ng/dL for testosterone.

female	**wild-type**			***Kiss1*$^{-/-}$**			***Gpr54*$^{-/-}$**		
	vehicle	**acyline**	**P**	**vehicle**	**acyline**	**P**	**vehicle**	**acyline**	**P**
FSH (ng/mL)	5.7 ± 2.6 (n=7)	2.7 ± 0.1 (n=4)	0.18	3.4 ± 0.2 (n=8)	2.6 ± 0 * (n=8)	0.007	3.4 ± 0.1 (n=3)	2.9 ± 0.5 (n=4)	0.53
LH (ng/mL)	0.62 ± 0.25 (n=7)	0.07 ± 0.02 (n=4)	0.07	0.61 ± 0.17 (n=10)	0.05 ± 0.01 (n=9)	0.01	0.96 ± 0.06 (n=3)	0.04 ± 0 * (n=4)	0.26
male									
FSH (ng/mL)	12.6 +/− 2.2 (n=8)	2.6 ± 0 * (n=8)	0.002	2.6 ± 0 * (n=7)	2.6 ± 0 * (n=6)	ND	2.6 ± 0 * (n=7)	2.6 ± 0 * (n=5)	ND
LH (ng/mL)	0.78 ± 0.46 (n=8)	0.04 ± 0 * (n=8)	0.15	0.73 ± 0.58 (n=7)	0.04 ± 0 * (n=6)	0.28	0.34 ± 0.14 (n=7)	0.04 ± 0 * (n=5)	0.07
testosterone (ng/dL)	90.4 ± 51.0 (n=7)	11.1 ± 2.6 (n=8)	0.17	79.0 ± 36.2 (n=7)	10.4 ± 2.6 (n=6)	0.11	22.1 ± 2.6 (n=7)	33.3 ± 12.4 (n=5)	0.42

Asterisks, all measurements were below the reportable range; ND, not determinable.