Gpr54−/− mice show more pronounced defects in spermatogenesis than Kiss1−/− mice and improved spermatogenesis with age when exposed to dietary phytoestrogens

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Abstract

Mice with mutations in the kisspeptin signaling pathway (Kiss1−/− or Gpr54−/−) have low gonadotrophic hormone levels, small testes, and impaired spermatogenesis. Between 2 and 7 months of age, however, the testes of the mutant mice increase in weight and in Gpr54−/− mice, the number of seminiferous tubules containing spermatids/spermatozoa increases from 17 to 78%. In contrast, the Kiss1−/− mice have a less severe defect in spermatogenesis and larger testes than Gpr54−/− mice at both 2 and 7 months of age. The reason for the improved spermatogenesis was investigated. Plasma testosterone and FSH levels did not increase with age in the mutant mice and remained much lower than in wild-type (WT) mice. In contrast, intratesticular testosterone levels were similar between mutant and WT mice. These data indicate that age-related spermatogenesis can be completed under conditions of low plasma testosterone and FSH and that intratesticular testosterone may contribute to this process. In addition, however, when the Gpr54−/− mice were fed a phytoestrogen-free diet, they showed no age-related increase in testes weight or improved spermatogenesis. Thus, both genetic and environmental factors are involved in the improved spermatogenesis in the mutant mice as they age although the mice still remain infertile. These data show that the possible impact of dietary phytoestrogens should be taken into account when studying the phenotype of mutant mice with defects in the reproductive axis.

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Introduction


Several transgenic mouse lines have been generated to study the role of kisspeptin signaling in the regulation of the reproductive axis (reviewed by Colledge (2009)). Gpr54−/− and Kiss1−/− mutant mice have broadly similar phenotypes, with hypogonadotrophic hypogonadism and infertility (Funes et al. 2003, Seminara et al. 2003, Dungan et al. 2006, d’Anglemont de Tassigny et al. 2007, Kauffman et al. 2007, Lapatto et al. 2007). Mutant male mice of either genotype have lower testicular weights and reduced serum testosterone levels compared to wild-type (WT). Spermatogenesis is severely impaired with spermatogenic arrest at the haploid spermatid stage and the absence of spermatozoa in the seminiferous tubules and epididymides of young...
mice. This phenotype is variable, however, with some mutant mice showing the ability to complete spermatogenesis and produce low numbers of spermatozoa (Lapatto et al. 2007). We have investigated the cause of this increase in spermatogenesis and found that residual signaling from the GPR54 receptor and dietary phytoestrogens may contribute to this process.

Results

Mutant mice show improved spermatogenesis with age

The Gpr54−/− and Kiss1−/− mice had significantly smaller testes, epididymides, and seminal vesicles than WT mice at both 2 and 7 months old while these organs were also heavier in the Kiss1−/− mice than the Gpr54−/− mice (Fig. 1A–C). Between 2 and 7 months of age, the weight of the testes increased for both WT and mutant mice. The weight increase in the WT mice probably reflects further sexual maturation of the reproductive system even though 2-month-old WT mice have full adult type spermatogenesis. If organ weights are expressed relative to body weight the same pattern of data is found for all three genotypes, eliminating any effects due to differences in body weight (data not shown).

Histological analysis showed that the increase in testicular weight in the Gpr54−/− mice was accompanied by improved spermatogenesis (Fig. 2A). Two-month-old Gpr54−/− mice had incomplete spermatogenesis with 44% of seminiferous tubules containing spermatocytes and 17% containing spermatids/spermatozoa. However, when the Gpr54−/− mice had reached 7 months of age, 95% of the seminiferous tubules contained spermatocytes and 78% contained spermatids/spermatozoa (Fig. 3A). In contrast, Kiss1−/− mice already had more complete spermatogenesis at 2 months of age (Fig. 2A) with 97% of seminiferous tubules containing spermatocytes and 76% containing spermatids/spermatozoa, which was not statistically different from 2-month-old WT mice (Fig. 3A).

Similar to the testes, the epididymides also showed a weight increase with age although this was not statistically significant for the Kiss1−/− mice due to the already higher weight at 2 months. At 2 months of age, the epididymides of the Gpr54−/− mice were significantly smaller than WT mice (Fig. 2B) and histological analysis showed that the epithelial cells were poorly developed with a narrow luminal area and no spermatozoa in the lumen (Fig. 2B). As the Gpr54−/− mice aged, the luminal epithelium cells enlarged and the size of the lumen increased. At 7 months, the majority of the lumina were still empty although occasionally a few spermatozoa could be found in individual lumina (Fig. 2B). In contrast, Kiss1−/− mice at both 2 and 7 months of age had a well-developed tubule structure within the epididymides and sperm in the majority of the lumina (Fig. 2B).

The WT mice showed an increase in the weight of the seminal vesicles between 2 and 7 months of age (Fig. 1C). In contrast, the growth of the seminal vesicles in Kiss1−/− or Gpr54−/− mice was not significant (Fig. 1C), reflecting the low serum testosterone levels in the mutant mice.


Figure 1 Age-related changes in sex organ weights. (A) Left and right testes, (B) left and right epididymides, and (C) seminal vesicles were weighed for each group of mice at 2 months (2m) and 7 months (7m). a versus b versus c: P<0.05 (one-way ANOVA followed with the Tukey–Kramer multiple comparisons test) among 2m WT, Gpr54−/−, and Kiss1−/− groups. A versus B versus C: P<0.05 (one-way ANOVA with the Tukey–Kramer multiple comparisons test) among 7m WT, Gpr54−/−, and Kiss1−/− groups. *P<0.05; **P<0.01; ****P<0.0001 between mice at 2 and 7m of individual genotypes (unpaired t-test with Welch's correction). The number of mice in each group was WT, 2m (5), 7m (11); Gpr54−/−, 2m (6), 7m (14); Kiss1−/−, 2m (6), 7m (14).
Older mutant mice are still infertile and have low sperm counts

As the Gpr54<sup>/−/−</sup> and Kiss1<sup>/−/−</sup> mice can produce mature spermatozoa at 7 months of age, the fertility of these mice was examined. Gpr54<sup>/−/−</sup> (n = 6) and Kiss1<sup>/−/−</sup> (n = 6) mice were housed separately with two sexually mature WT male mice for 3 weeks. No copulatory plugs were observed during this period and no pregnancies occurred. As a control group, 90% of female mice housed with WT male mice (n = 5) became pregnant and delivered litters. The number of spermatozoa in the epididymides and vas deferens of the 7-month-pregnant and delivered litters. The number of spermatozoa found in WT mice (2.5 × 10⁶ spermatozoa/epididymis, which was around 5% of the number found in WT mice (2.5 × 10⁶ spermatozoa/epididymis, Fig. 3B). Although the Kiss1<sup>/−/−</sup> mice had more spermatozoa (4 × 10⁵ spermatozoa/epididymis) than Gpr54<sup>/−/−</sup> mice, this was significantly less than WT (Fig. 3B). Similarly, low sperm numbers were also observed in the vas deferens (Fig. 3C). The Gpr54<sup>/−/−</sup> mice had only 8% of the spermatozoa found in WT and the Kiss1<sup>/−/−</sup> mice had about 13% of the spermatozoa found in WT.

Improved spermatogenesis is not caused by hormonal changes

We hypothesized that dietary phytoestrogens might contribute to the change in spermatogenesis in the mutant mice. Both Gpr54<sup>/−/−</sup> and Kiss1<sup>/−/−</sup> mice fed with phytoestrogen-free food (AIN-93M diet) showed no significant increase in testicular weight between 2 and 7 months of age. In contrast, the mutant mice that were maintained on normal food (PM3 diet) showed a significant increase in testicular weight during this period (Fig. 5A). Histological analysis revealed that spermatogenesis was still arrested at the spermatocyte stage in the 7-month-old Gpr54<sup>/−/−</sup> mice on the phytoestrogen-free diet. In contrast, the 7-month-old Gpr54<sup>/−/−</sup> mice on a normal diet completed spermatogenesis and produced spermatids/spermatozoa (Fig. 5B). It has been shown by others that a phytoestrogen-free diet does not inhibit spermatogenesis in WT rodents (Atanassova et al. 2000, Robertson et al. 2002) so we did not test the effect of the two diets on WT mice. Quantitation of the percentage of seminiferous tubules containing different cell types (spermatogonia, spermatocyte, and spermatid/spermatozoa) confirmed these observations (Fig. 5C). Two-month-old Gpr54<sup>/−/−</sup> mice on a normal diet had 44% of seminiferous tubules containing spermatocytes and 17% containing spermatids/spermatozoa. Seven-month-old Gpr54<sup>/−/−</sup> mice on a normal diet had 95% of seminiferous tubules containing spermatocytes and 78% containing spermatids/spermatozoa, both of which were significantly
Gpr54

deferens of 7-month-old mice; the Tukey–Kramer multiple comparisons test) between two groups as containing spermatocytes. A versus B: using the Kruskal–Wallis test) for 2m WT, * (B) The number of spermatozoa in the epididymis of 7-month-old WT, higher than those found in 2-month-old mice. In contrast, the 7-month-old Gpr54−/− mice had well-developed testes and showed more extensive spermatogenesis than the Gpr54−/− mice at both 2 and 7 months on the phytoestrogen-free diet (Fig. 5B and C).

Figure 3  Age-related changes in the percentage of seminiferous tubules containing different spermatogenic cell types in the epididymides and vas deferens. (A) The percentage of seminiferous tubules containing spermatagonia, spermatocytes, or spermatids/spermatozoa were counted for each group of mice (n = 5 for WT at 2 and 7 months, n = 6 for Gpr54−/− and Kiss1−/− at 2 and 7 months), a versus b: P < 0.05 (nonparametric ANOVA using the Kruskal–Wallis test) for 2m WT, Gpr54−/−, or Kiss1−/− for the percentage of seminiferous tubules containing spermatocytes. A versus B: P < 0.05 (nonparametric ANOVA using the Kruskal–Wallis test) for 2m WT, Gpr54−/−, or Kiss1−/− for the percentage of seminiferous tubules containing spermatids/spermatozoa. **P < 0.05 (the Mann–Whitney U test) between 2- and 7-month-old Gpr54−/− mice for the percentage of seminiferous tubules containing spermatocytes and spermatid/spermatozoa. (B) The number of spermatogonial in the epididymis of 7-month-old WT, Gpr54−/−, and Kiss1−/− mice; n = 12 for 7m WT, n = 14 for 7m Gpr54−/−, n = 10 for 7m Kiss1−/−. **P < 0.01 (one-way ANOVA with the Tukey–Kramer multiple comparisons test) between two groups as indicated in the figure. (C) The number of spermatozoa in the vas deferens of 7-month-old mice; n = 12 for 7m WT, n = 14 for 7m Gpr54−/−, n = 12 for 7m Kiss1−/−. ***P < 0.001 (one-way ANOVA with the Tukey–Kramer multiple comparisons test) between two groups as indicated in the figure.

higher than those found in 2-month-old mice. In contrast, the 7-month-old Gpr54−/− mice on the phytoestrogen-free diet had 73% of seminiferous tubules containing spermatocytes and 30% containing spermatids/spermatozoa, which were not significantly different from the 2-month-old Gpr54−/− mice and significantly lower than those in the 7-month-old Gpr54−/− mice on the normal diet (Fig. 5C). The Kiss1−/− mice had well-developed testes and showed more extensive spermatogenesis than the Gpr54−/− mice at both 2 and 7 months on the phytoestrogen-free diet (Fig. 5B and C).

Discussion

Transgenic mice with inactivating mutations in the Gpr54 or Kiss1 genes are sterile and males show defects in spermatogenesis with impaired production of spermatozoa. It has been reported that the severity of this spermatogenic defect is variable with some mice showing a limited capacity to produce spermatozoa (Lapatto et al. 2007, Chan et al. 2009). We have investigated the cause of this variation and have found that the defect in spermatogenesis is more pronounced in Gpr54−/− mice than in Kiss1−/− mice. Sperm production was higher in the 2-month-old Kiss1−/− mice than in the Gpr54−/− mice. Kiss1−/− mice had bigger testes, epididymides, and seminal vesicles than Gpr54−/− mice at both 2 and 7 months old. Histological analysis showed that at 2 months old, the Kiss1−/− mice had a majority (97%) of seminiferous tubules containing spermatids/spermatozoa while only 17% of seminiferous tubules in Gpr54−/− mice contained spermatids/spermatozoa. As the Gpr54−/− mice age, however, they also acquired the capacity to produce lower numbers of spermatozoa.

We initially tested the hypothesis that the improved spermatogenesis in the Gpr54−/− mice was due to increased hormone levels with age. No significant increase in serum FSH or testosterone levels was found in the Gpr54−/− mice between 2 and 7 months of age, but intratesticular testosterone levels were similar between all the groups. Thus, the improved spermatogenesis with age in the Gpr54−/− mice did not correlate with increased serum hormone levels but it is possible that the intratesticular testosterone contributes to this process. Complete spermatogenesis is still found in rats where intratesticular testosterone levels have been reduced to 0.006 ng/mg tissue (Cunningham & Huckins 1979), which is lower than the intratesticular testosterone levels (0.04 ng/mg) found in the Gpr54−/− mice. Moreover, low levels of intratesticular testosterone have been found to stimulate age-onset development of spermatogenesis in mice with a targeted disruption of the LH receptor (Zhang et al. 2003). In LH receptor null mice, spermatogenesis is arrested at the spermatid stage in 2-month-old animals but by 12 months of age, these mice are capable of qualitatively full spermatogenesis with elongated spermatid production. This effect is blocked by treatment of the mutant mice with the antiandrogen flutamide, indicating that the low level of intratesticular androgens found in these mutant mice is required for this effect. An important difference between the LH receptor knockout mice and the Gpr54−/− or Kiss1−/− mice, however, is that the former have high levels of FSH, which can act synergistically with any intratesticular androgens to stimulate spermatogenesis (Haywood et al. 2003). The low levels of FSH in the Gpr54−/− and Kiss1−/− mice would not favor a synergistic action but the exact contribution of
intratesticular androgens in promoting spermatogenesis in these mutant mice needs to be investigated by flutamide treatment.

The Gpr54<sup>−/−</sup> and Kiss1<sup>−/−</sup> mice show qualitatively normal spermatogenesis even when plasma FSH levels are very low. This is consistent with the phenotype of male mice in which the FSH receptor (Dierich et al. 1998) or FSH β-subunit (Kumar et al. 1997) has been disrupted and are fertile with only partial spermatogenic failure. While FSH maintains germ cell numbers and promotes Sertoli cells maturation and germ cell progression through meiosis, it is not required for qualitatively normal spermatogenesis.

There are increasing data to support a role of estradiol in spermatogenesis. Transgenic mice deficient in aromatase (Robertson et al. 1999) or estrogen receptor α (ESR1; Eddy et al. 1996) show impaired spermatogenesis. Neonatal administration of weak environmental estrogens stimulates the first wave of spermatogenesis at puberty in rats (Atanassova et al. 2000). Chronic treatment of the GnRH-deficient (Hpg) mice with estradiol implants can increase the size of the testis and stimulate complete spermatogenesis qualitatively (Ebling et al. 2000, Baines et al. 2005, Ebling et al. 2006).

We hypothesized that estrogenic compounds found in normal mouse food may contribute to the increase in spermatogenesis found in Gpr54<sup>−/−</sup> and Kiss1<sup>−/−</sup> mice as they age. The maintenance diet of our mice contained a source of phytoestrogens, mainly genistein and daidzein from soya (around 100 μg/g food), which can mimic the function of estrogen in animals (Strauss et al. 1998), raising the possibility that dietary phytoestrogens may stimulate the completion of spermatogenesis with age. Assuming 4 g of food consumption per day, each mouse would ingest around 0.4 mg phytoestrogens/day (16 mg/kg per day), which is within the range that phytoestrogens can exert physiological effects on the male reproductive axis (Strauss et al. 1998). We found that the increase in testes weight in the Gpr54<sup>−/−</sup> mice between 2 and 7 months old did not occur when the mice were fed with the phytoestrogen-free food. Histological analysis showed that the 7-month-old Gpr54<sup>−/−</sup> mice on the phytoestrogen-free diet still had delayed spermatogenesis at mainly the spermatocyte stage, but on a normal diet could complete full spermatogenesis and produce mature spermatozoa. Spermatogenesis in adult WT mice is not inhibited by the absence of phytoestrogens in the diet (Robertson et al. 1999). These results suggest that phytoestrogens contribute to stimulating the completion of spermatogenesis under conditions of low plasma testosterone and FSH levels.

The mechanisms by which dietary phytoestrogens stimulate spermatogenesis in the mutant mice are not known. It is likely that they exert their actions through estrogen receptors but additional studies are required to define which classes are involved. Phytoestrogens can bind to both ESR1 and ESR2 (ERβ) so the pathways involved in the phytoestrogen responses will have to be defined by using selective estrogen receptor antagonists or by analysis of transgenic mice lacking both GPR54 and ESR1 or ESR2.

Other studies have also shown that dietary phytoestrogens can act to stimulate spermatogenesis in the mouse. Aromatase knockout (Ar<sup>−/−</sup>) mice (Robertson et al. 1999) cannot convert testosterone into estradiol and are initially fertile but show progressive loss of fertility with postmeiotic defects in spermatogenesis around 18 weeks old. The decline in spermatogenesis is ameliorated by providing the mice with chow containing phytoestrogens.

Figure 4 Age-related changes in hormone levels. (A) Plasma testosterone, (B) intratesticular testosterone, and (C) plasma FSH were measured by ELISA. a versus b: P<0.05 (nonparametric ANOVA using the Kruskal–Wallis test) for 2m WT, Gpr54<sup>−/−</sup>, and Kiss1<sup>−/−</sup> groups. A versus B: P<0.05 (nonparametric ANOVA using the Kruskal–Wallis test) for 7m WT, Gpr54<sup>−/−</sup>, and Kiss1<sup>−/−</sup> groups *P<0.05; **P<0.01 between mice at 2 months and mice at 7 months for individual genotypes using a Mann–Whitney U test. The number of mice in each group for plasma testosterone measurement was WT, 2m (10), 7m (13); Gpr54<sup>−/−</sup>, 2m (6), 7m (6); Kiss1<sup>−/−</sup>, 2m (11), 7m (7). The number of mice in each group for intratesticular testosterone and plasma FSH measurement was WT, 2m (5), 7m (5); Gpr54<sup>−/−</sup>, 2m (6), 7m (6); Kiss1<sup>−/−</sup>, 2m (6), 7m (6).
(Chan et al. 2009). As no major changes in the LH or FSH levels were found in these mice, the authors suggest that the phytoestrogens probably act directly on the testes rather than stimulating the pituitary. Our data are consistent with this as we observe improved spermatogenesis in the Gpr54−/− mice exposed to dietary phytoestrogens without any change in the testosterone or FSH level.

It has been proposed that estrogen stimulates spermatogenesis by increasing FSH secretion from the pituitary, which acts synergistically with testosterone (Baines et al. 2005). E2-induced spermatogenesis in Hpg mice is prevented by an androgen receptor antagonist (Baines et al. 2005) and requires ESR1 but not ESR2 signaling (Allan et al. 2010). It is not clear whether phytoestrogens stimulate spermatogenesis by a similar mechanism. We did not observe an increase in FSH levels in mutant mice maintained on a diet containing phytoestrogens similar to the increase in FSH reported for E2-treated Hpg mice. We have not tested whether estrogen can produce effects similar to phytoestrogens in our mutant mice or whether estrogens and phytoestrogens act differently on increasing FSH levels. Since phytoestrogens and estrogens have very different potencies in vivo, these experiments will require careful dose–response studies to estrogen in the mutant mice.

Most studies that have examined the effects of phytoestrogens on spermatogenesis have focussed on the possible detrimental effects of exposure during embryonic development or following chronic exposure
in adults (for review, see Cederroth et al. (2010)). These studies have produced inconsistent results with chronic exposure, causing reduced spermatogenesis and increased germ cell apoptosis in some cases (Assinder et al. 2007) but with little effect in other studies (Roberts et al. 2000). These differences may reflect differences in exposure period, type of phytoestrogen used, or end points assed. In contrast, our data show that under specific conditions of low testosterone and gonadotrophic hormone levels, dietary phytoestrogens can actually stimulate spermatogenesis.

Irrespective of the age or dietary regime, the mutant mice remained infertile even when they produced mature spermatozoa. The mutant mice never generated a copulatory plug when paired with females. This is most likely because the mutant mice do not show any mating behavior but could also be that the seminal vesicles are too small to form a plug. We favor the former explanation since others have reported that Gpr54−/− males do not exhibit normal sexual behavior such as mounts, thrusts, and intromissions when paired with females. Sexual behavior can be restored in Gpr54−/− mice by testosterone treatment (Kauffman et al. 2007). In addition, the low sperm count in the 7-month-old mutant males will also contribute to their continued sterility. The low sperm count in the vas deferens and epididymis is probably caused by lower sperm production in the testes and impaired fluid movement out of the testes due to the low testosterone levels in the mutants.

There are several unresolved questions that need to be addressed. The Kiss1−/− mice have earlier spermatogenesis and development of secondary sex organs than Gpr54−/− mice, which might be due to the higher levels of plasma testosterone reported for the Kiss1−/− mice (Chan et al. 2009) although this did not achieve statistical significance. However, the underlying mechanism of these differences between Kiss1−/− and Gpr54−/− mice on spermatogenesis and hormonal levels is still unknown. It has been previously reported that Kiss1−/− female mice exhibit two distinct phenotypes; half have severe gonadal defects, which are similar to Gpr54−/− mice, and the other half have gonadal weights comparable with those of WT females (Lapatto et al. 2007). However, no bimodal difference between these two knockouts has been reported for male mice. Our data indicate a subtle difference between Gpr54−/− and Kiss1−/− mutant male mice. This may indicate that the Kiss1−/− mice retain some residual signaling through the GPR54 receptor that is sufficient to stimulate the reproductive axis. This may be caused by ligand-independent activation of the GPR54 receptor (Levoye et al. 2006) or stimulation by another ligand, although so far none have been identified. GPR54 shows around 5% basal signaling activity independent of kisspeptin binding when expressed in tissue culture cells (Pampillo et al. 2009) so it is possible that Kiss1−/− mice may retain some GPR54 signaling activity. It has also been shown that there is some GNRH release in the Gpr54−/− and Kiss1−/− mice that may contribute to gonadal maturation (Chan et al. 2009). In the study by Chan and colleagues, treatment of Gpr54−/− or Kiss1−/− mice with the GNRH antagonist acyline prevented testicular weight increase and acquisition of sperm production. Thus, the Gpr54−/− and Kiss1−/− mice also are capable of GNRH release, independent of kisspeptin signaling through GPR54.

In summary, Kiss1−/− mice show a less severe defect in spermatogenesis than Gpr54−/− mice, suggesting a low level of kisspeptin-independent GPR54 signaling in the Kiss1−/− mice. As the mutant mice age, however, both genotypes show improved spermatogenesis caused by GPR54/kisspeptin-independent GNRH release and/or exposure to environmental phytoestrogens, although this does not restore fertility. These data show that consideration must be made to dietary phytoestrogens when studying the phenotype of mutant mice with defects in the reproductive axis.

**Materials and Methods**

**Mouse lines and maintenance**

Mice with targeted disruption of Gpr54 or Kiss1 were generated by our group as described previously (Seminara et al. 2003, d’Anglemont de Tassigny et al. 2007). The mice were maintained as a pure inbred 129S6/Sv/Ev line and mutant mice were obtained from heterozygote crosses. WT mice were littersmates of the mutant mice. Mice were maintained on a 12 h light:12 h darkness cycle (light on between 0630 and 1830 h) with ad libitum access to food and water. Experimental procedures were performed under the authority of a Home Office Project Licence and approved by a local ethics committee.

**Histology and quantitation of spermatogenesis**

Mice were killed by CO2 asphyxiation and tissues (including the left and right testes, left and right epididymides and seminal vesicles) were weighed and fixed in 4% paraformaldehyde in PBS (pH = 7.6) overnight at 4 °C. Tissues were dehydrated with gradient ethanol, paraffin embedded, and sectioned at 7 μm. The sections were stained with haematoxylin and eosin. Digital photomicrographs of sections were enlarged on a computer and each seminiferous tubule was scored for the presence of spermatogonia, spermatocytes, and spermatids/spermatozoa. The seminiferous tubules containing different cell types were counted and their proportions to the total number of seminiferous tubules were calculated.

**Sperm count in epididymides and vas deferens**

The weight of each epididymis and vas deferens was recorded and the length of the vas deferens was measured. Each epididymis or vas deferens was placed into 0.5 ml PBS and torn into pieces to release the spermatozoa. Spermatozoa were...
counted in a hemocytometer and the number was standardized to the length of the vas deferens.

**Intratesticular testosterone extraction**

One of the testes from each mouse was cut into smaller pieces in 1 ml PBS and homogenized on ice (PRO200, PRO Scientific, Inc., Oxford, CT, USA) at 12 000 g for 1 min. Samples were centrifuged at 12 000 g for 10 min at 4 °C. The supernatant was taken into a fresh tube and the volume was recorded. In all, 500 μl of the supernatant was extracted with 1 ml diethyl ether three times; 3 ml diethyl ether were pooled and evaporated overnight. The residue was dissolved in 200 μl of the zero testosterone standard from the testosterone ELISA kit (EIA-1559; DRG Instruments GmbH, Marburg, Germany) and stored in −20 °C until assayed.

**Hormone assays**

Animals were killed by CO₂ asphyxiation and the blood was collected into heparinized tubes from the vena cava and centrifuged. Plasma samples were stored in −20 °C until assayed. Plasma testosterone and intratesticular testosterone levels were measured using a testosterone ELISA kit (EIA-1559; DRG Instruments GmbH) and FSH levels were determined using a rat FSH ELISA kit (AER004; Immunodiagnostic Systems Ltd, Boldon, Tyne & Wear, UK) according to the manufacturer’s instructions. The testosterone ELISA kit had a sensitivity of 0.083 ng/ml, an interassay variation of 7%, and an intra-assay variation of 3%. The FSH ELISA kit had a detection sensitivity of 0.2 ng/ml, an interassay variation of 12%, and an intra-assay variation of 4%.

**Mice on phytoestrogen-free diet**

*Kiss1−/−* and *Gpr54−/−* mice were maintained on a phytoestrogen-free diet (AIN93M, Special Diets Services, Essex, UK) from weaning at 1 month old and the control group was given a normal mouse diet (RM3, Special Diets Services), which contained a source of phytoestrogens (~100 p.p.m. genistein and daidzein) from soya. These diets also differ in their exact formulations but these differences are unlikely to be relevant as both diets are routinely used for long-term rodent maintenance without adversely affecting male reproductive parameters. The metabolizable energy levels are 11.2 MJ/kg for RM3 and 15.1 MJ/kg for AIN93M. Mice were killed at 7 months old and tissues, including the testes, epididymides, and seminal vesicles, were weighed and fixed in 4% paraformaldehyde overnight for histological processing.

**Statistical analysis**

WT, *Kiss1−/−*, and *Gpr54−/−* mice of the same age were compared. If the sample values in each group followed a Gaussian distribution, one-way ANOVA was applied using the Tukey–Kramer multiple comparisons test. If the sample values failed to show a Gaussian distribution, a nonparametric ANOVA was applied using the Kruskal–Wallis test. The comparison between the 2- and 7-month group of mice of the same genotype was conducted separately. If the sample values followed a Gaussian distribution, an unpaired t-test with Welch’s correction was used. If the sample values failed to show a Gaussian distribution, a Mann–Whitney U test was applied. Statistical methods used for individual analyses are provided in the figure legends.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**References**


 Phytoestrogens can enhance spermatogenesis


Cunningham GR & Hucks C 1979 Persistence of complete spermatogenesis in the presence of low intratesticular concentrations of testosterone. Endocrinology 105 177–186. (doi:10.1210/endo-105-1-177)


Dungan HM, Clifton DK & Steiner RA 2006 Kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion. Endocrinology 147 1154–1159. (doi:10.1210/endo-147-4-1154)


Kumar TR, Yang W, Lu N & Matzuk MM 1997 Kisspeptin-stimulating hormone is required for ovarian follicle maturation but not male fertility. Nature Genetics 15 201–204. (doi:10.1038/ng0227-201)


Zhang FP, Pakarainen T, Poutanen M, Toppari J & Huhtaniemi I 2003 The low gonadotropin-independent constitutive production of testicular testosterone is sufficient to maintain spermatogenesis. PNAS 100 13692–13697. (doi:10.1073/pnas.2232815100)

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