***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

Kisspeptins are an overlapping family of amidated peptides, encoded by the *Kiss1* gene, that have a crucial role in regulating the mammalian reproductive axis (Oakley *et al.* 2009). Kisspeptins bind to the *GPR54* (*KISS1R*) receptor and stimulate GnRH secretion from the hypothalamus (Messenger *et al.* 2005, d'Anglemont de Tassigny *et al.* 2008). Consequently, kisspeptins stimulate gonadotrophic hormone (LH and FSH) secretion in many mammalian species (Gottsch *et al.* 2004, Irwig *et al.* 2004, Matsui *et al.* 2004, Navarro *et al.* 2004a, Thompson *et al.* 2004, Dhillo *et al.* 2005, 2007, Plant *et al.* 2006, Seminara *et al.* 2006, Caraty *et al.* 2007, Lents *et al.* 2008, Hashizume *et al.* 2010) and can advance the onset of puberty in female rats (Navarro *et al.* 2004b). Humans with inactivating mutations in *GPR54* fail to progress through puberty (de Roux *et al.* 2003, Seminara *et al.* 2003, Lanfranco *et al.* 2005, Semple *et al.* 2005) while activating mutations in kisspeptin signaling are associated with precocious

puberty (Teles *et al.* 2008, Silveira *et al.* 2010). In addition to activation of the reproductive axis at puberty, kisspeptin signaling is also required for the preovulatory LH surge in females (Kinoshita *et al.* 2005, Adachi *et al.* 2007, Clarkson *et al.* 2008) and the development of some sexual dimorphisms in the hypothalamus (for review, see Kauffman (2010)).

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 epithelial 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

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).

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 2 Representative histological changes with age in the testes and epididymides. (A) Testes and (B) epididymides from WT, *Gpr54*^{-/-}, and *Kiss1*^{-/-} mice on a normal diet at 2 months (2m) and 7 months (7m) of age. The representative histology was chosen from samples with the weight closest to the average value of each group. Arrows point at the spermatocytes in the testes of 2-month-old *Gpr54*^{-/-} mice. Scale bar represents 100 µm.

Older mutant mice are still infertile and have low sperm counts

As the *Gpr54*^{-/-} and *Kiss1*^{-/-} mice can produce mature spermatozoa at 7 months of age, the fertility of these mice was examined. *Gpr54*^{-/-} ($n=6$) and *Kiss1*^{-/-} ($n=6$) mice were housed separately with two sexually mature WT female 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-old mice was counted. The *Gpr54*^{-/-} mice had 1.3×10^5 spermatozoa/epididymis, which was around 5% of the number found in WT mice (2.5×10^6 spermatozoa/epididymis, Fig. 3B). Although the *Kiss1*^{-/-} mice had more spermatozoa (4×10^5 spermatozoa/epididymis) than *Gpr54*^{-/-} mice, this was significantly less than WT (Fig. 3B). Similarly, low sperm numbers were also observed in the vas deferens (Fig. 3C). The *Gpr54*^{-/-} mice had only 8% of the spermatozoa found in WT and the *Kiss1*^{-/-} mice had about 13% of the spermatozoa found in WT.

Improved spermatogenesis is not caused by hormonal changes

We hypothesized that the increase in sperm production with age might be caused by increased levels of testosterone. The *Gpr54*^{-/-} mice had significantly lower plasma testosterone levels than WT mice at both 2 and 7 months of age (Fig. 4A). Within the WT and *Kiss1*^{-/-} groups, some animals had testosterone levels that were considerably higher than the average value of the group. This variation was not observed in the *Gpr54*^{-/-} group. Intratesticular testosterone concentrations were similar for all genotype groups although

the *Kiss1*^{-/-} mice showed a decline between 2 and 7 months of age (Fig. 4B). Plasma FSH levels were significantly less in *Gpr54*^{-/-} and *Kiss1*^{-/-} mice at both 2 and 7 months of age compared to WT (Fig. 4C). WT and *Kiss1*^{-/-} mice showed a significant reduction in FSH between 2 and 7 months of age (Fig. 4C).

Dietary phytoestrogens may contribute to improved spermatogenesis

We hypothesized that dietary phytoestrogens might contribute to the change in spermatogenesis in the mutant mice. Both *Gpr54*^{-/-} and *Kiss1*^{-/-} 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*^{-/-} mice on the phytoestrogen-free diet. In contrast, the 7-month-old *Gpr54*^{-/-} 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*^{-/-} mice on a normal diet had 44% of seminiferous tubules containing spermatocytes and 17% containing spermatids/spermatozoa. Seven-month-old *Gpr54*^{-/-} mice on a normal diet had 95% of seminiferous tubules containing spermatocytes and 78% containing spermatids/spermatozoa, both of which were significantly

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 spermatogonia, 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 spermatozoa 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 normal diet and also at 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 low 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 anti-androgen 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

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*^{-/-}, and *Kiss1*^{-/-} groups. A versus B: $P < 0.05$ (nonparametric ANOVA using the Kruskal–Wallis test) for 7m WT, *Gpr54*^{-/-}, and *Kiss1*^{-/-} 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*^{-/-}, 2m (6), 7m (6); *Kiss1*^{-/-}, 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*^{-/-}, 2m (6), 7m (6); *Kiss1*^{-/-}, 2m (6), 7m (6).

intratesticular androgens in promoting spermatogenesis in these mutant mice needs to be investigated by flutamide treatment.

The *Gpr54*^{-/-} and *Kiss1*^{-/-} 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*^{-/-} and *Kiss1*^{-/-} mice as they age. The maintenance diet of our mice contained a source of phytoestrogens, mainly genistein and daidzein from soya (around 100 μ g/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*^{-/-} 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*^{-/-} 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 (ER β) 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*^{-/-}) 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 5 Effect of dietary phytoestrogens on testicular weight gain and histological changes with age. *Gpr54*^{-/-} ($n=6$) and *Kiss1*^{-/-} ($n=7$) mice were fed with phytoestrogen-free (P-F) food after weaning and were killed at 7m. *Gpr54*^{-/-} and *Kiss1*^{-/-} mice fed with a normal diet (N) were killed at 2 and 7m ($n=6, 14, 6$, and 14 respectively for 2m *Gpr54*^{-/-}, 7m *Gpr54*^{-/-}, 2m *Kiss1*^{-/-}, and 7m *Kiss1*^{-/-}) respectively as comparison. Testes were weighed and sectioned for histology. (A) Weight of testes in 2- and 7-month-old mutant mice on a normal diet and 7-month-old mutant mice on a phytoestrogen-free diet. * $P<0.05$ (one-way ANOVA with the Tukey–Kramer multiple comparisons test) between two groups of mice as indicated in the figure. (B) Representative histology of testes from 2- and 7-month-old mutant mice fed with a normal diet and from 7-month-old mutant mice fed with a phytoestrogen-free diet. The representative histology was chosen from samples with the weight closest to the average value of each group. Scale bar represents $100\ \mu\text{m}$. (C) The percentage of seminiferous tubules containing spermatogonia, spermatocytes, or spermatids/spermatozoa were counted for each group of mice. a versus b: $P<0.05$ (nonparametric ANOVA using the Kruskal–Wallis test) for 2 and 7m *Gpr54*^{-/-} mice on normal diet and 7m *Gpr54*^{-/-} mice on phytoestrogen-free diet on the percentages of seminiferous tubules containing spermatocyte. A versus B: $P<0.05$ (nonparametric ANOVA using the Kruskal–Wallis test) for 2 and 7m *Gpr54*^{-/-} mice on normal diet and 7m *Gpr54*^{-/-} mice on phytoestrogen-free diet on the percentage of seminiferous tubules containing spermatids/spermatozoa.

(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). E_2 -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 E_2 -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 estrogen 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 assayed. 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 epididymides 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/SvEv line and mutant mice were obtained from heterozygote crosses. WT mice were littermates 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 ethanols, paraffin embedded, and sectioned at 7 µm. The sections were stained with hematoxylin 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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