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Gonadal hormone-dependent vs. -independent effects of kisspeptin signaling in the control of body weight and metabolic homeostasis

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ABSTRACT

Background: Kisspeptins, encoded by *Kiss1*, have emerged as essential regulators of puberty and reproduction by primarily acting on GnRH neurons, via their canonical receptor, Gpr54. Mounting, as yet fragmentary, evidence strongly suggests that kisspeptin signaling may also participate in the control of key aspects of body energy and metabolic homeostasis. However, characterization of such metabolic dimension of kisspeptins remains uncomplete, without an unambiguous discrimination between the primary metabolic actions of kisspeptins vs. those derived from their ability to stimulate the secretion of gonadal hormones, which have distinct metabolic actions on their own. In this work, we aimed to tease apart primary vs. secondary effects of kisspeptins in the control of key aspects of metabolic homeostasis using genetic models of impaired kisspeptin signaling and/or gonadal hormone status.

Methods: Body weight (BW) gain and composition, food intake and key metabolic parameters, including glucose tolerance, were comparatively analyzed, in lean and obesogenic conditions, in mice lacking kisspeptin signaling due to global inactivation of Gpr54 (displaying profound hypogonadism; $Gpr54^{-/-}$) vs. Gpr54 null mice with selective re-introduction of Gpr54 expression only in GnRH cells ($Gpr54^{-/-}$Tg), where kisspeptin signaling elsewhere than in GnRH neurons is ablated but gonadal function is preserved.

Results: In male mice, global elimination of kisspeptin signaling resulted in decreased BW, feeding suppression and increased adiposity, without overt changes in glucose tolerance, whereas $Gpr54^{-/-}$ female mice displayed enhanced BW gain at adulthood, increased adiposity and perturbed glucose tolerance, despite reduced food intake. $Gpr54^{-/-}$Tg rescued mice showed altered postnatal BW gain in males and mildly perturbed glucose tolerance in females, with intermediate phenotypes between control and global KO animals. Yet, body composition and leptin levels were similar to controls in gonadal-rescued mice. Exposure to obesogenic insults, such as high fat diet (HFD), resulted in exaggerated BW gain and adiposity in global $Gpr54^{-/-}$ mice of both sexes, and worsening of glucose tolerance, especially in females. Yet, while rescued $Gpr54^{-/-}$Tg males displayed intermediate BW gain and feeding profiles and impaired glucose tolerance, rescued $Gpr54^{-/-}$Tg females behaved as controls, except for a modest deterioration of glucose tolerance after ovariectomy.

Conclusion: Our data support a global role of kisspeptin signaling in the control of body weight and metabolic homeostasis, with a dominant contribution of gonadal hormone-dependent actions. However, our results document also discernible primary effects of kisspeptin signaling in the regulation of body weight gain, feeding and responses to obesogenic insults, which occur in a sexually-dimorphic manner.

Summary of translational relevance: Kisspeptins, master regulators of reproduction, may also participate in the control of key aspects of body energy and metabolic homeostasis; yet, the nature of such metabolic actions remains debatable, due in part to the fact that kisspeptins modulate gonadal hormones, which have metabolic

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actions on their own. By comparing the metabolic profiles of two mouse models with genetic inactivation of kisspeptin signaling but different gonadal status (hypogonadal vs. preserved gonadal function), we provide herein a systematic dissection of gonadal-dependent vs. -independent metabolic actions of kisspeptins. Our data support a global role of kisspeptin signaling in the control of body weight and metabolic homeostasis, with a dominant contribution of gonadal hormone-dependent actions. However, our results document also discernible primary effects of kisspeptin signaling in the regulation of body weight gain, feeding and responses to obesogenic insults, which occur in a sexually-dimorphic manner. These data pave the way for future analyses addressing the eventual contribution of altered kisspeptin signaling in the development of metabolic alterations, especially in conditions linked to reproductive dysfunction.

1. Introduction

Kisspeptins, a family of peptides encoded by *Kiss1* that operate via the G protein coupled receptor, Gpr54 (also named Kiss1R), have been recognized in recent years as master regulators puberty and gonadal function [1,2]. This fundamental role is illustrated by the fact that humans and mice with genetic inactivation of *Kiss1* or *Gpr54* suffer from hypogonadism of central origin [3,4]. The primary site of action of kisspeptins in the central control of reproduction is the hypothalamus, where they are known to potently stimulate GnRH secretion, and thereby gonadotropin secretion, thus ultimately controlling gonadal function [1,2]. In fact, GnRH neurons express Gpr54 and kisspeptins can potently activate GnRH neurons, as documented by the induction of c-Fos, electrical firing and neurosecretion [5]. Hence, Kiss1 neurons operate as upstream regulators of GnRH neurons, responsible for the transmission of at least part of the modulatory actions of key factors affecting reproduction, including metabolic cues [6].

In spite of the conclusive data of the direct action of kisspeptins on GnRH neurons, there is growing evidence suggesting that kisspeptins are capable to activate non-GnRH cells, either in the brain or elsewhere in the body, and may contribute to the regulation of various non-reproductive homeostatic systems [7,8]. In this context, the possibility that kisspeptins may also participate in the control of key aspects of metabolism has recently gained momentum [8]. Thus, although initial pharmacological data failed to demonstrate any detectable effect of kisspeptin on body weight in rats [9], more recent studies suggested that kisspeptins activate POMC neurons [10], which express Gpr54 in a substantial fraction [11], as putative mechanism to suppress feeding [12]. In addition, Kiss1 and AgRP neurons have been shown to reciprocally inhibit each other [13,14]. However, the physiological relevance of these complex interactions is yet to be fully demonstrated and may involve transmitters other than kisspeptins, such as glutamate [13,15]. In addition, a very recent study has suggested that Kiss1 neurons in the hypothalamic arcuate nucleus (ARC) participate in the daily timing of food intake, together with the rhythmic regulation of body temperature and locomotor activity [16]. Moreover, a role for ARC Kiss1 neurons in the control of body weight responses to obesogenic insults has been also suggested, since toxin-mediated ablation of at least a fraction of Kiss1 cells in the ARC has been shown to prevent the body weight gain following gonadectomy in female rats, and to abolish the body weight suppressing effects of estrogen. These effects are likely mediated via the co-transmitter, neurokinin B (NKB) [17]. In fact, since ARC Kiss1 neurons do express other neuropeptides and transmitters, the specific contribution of kisspeptin signaling to the latter phenomenon remains unsolved.

Expression of *Kiss1* and *Gpr54* has been documented also in a number of peripheral metabolic tissues, including fat, liver and pancreas, therefore suggesting additional roles for kisspeptins in metabolic homeostasis [8]. To date, a limited number of studies have reported the expression of *Kiss1* in the white adipose tissue [18,19], but the physiological relevance of such expression remains unknown. Likewise, pharmacological studies have pointed out potential actions of kisspeptins on pancreatic function, although both stimulatory and inhibitory effects on insulin secretion, depending on the prevailing glycaemia, have been reported [20,21]. In this context, liver-born kisspeptins have been suggested to operate as inhibitory signals on pancreatic insulin secretion; a phenomenon that was proposed to contribute to the pathogenesis of type 2 diabetes [22]. Notwithstanding, the evidence for a genuine metabolic function of kisspeptins remains, in many cases, inconclusive. This makes mandatory the use of additional, more incisive approaches, to fully expose the physiological actions, and eventual pathogenic roles, of kisspeptin signaling in the control of key facets of metabolism.

In an attempt to surface the physiological roles of kisspeptins in the control of metabolism, functional genomic studies have been applied also. Pioneering studies in mice revealed that whole-body elimination of Gpr54 resulted in increased body weight and impaired glucose homeostasis [23], specifically in females. In addition, a suppression of feeding, locomotor activity and energy expenditure was also noticed in Gpr54 KO mice [23]. These initial studies were later extended by analyses in Gpr54 KO female mice at 6-wk of age, i.e., before differences in body weight were detected. These studies revealed that increased adiposity and decreased energy expenditure, but not impaired glucose homeostasis, can be detected before the onset of obesity in female mice with congenital ablation of kisspeptin signaling [24].

It must be noted, however, that due to complete absence of kisspeptin signaling, Gpr54 null mice suffer also a profound state of hypogonadism, which manifests already postnatally; e.g., global Gpr54 null mice do not display any of the manifestations of puberty onset, such as preputial separation in males or vaginal opening in females, reflecting early-onset sex steroid deficiency [3]. In this context, it is well known that gonadal steroids per se have important metabolic roles, with a discernible impact on feeding, energy expenditure and glucose homeostasis [25]. This raises the possibility that part of the metabolic phenotype of Gpr54 KO mice might be secondary to their hypogonadal state. Admittedly, studies in Gpr54 KO mice aimed to control for this key confounding factor by analyzing also gonadectomized (GNX) animals. However, due to operational reasons, GNX could only be performed at 2.5 weeks postpartum [23], which hampers full discrimination of the actual contribution of early-onset hypogonadism to the metabolic perturbations seen in whole-body Gpr54 null mice.

In the present study, we have taken advantage of a recently generated genetically-modified mouse model in which, upon a global Gpr54 deficient background, expression of Gpr54 was selectively rescued in GnRH-expressing cells; a mouse line named Gpr54$^{-/-}$Tg rescued mouse [26]. By virtue of such GnRH-specific Gpr54 (re)expression, the functionality of the gonadal axis is globally preserved, despite absence of Gpr54-mediated signaling elsewhere than in GnRH neurons [26]. This model offers a unique setting where to tease apart genuine direct kisspeptin actions on metabolism vs. those secondary to kisspeptin-derived changes of gonadal hormone secretion.

2. Materials and methods

2.1. Generation of Gpr54$^{-/-}$Tg (rescued) mouse line

A GnRH cell-specific Gpr54 expressing (rescued) mouse line, generated using BAC transgenesis, and validated by the groups of G. Schuzt

and M. Kirilov (German Cancer Research Center, Heidelberg, Germany) and A.E. Herbison (Centre of Neuroendocrinology, University of Otago, NZ) [26], was transferred to our laboratory for extensive reproductive phenotyping [27]. In this mouse line, named hereafter Gpr54$^{-/-}$Tg, the selective (re)expression of Gpr54 transgene in GnRH cells, over a Gpr54 null background, permits full rescue of gonadal function at postnatal and young adult ages [26,27]. Genotyping was conducted by PCR analyses on isolated genomic DNA from tail biopsies [27]. The primers used for detection the wild-type (WT) allele were: T1003E: 5′-GCC TAA GTT TCT CTG GTG GAG GAT G-3′ and T1003TE: 5′-CGC GTA CCT GCT GGA TGT AGT TGA C-3′, while the primer pair used to detect the mutated allele was: NeoT: 5′-GGG TGG GAT TAG ATA AAT GCC TGC TCT-3′ and T1003TE: 5′-CGC GTA CCT GCT GGA TGT AGT TGA C-3′. To evaluate the presence of the Gpr54 transgene, with directed expression to GnRH cells, the primers used were GnRH_F: 5′-GGT TTC AGG GAA CCC AAA TTA-3′ AND GPR54_R: 5′-ACC AAT GAG TTT CCG ACC AG-3′. The PCR conditions and amplicons produced have been described in detail elsewhere [27]. As breeding strategy, heterozygous Gpr54$^{+/-}$ male mice harboring the GnRH-driven Gpr54 Tg were crossed with Gpr54$^{+/-}$ female mice without the Tg (Suppl. Fig. S1). Accordingly, in addition to heterozygous animals, four genotypes homozygous for the Gpr54 allele were generated: Gpr54$^{+/+}$(WT), Gpr54$^{+/+}$Tg, Gpr54$^{-/-}$ (KO) and Gpr54$^{-/-}$Tg (rescued). Comparison of metabolic features of Gpr54$^{+/+}$ and Gpr54$^{+/+}$Tg mice of both sexes revealed no differences between these two control lines regarding key parameters, as body weight, food intake and basal glucose levels (Suppl. Fig. S1). Thus, in line with previous studies [27], the genotypes considered for analysis were Gpr54$^{+/+}$ (WT; control), Gpr54$^{-/-}$ (KO) and Gpr54$^{-/-}$Tg (rescued).

2.2. Drugs, reagents and diets

Ghrelin (cat# H-4862) was obtained from Bachem (Bubendorf, Switzerland). Glucose (cat# G5767) and insulin (cat# 12643) were purchased from Sigma Aldrich (Saint Louis, MI, USA). The drugs were dissolved in saline (0.9% NaCl). Mice were fed ad libitum with either chow diet (≪5% calories from fat) or a standard high fat diet (HFD, D12451; 45% of calories from fat, 20% from protein, and 35% from carbohydrate; Research Diets Inc. New Brunswick, NJ).

2.3. Experimental design

2.3.1. General procedures

All the experimental protocols were approved by the Córdoba University Ethical Committee of animal experimentation and conducted in accordance with the European Union guidelines for use of experimental animals. For hormonal analyses, blood samples were obtained by jugular venipuncture using standard procedures, routinely running in our laboratory [28–30]. For specific pharmacological studies, protocols of intracerebroventricular (icv) administration were conducted. Cannulas (INTRADEMIC polyethylene Tubing, Becton Dickinson, Sparks, MD, USA) were implanted 24-h before the beginning of the test, at the following coordinates: cannulas were lowered to a depth of 2 mm beneath the surface of the skull; the insertion point being 1 mm posterior and 1.2 mm lateral to bregma, in keeping with standard procedures at our group [28–30]. A mouse brain atlas was used for reference purposes [31]. After cannulation, the mice were housed in individual cages until the end of the experiments. At the end of the experimental procedures, mice were anesthetized and euthanized by decapitation. The tissues used for RNA isolation and PCR analyses, which included the hypothalamus, brown adipose tissue (BAT) and subcutaneous white adipose tissue (WAT), were dissected and stored at −80 °C until further processing and assays. Hypothalamic dissection was conducted as described previously elsewhere [30].

2.3.2. Exp. study 1: Evaluation of metabolic parameters in Gpr54$^{-/-}$ and Gpr54$^{-/-}$ Tg mice in lean conditions

To discriminate the primary physiological role of kisspeptin signaling in the control of key metabolic parameters, body weight (BW) and composition, leptin levels (as additional marker of adiposity) and glucose tolerance were comparatively assessed in wild-type (WT) mice (Gpr54$^{+/+}$), Gpr54$^{-/-}$ null mice and Gpr54$^{-/-}$Tg rescued mice. Studies were conducted in both sexes in mice fed ad libitum with chow diet. BW gain was monitored on a weekly basis in groups of male and female mice of the three genotypes, from weaning (at 3-wk; PND21) until the 18-wk of age. Group sizes were as follows. Males: Gpr54$^{+/+}$ (N = 10), Gpr54$^{-/-}$Tg (N = 7); and Gpr54$^{-/-}$ (N = 12); Females: Gpr54$^{+/+}$ (N = 16), Gpr54$^{-/-}$Tg (N = 6); and Gpr54$^{-/-}$ (N = 9). Differences in final group sizes were mainly due to variable generation of the different genotypes; in any event, a minimal number of 6–7 individuals per group was secured in all studies to guarantee sufficient statistical robustness. At the end of the experiment (20-wk of age), body composition analyses were conducted. Both fat and lean mass were measured by quantitative magnetic resonance (QMR) using the EchoMRI™ 700 analyzer (Houston, TX, software v.2.0). In addition, circulating leptin levels were measured in the three genotypes, in groups of 18–20 week-old male and female mice. Blood samples were collected as described above, and sera were stored at −20 °C until used for hormone analyses. Group sizes were as follows. Males: Gpr54$^{+/+}$ (N = 12); Gpr54$^{-/-}$Tg (N = 13); and Gpr54$^{-/-}$ (N = 12); Females: Gpr54$^{+/+}$ (N = 8); Gpr54$^{-/-}$Tg (N = 10); and Gpr54$^{-/-}$ (N = 6). In addition, in order to evaluate the primary roles of kisspeptin signaling in glucose homeostasis, adult male and female mice of the three genotypes (N = 8–12 animals/group) were subjected to glucose tolerance test (GTT). Mice were fasted for 4 h and subsequently received an intraperitoneal (ip) bolus of glucose (2 g/kg BW). Glucose levels were determined before (0′) and at 20, 60, and 120-min post administration. Glucose concentrations were measured using a handheld glucometer (ACCU-CHECK Aviva; Roche Diagnostics). Integral glucose changes were estimated by the trapezoidal rule method as the area under the curve (AUC) during the 120 min period. When relevant, increment AUC values over basal levels were calculated. Finally, since perturbations of GTT were selectively detected in females, an insulin tolerance test (ITT) was conducted specifically in adult female mice of the three genotypes. Mice (N = 9–12 animals/group) were fasted 4 h and glucose levels were measured before (0′) and at 20, 60, and 120-min after ip injection of insulin (0,75 UI). Integral glucose changes were estimated by trapezoidal method as AUC during the 120 min following insulin administration.

2.3.3. Exp. study 2: Feeding patterns in Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg mice in lean conditions

To tease apart the primary roles of kisspeptin signaling on food intake vs. the potential impact of gonadal hormones, spontaneous feeding was monitored in Gpr54$^{-/-}$ null and Gpr54$^{-/-}$Tg rescued mice, under lean conditions (i.e., receiving chow diet). Food intake was recorded manually, in parallel by two experienced researchers following standard procedures at our laboratory [30], during a 24 h period; accumulated feeding was calculated during the light (from 9:00 to 19:00 h) and dark phases (from 19:00 to 9:00 h), with the following group sizes: Males = 16 mice/group; Females = 9–12 mice/group. In addition, BW was measured before and after the food intake test to estimate the efficiency of food assimilation. In addition, the hypothalamic gene expression of key neuropeptides controlling feeding was monitored in independent groups of male and female mice of the three genotypes by means of RT-qPCR (N = 5 mice/group). Finally, to further interrogate the differences in the feeding patterns observed between Gpr54$^{-/-}$Tg and Gpr54$^{-/-}$ mice, feeding responses to an acute bolus of the orexigenic hormone, ghrelin, were explored in male mice. To this end, icv administration of a single bolus of ghrelin (5 μg in 5 μl) was conducted, and food intake was monitored at 1-, 3-, 6-, 10- and 24-h after injection. Group sizes were: Gpr54$^{+/+}$ (N = 8); Gpr54$^{-/-}$Tg (N = 10); and Gpr54$^{-/-}$ (N = 5).

2.3.4. Exp. Study 3: Evaluation of metabolic parameters in Gpr54$^{-/-}$ and Gpr54$^{-/-}$ Tg mice in obese conditions

To assess the impact of obesogenic insults on the putative functions of kisspeptins in the control of key metabolic parameters, analyses similar to those in studies 1 and 2 were conducted in male and female mice of the three genotypes, subjected to a high fat content diet from weaning (PND21) until the 18-wk of age. Experiments were conducted under strictly similar conditions as in studies 1 and 2, thus allowing the proper comparison of data. BW gain was monitored on a weekly basis and body composition analyses were carried out at the end of the experimental period. GTT and food intake monitoring were conducted 11-wks after initiation of the HFD. The group sizes for the different analyses were as follows: BW and body composition analyses – Males: Gpr54$^{+/+}$ ($N = 14$); Gpr54$^{-/-}$Tg ($N = 9$); Gpr54$^{-/-}$ ($N = 18$); Females: Gpr54$^{+/+}$ ($N = 6$); Gpr54$^{-/-}$T (N = 6); Gpr54$^{-/-}$ ($N = 8$). GTT – Males: $N = 7$–8 mice/group; Females: N = 6–8 mice/group. FI – Males: N = 8 mice/group; Females: N = 6–8 mice/group.

In addition, the impact of another obesogenic insult, namely, ovariectomy (OVX) [32], was evaluated in Gpr54$^{-/-}$Tg and Gpr54$^{-/-}$ null mice, either alone or in combination with HFD. Admittedly, the impact of adult OVX might vary across the genotypes, depending on the prevailing gonadal status, which is preserved in control and Gpr54$^{-/-}$Tg rescued mice, but suppressed in Gpr54$^{-/-}$ KO mice. Adult female mice of the three genotypes were subjected to bilateral OVX, following standard protocols in our laboratory [32]. Subgroups of OVX females were fed HFD or chow for 11 weeks, and BW gain (OVX, $N = 7$; OVX + HFD, $N = 6$–9); glucose tolerance (OVX, $N = 8$–15; OVX + HFD, N = 8–12); and food intake (OVX, N = 7; OVX + HFD, N = 6–9) were analyzed, using procedures similar to those described for the experimental studies 1–2.

2.4. Quantitative Real time RT-PCR

Real time PCR was performed on a CFX Real-Time System and C1000TM Thermal-Cycler (Bio-Rad). For PCR, we used SYBR Green qPCR Master Mix (Promega). For analyses of gene expression, the primer pairs used were as follow: *Pomc* forward (5′-TGA AAA CCC CCG GAA GTA CG-3′), *Pomc* reverse (5′-ACG TTG GGG TAC ACC TTC AC-3′); *Cart* forward (5′-ATC GGG AAG CTG TGT GAC TG-3′), *Cart* reverse (5′-TCT CTG AGG GGA ACG CAA AC -3′); *Npy* forward (5′-CAG AAA ACG CCC CCA GAA CAA GG-3′), *Npy* reverse (5′-GGC AGA CTG GTT TCA GGG GAT GGA T-3′); *Agrp* forward (5′-CAG AAA ACG CCC CCA GAA CAA-3′), *Agrp* reverse (5′-GGC AGA CTG GTT TCA GGG GAT GGA T-3′); *Ucp1* forward (5′-GCC GGG TTT TGC ACC ACA CT-3′), *Ucp1* reverse (5′-CCA GGA CCC GAG TCG CAG AA-3′); and *Ucp3* forward (5′-ACC ATG GTT GGA CTT CAG CC-3′), *Ucp3* reverse (5′-TGG GTT CTC CCC TTG GAT CT-3′). The PCR conditions used were: denaturing for 5 min at 95 °C, followed by 40 cycles consisting of denaturing at 95 °C for 30 s, annealing at 57 °C (except for Npy, for which annealing was at 62 °C, and Ucp1 and Ucp3, with an annealing temperature of 60 °C) for 30 s and extension at 72 °C for 1 min. Relative standard curves were constructed from serial dilutions of a pool of cDNAs generated by mixing equal amounts of cDNA from each sample. The C_T from each sample was referred to the relative standard curve to estimate the mRNA content/sample. The values obtained were normalized by using S11 ribosomal protein mRNA with the following primer pair: RP-S11 forward (5′-CAT TCA GAC GGA GCG TGC TTA C-3′) and RP-S11 reverse (5′-TG CAT CTT CAT CTT CGT CAC-3′). The PCR conditions were: initial denaturation and enzyme activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 70 °C for 10 s.

2.5. Hormonal analyses

Serum levels of LH and FSH levels were measured using RIA kits supplied by the National Institutes of Health (Dr. A. F. Parlow, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labeled with ^{125}I by the chloramine-T method. LH and FSH concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2, respectively, as standards. Intra- and inter-assay coefficients of variation were ≪8% and ≪10% for LH and ≪6% and ≪9% for FSH, respectively. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. Serum levels of leptin were quantified using commercial radioimmunoassay (RIA) kits from Merck (Multi-species leptin RIA kit. Cat# XL-85K); the sensitivity of the assay was 0.5 ng/ml. Insulin levels were measured using RIA kits from LINCO Research, with a sensitivity of 0.1 ng/ml. In addition, sex steroid levels were measured in serum samples using a previously validated, sensitive gas chromatography-tandem mass spectrometry (GC–MS/MS) method, as described in detail elsewhere [33]. To assess the state of gonadal function in male and female mice of the three genotypes, testosterone (T) and dihydrotestosterone (DHT) were assayed in males, while progesterone (P) and estradiol (E_2) were measured in females. In addition, androstenedione levels were also determined. The lower detection limits of the assay were: 4 pg/ml for T, 1.6 pg/ml for DHT, 0.3 pg/ml for E_2, 8 pg/ml for P, and 4 pg/ml for androstenedione, in keeping with previous references [33].

2.6. Presentation of data and statistic

Statistical analyses were achieved using Prism software (GraphPad Prism 5.0, GraphPad Software, La Jolla, California, USA, www.graphpad.com). All data are presented as mean ± SEM for each group. Parametric tests were applied for assessment of statistically significant differences between groups. In detail, unpaired Student *t*-tests (for comparisons between two groups) and ANOVA followed by post hoc Student-Newman-Keuls tests (for comparisons of more than two groups) were used to assess variation between experimental groups. The significance level was set at $P \leq 0.05$.

3. Results

3.1. Gonadal-dependent vs. independent effects of kisspeptin signaling manipulation in lean mice

Initial hormonal analyses were conducted in Gpr54$^{+/+}$ (WT; control), Gpr54$^{-/-}$ (KO) and Gpr54$^{-/-}$Tg (rescued) mice, in order to assess the functional status of the gonadotropic/gonadal axis in each genotype. Compared to Gpr54$^{+/+}$ mice, global Gpr54$^{-/-}$ KO animals displayed significantly suppressed LH levels, in both males and females. Alike, circulating FSH levels were reduced in Gpr54 null animals, although the inhibition in female Gpr54$^{-/-}$ mice fell shortly below the level of statistical significance (Suppl. Fig. S2 A–B and E–F). In addition, gonadal steroid levels were significantly lower in Gpr54$^{-/-}$ mice of both sexes. Thus, in Gpr54$^{-/-}$ male mice, serum concentrations of testosterone and DHT were suppressed vs. control levels in Gpr54$^{+/+}$ males (Suppl. Fig. S2 C—C), while in Gpr54$^{-/-}$ females, serum levels of estradiol and progesterone were significantly reduced (Suppl. Fig. S2 G—H). In clear contrast, neither the circulating levels of LH and FSH, nor the serum concentrations of sex steroids were different between control (Gpr54$^{+/+}$) and Gpr54$^{-/-}$Tg mice, therefore confirming the efficacy of our rescue strategy to reverse the profound central hypogonadism caused by whole-body ablation of Gpr54. Of note, despite the profound suppression of gonadal steroid levels in Gpr54$^{-/-}$ KO mice, the levels of the adrenal-born androgen, androstenedione, were similar in males of the three genotypes (Gpr54$^{+/+}$: 33.66 ± 3.65; Gpr54$^{-/-}$: 39.77 ± 2.74; and Gpr54$^{-/-}$Tg: 43.05 ± 4.31 pg/mL), therefore confirming the central nature of the hypogonadal state of Gpr54$^{-/-}$ mice, and its rescue in Gpr54$^{-/-}$Tg mice.

Compared to Gpr54$^{+/+}$ mice, BW gain was markedly attenuated throughout the study period (between PND21 and 18-wks postpartum) in Gpr54$^{-/-}$ male mice, while Gpr54$^{-/-}$Tg rescued males displayed an intermediate phenotype (Fig. 1A). Considering the genuine differences in body weight gain and growth between the pre/peripubertal period

(considered as ≤6-wk postpartum) and adulthood (≫6-wk), accumulated BW gain was independently calculated for both periods, either as increase of integral BW gain calculated as AUC (Fig. 1B), or as net difference in terms of absolute BW between the start and end of the study periods (Fig. 1C). These analyses confirmed the intermediate phenotype of Gpr54$^{-/-}$Tg males in terms of BW gain during the pre/peripubertal period, as they showed greater BW gain than Gpr54 null mice, but smaller than Gpr54$^{+/+}$ controls. In contrast, the absolute increase of BW during the adult period (≫6-wk) was not statistically different between the three genotypes (Fig. 1C). In addition, body composition analyses revealed that Gpr54$^{-/-}$ male mice, but not Gpr54$^{-/-}$Tg males, show increased fat mass and decreased lean body mass at 18-wks of age, together with a significant increase in circulating leptin (Fig. 1D–E). Finally, neither Gpr54$^{-/-}$ nor Gpr54$^{-/-}$Tg males displayed significant changes of glucose tolerance, as revealed by GTT in adulthood (Fig. 1F–G).

In females, differences in the BW gain curves were less marked than in males (Fig. 2A), with clear differences between the pre/peripubertal and adult periods. Thus, BW gain was suppressed in both Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg females in the ≤6-wk period, when calculated as integral (AUC) gain, whereas no difference was detected during adulthood (≫6 wks.; Fig. 2B). In contrast, when calculated as net difference between initial and final BW, a significant increase was detected in the adult period, but only for the global Gpr54$^{-/-}$ female mice (Fig. 2C). In line with this observation, terminal determinations of body composition and leptin levels revealed increased adiposity and leptin concentrations, and decreased lean mass, in Gpr54$^{-/-}$, but not in Gpr54$^{-/-}$Tg female mice (Fig. 2D–E). In addition, in contrast to males, both Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg female mice displayed perturbed glucose tolerance, albeit with differences between the genotypes. Thus, while Gpr54$^{-/-}$ females showed elevated basal glucose levels and worse glycemic profiles at 20-, 60- and 120-min after the glucose bolus administration, Gpr54$^{-/-}$Tg female mice had an intermediate phenotype, with significantly higher glucose levels than controls only at 20-min of GTT; a profile that was also reflected as integral (AUC) glucose responses, whose magnitude was: Gpr54$^{+/+}$ ≪ Gpr54$^{-/-}$Tg ≪ Gpr54$^{-/-}$ (Fig. 2F–G). However, neither Gpr54$^{-/-}$ nor Gpr54$^{-/-}$Tg female mice suffered detectable perturbations of basal insulin levels, despite a subtle trend from decreased concentrations in Gpr54$^{-/-}$ (Suppl. Fig. S3 A), neither did they show perturbed insulin sensitivity, as evaluated by ITT (Suppl. Fig. S3 B).

3.2. Food intake patterns in Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg male and female mice

The 24-h patterns of food intake were monitored in both global Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued mice. Cumulative food intake during light and dark phases, as well as for a total 24-h period, was recorded in both males and females. In males, only Gpr54$^{-/-}$ mice displayed a consistent suppression of feeding, compared to Gpr54$^{+/+}$, both during the light and dark periods, whereas Gpr54$^{-/-}$Tg males had food intake patterns indistinguishable from WT animals (Fig. 3A). In contrast, feeding was also partially suppressed in Gpr54$^{-/-}$Tg female mice, which ate less than control mice all through the study period, with an intermediate phenotype between control and global Gpr54$^{-/-}$ mice during the dark phase and for the total 24-h period (Fig. 3B). This intermediate profile was also detected when the ratio between 24-h feeding and BW was calculated for the three genotypes. Interestingly, expression analyses of food intake controlling genes revealed a significant increase of hypothalamic *Pomc* gene expression in Gpr54$^{-/-}$ of both sexes, which in the case of females was associated with a rise of *Cart* expression levels also (Fig. 3C–D). In contrast, neither *Pomc* nor *Cart* expression was altered in the hypothalamus of Gpr54$^{-/-}$Tg mice, nor was the hypothalamic expression of the orexigenic genes, *Npy* and *Agrp*, changed in either Gpr54$^{-/-}$ or Gpr54$^{-/-}$ Tg mice of both sexes (*data not shown*).

To further interrogate the divergence of feeding patterns between Gpr54$^{-/-}$ (suppressed food intake) and Gpr54$^{-/-}$Tg mice (feeding

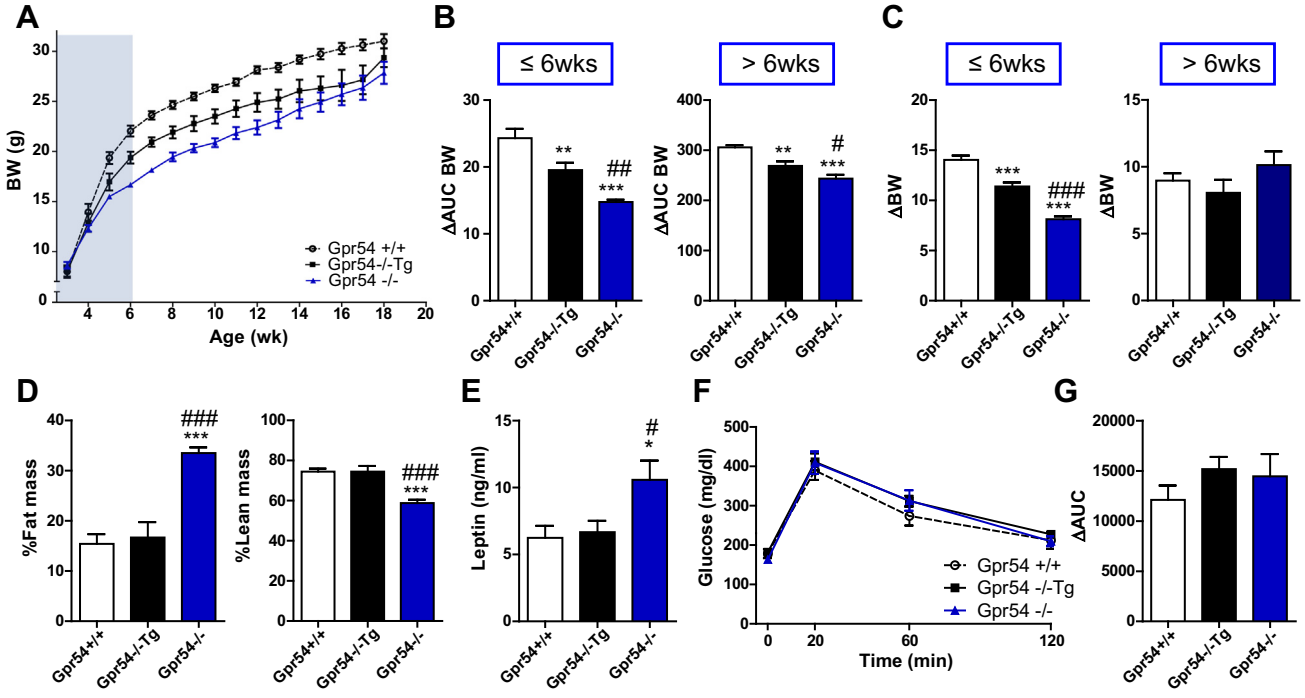


Fig. 1. Basic metabolic parameters in male Gpr54$^{-/-}$ null and Gpr54$^{-/-}$Tg rescued mice. (A) Postnatal evolution of body weight (BW) gain from weaning (PND21) to adulthood (18-wk of age) is presented from WT (Gpr54$^{+/+}$), global Gpr54 KO (Gpr54$^{-/-}$) and Gpr54$^{-/-}$Tg rescued male mice. In addition, BW gain analyses were split into two age-windows: ≤6-wk and ≫6-wk of age; for both periods, integral BW gain (represented as Δ AUC BW in B) and net differences in terms of absolute BW between the start and end of each study period (shown as Δ BW, in C) are presented. Body composition analyses (D) and serum leptin levels (E) are also shown for the three genotypes at adulthood (18-wk of age). Finally, glucose tolerance tests (GTT) are presented for Gpr54$^{+/+}$, Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued male mice, both as 120-min time-course profiles (F) and net increment of integral glucose levels after ip injection of the glucose bolus (represented as ΔAUC in G). Data are presented as mean ± SEM. *P ≪ 0.05, **P ≪ 0.01 and ***P ≪ 0.001 vs. corresponding WT (Gpr54$^{+/+}$) groups; #P ≪ 0.05, ##P ≪ 0.01 and ### P ≪ 0.001 vs. corresponding Gpr54$^{-/-}$Tg rescued mice (ANOVA followed by Student-Newman-Keuls multiple range test).

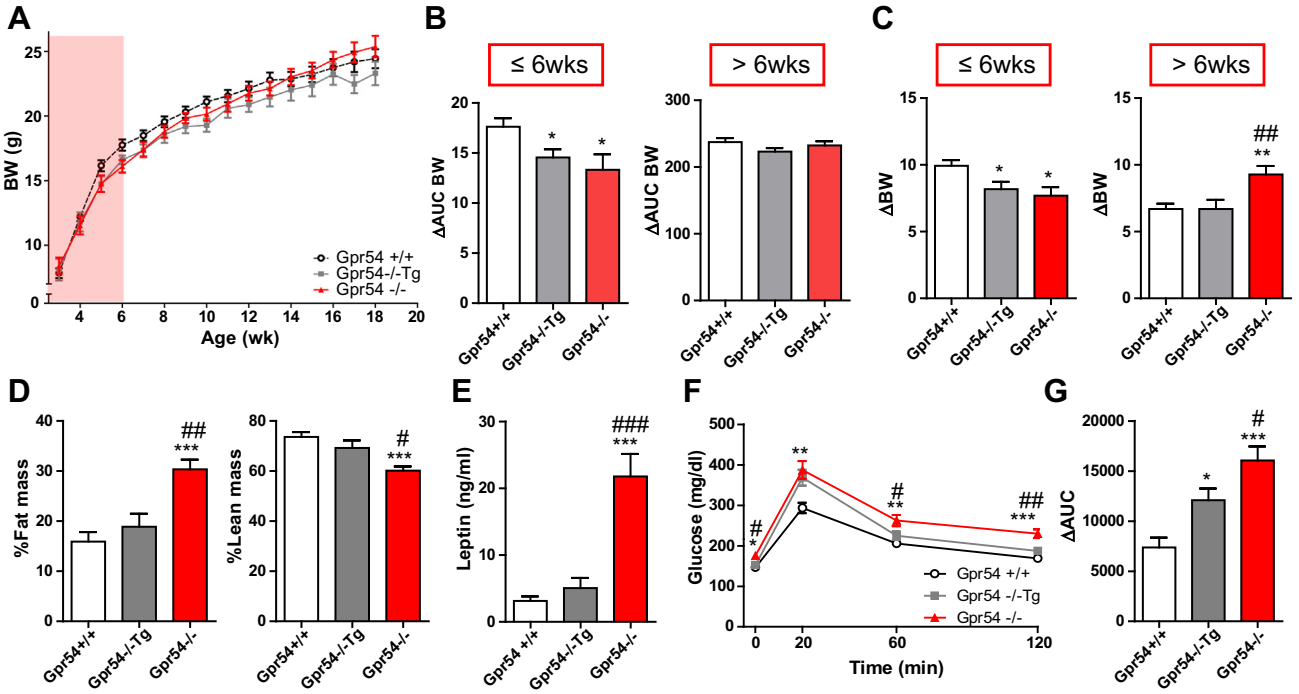


Fig. 2. Basic metabolic parameters in female Gpr54$^{-/-}$ null and Gpr54$^{-/-}$Tg rescued mice. (A) Postnatal evolution of body weight (BW) gain from weaning (PND21) to adulthood (18-wk of age) is presented from WT (Gpr54$^{+/+}$), global Gpr54 KO (Gpr54$^{-/-}$) and Gpr54$^{-/-}$Tg rescued female mice. In addition, BW gain analyses were split into two age-windows: ≤6-wk and ≫6-wk of age; for both periods, integral BW gain (represented as Δ AUC BW in B) and net differences in terms of absolute BW between the start and end of each study period (shown as Δ BW, in C) are presented. Body composition analyses (D) and serum leptin levels (E) are also shown for the three genotypes at adulthood (18-wk of age). Finally, glucose tolerance tests (GTT) are presented for Gpr54$^{+/+}$, Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued female mice, both as 120-min time-course profiles (F) and net increment of integral glucose levels after ip injection of the glucose bolus (represented as Δ AUC in G). Data are presented as mean ± SEM. *P ≪ 0.05, **P ≪ 0.01 and ***P ≪ 0.001 vs. corresponding WT (Gpr54$^{+/+}$) groups; #P ≪ 0.05, ##P ≪ 0.01 and ###P ≪ 0.001 vs. corresponding Gpr54$^{-/-}$Tg rescued mice (ANOVA followed by Student-Newman-Keuls multiple range test).

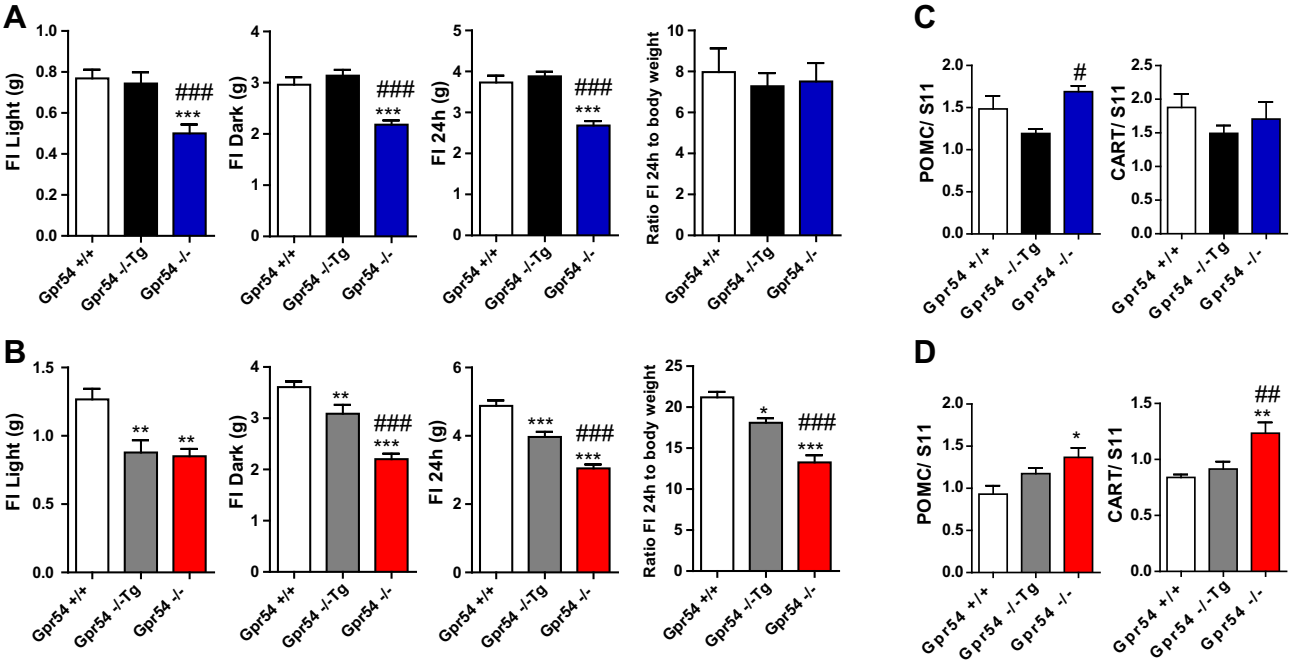


Fig. 3. Feeding patterns in Gpr54$^{-/-}$ null and Gpr54$^{-/-}$Tg rescued mice of both sexes. Spontaneous food intake was evaluated, after overnight fasting (≪12-h), during a 24-h period in WT (Gpr54$^{+/+}$), global Gpr54 KO (Gpr54$^{-/-}$) and Gpr54$^{-/-}$Tg rescued mice of both sexes (males in A; females in B). Food intake data during the light and dark phases, as well as the integral feeding during the 24-h period, are presented, together with the food intake/BW ratios. In addition, the expression levels of the anorectic genes, *Pomc* and *Cart* were measured in the mediobasal hypothalamic tissue from male (C) and female (D) mice of the three genotypes. Data are presented as mean ± SEM. *P ≪ 0.05, **P ≪ 0.01 and ***P ≪ 0.001 vs. corresponding WT (Gpr54$^{+/+}$) groups; #P ≪ 0.05, ##P ≪ 0.01 and ###P ≪ 0.001 vs. corresponding Gpr54$^{-/-}$Tg rescued mice (ANOVA followed by Student-Newman-Keuls multiple range test).

similar to controls) specifically in males, cumulative food intake was evaluated after challenge with the orexigenic compound, ghrelin, in this sex. Of note, similar ghrelin tests were not conducted in females, since a suppression of spontaneous food intake was already observed in Gpr54$^{-/-}$Tg female mice in basal conditions. In keeping with the proven capacity of ghrelin to stimulate feeding [34], icv injection of an effective dose of ghrelin evoked consistent increases of food intake during the first 10-h after the administration in Gpr54$^{+/+}$ mice. A strictly similar response to ghrelin was detected in Gpr54$^{-/-}$Tg mice. In clear contrast, Gpr54$^{-/-}$ male mice were resistant to the acute orexigenic effects of ghrelin (during the first 10-h), but showed a delayed increase of feeding, detectable during the 24-h period after icv injection of ghrelin (Suppl. Fig. S4).

3.3. Expression of thermogenic markers in adipose tissue of Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg mice

The mRNA levels of the genes encoding the thermogenic proteins, UCP1 and UCP3 [35], were assessed in brown (BAT) and subcutaneous white adipose (WAT) tissue samples from Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued mice, of both sexes. No significant differences in the expression levels of UCP1 or UCP3 were detected in either BAT or WAT of male mice of the three genotypes, except for a modest increase in UCP1 mRNA levels in the BAT of Gpr54$^{-/-}$Tg male mice. In contrast, in female mice, a significant suppression of UCP1 mRNA levels was detected in the BAT of global Gpr54$^{-/-}$ KO mice vs. control values, whereas in Gpr54$^{-/-}$Tg females intermediate UCP1 mRNA levels were detected (Suppl. Fig. S5). Concerning UCP3, no significant differences in its mRNA levels were observed between Gpr54$^{+/+}$, Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg female mice, neither in the BAT or WAT (Suppl. Fig. S5).

3.4. Metabolic responses to obesogenic insults in Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg male and female mice

In addition to metabolic parameters of Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg mice in lean conditions, changes in body weight gain and composition, as well as glucose homeostasis, were comparatively explored in both genotypes and sexes after a challenge with well-defined obesogenic insults; namely, HFD in males and females, and ovariectomy (OVX) in females.

Control males fed with 45% HFD from weaning onwards displayed an increase in BW that became overtly detectable after 10-wks of exposure to HFD and resulted in nearly doubled fat mass compared to Gpr54$^{+/+}$ males receiving a control diet. Male mice with global inactivation of Gpr54 showed greater BW gain responses to HFD, with an increase in BW over corresponding null mice fed a control diet that occurred much earlier and nearly doubled the weight gain induced by HFD in control, Gpr54$^{+/+}$ animals. In fact, the fat mass of Gpr54$^{-/-}$ mice fed with HFD almost tripled that of mice fed with a low fat diet. Finally, Gpr54$^{-/-}$Tg male mice showed intermediate BW gain responses to HFD, both in terms of time-course profiles and total BW gain. However, the fat mass gain of Gpr54$^{-/-}$Tg mice fed with HFD was similar to that of WT controls (Fig. 4A–C).

The same intermediate phenotype was detected for feeding patterns in Gpr54$^{-/-}$Tg male mice exposed to HFD, with a significant attenuation of food intake during the dark phase and the 24-h period vs. WT males (Fig. 4D). Finally, glucose tolerance was significantly worsened by HFD in both Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg male mice, but no difference in the magnitude of GTT alterations was detected between males of the two genotypes when exposed to HFD (Fig. 4E–F).

In females, control mice fed with 45% HFD from weaning onwards showed also an elevation of BW, detectable already 6-wks after the initiation of HFD exposure. In good agreement, the increase of fat mass

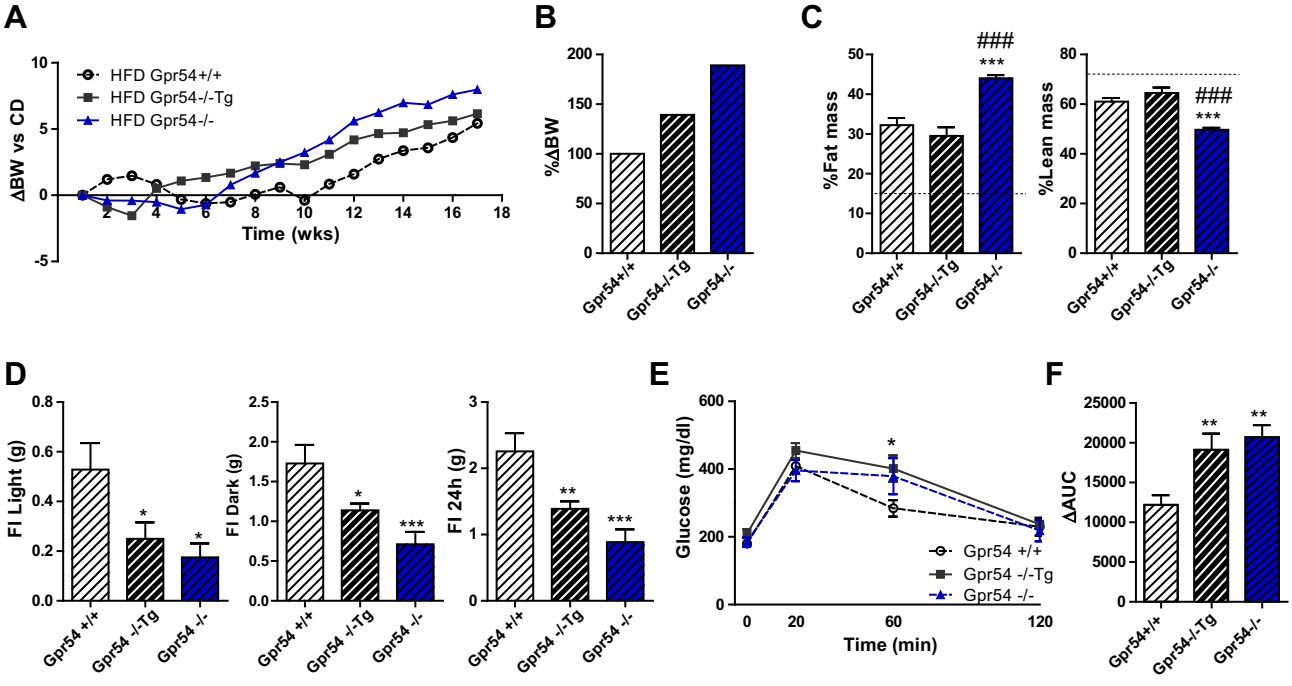


Fig. 4. Metabolic responses to HFD in male Gpr54$^{-/-}$ null and Gpr54$^{-/-}$Tg rescued mice. (A) Body weight (BW) gain in response to exposure to HFD, from weaning (PND21) to adulthood (18-wk of age), is presented for WT (Gpr54$^{+/+}$), global Gpr54 KO (Gpr54$^{-/-}$) and Gpr54$^{-/-}$Tg rescued male mice, as normalized ΔBW over the basal body weight values in the corresponding genotype groups fed with control (chow) diet. The % of ΔBW caused by HFD during the 11-wk exposure, normalized by the increment in WT mice, is also shown in B. In addition, body composition analyses in the three genotypes are displayed in C, with % fat and lean mass values (for reference purposes, mean levels in WT mice fed with control diet are shown as dotted lines). In panel D, spontaneous feeding patterns in Gpr54$^{+/+}$, Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued male mice over a 24-h period, are shown. Finally, glucose tolerance tests (GTT) are presented for Gpr54$^{+/+}$, Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued male mice fed with HFD, both as 120-min time-course profiles (E) and net increment of integral glucose levels after ip injection of the glucose bolus (represented as ΔAUC in F). Data are presented as mean ± SEM. *P ≪ 0.05, **P ≪ 0.01 and ***P ≪ 0.001 vs. corresponding WT (Gpr54$^{+/+}$) groups; ###P ≪ 0.001 vs. corresponding Gpr54$^{-/-}$Tg rescued mice (ANOVA followed by Student-Newman-Keuls multiple range test).

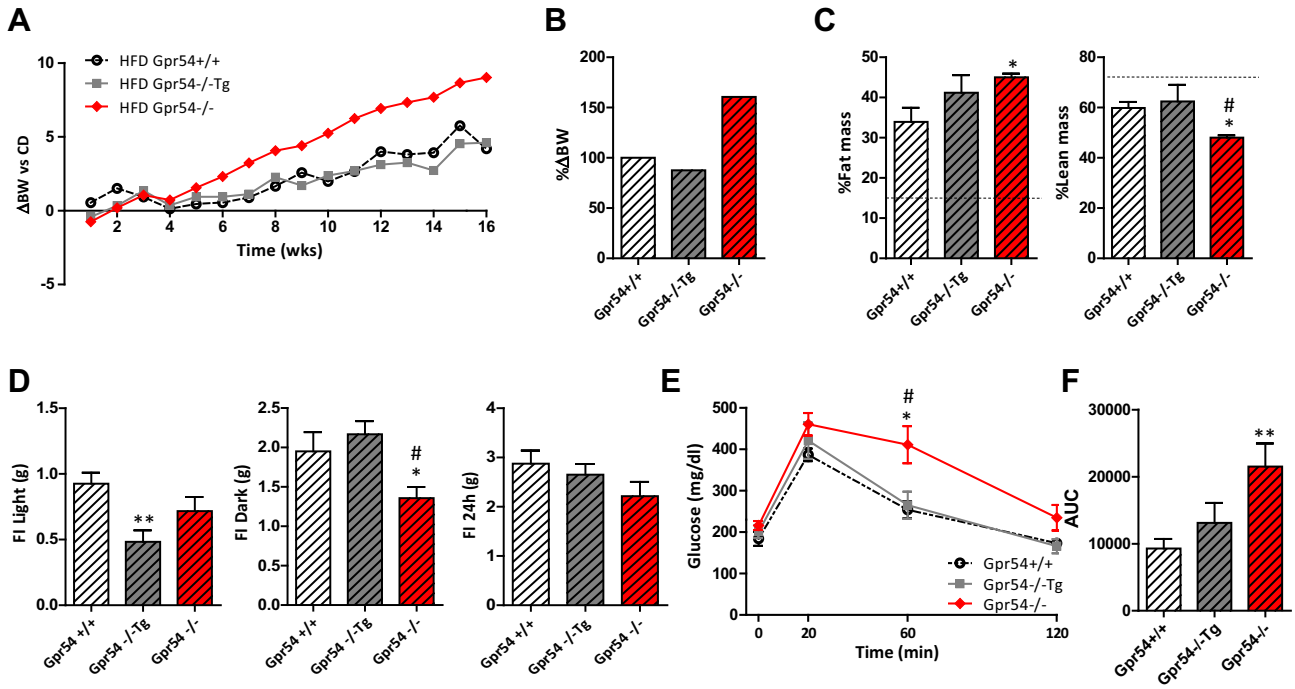


Fig. 5. Metabolic responses to HFD in female Gpr54$^{-/-}$ null and Gpr54$^{-/-}$Tg rescued mice. (A) Body weight (BW) gain in response to exposure to HFD, from weaning (PND21) to adulthood (18-wk of age), is presented for WT (Gpr54$^{+/+}$), global Gpr54 KO (Gpr54$^{-/-}$) and Gpr54$^{-/-}$Tg rescued female mice, as normalized ΔBW over the basal body weight values in the corresponding genotype groups fed with control (chow) diet. The % of ΔBW caused by HFD during the 11-wk exposure, normalized by the increment in WT mice, is also shown in B. In addition, body composition analyses in the three genotypes are displayed in C, with % fat and lean mass values (for reference purposes, mean levels in WT mice fed with control diet are shown as dotted lines). In panel D, spontaneous feeding patterns in Gpr54$^{+/+}$, Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued female mice over a 24-h period, are shown. Finally, glucose tolerance tests (GTT) are presented for Gpr54$^{+/+}$, Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued female mice fed with HFD, both as 120-min time-course profiles (E) and net increment of integral glucose levels after ip injection of the glucose bolus (represented as ΔAUC in F). Data are presented as mean ± SEM. *P ≪ 0.05 and **P ≪ 0.01 vs. corresponding WT (Gpr54$^{+/+}$) groups; #P ≪ 0.05 vs. corresponding Gpr54$^{-/-}$Tg rescued mice (ANOVA followed by Student-Newman-Keuls multiple range test).

doubled that of Gpr54$^{+/+}$ females receiving a control diet. Gpr54$^{-/-}$ female mice displayed substantially increased BW gain responses to HFD, with an earlier rise of BW compared to null females fed a control diet, and ≫50% increase of BW gain over WT animals fed HFD. This was reflected also in a significant increase of the percentage of fat mass. However, in contrast to males, Gpr54$^{-/-}$Tg female mice presented BW responses to HFD that were strictly similar to WT controls, in all parameters studied (Fig. 5A–C). Likewise, food intake during the dark phase was suppressed in Gpr54$^{-/-}$ female mice, while a non-significant trend for reduced 24-h feeding was also detected. In contrast, 24-h and dark-phase feeding profiles were similar between control and Gpr54$^{-/-}$ Tg female mice fed HFD. However, feeding during the light phase was partially attenuated in Gpr54$^{-/-}$Tg females (Fig. 5D). Nonetheless, no changes in the expression levels of POMC or CART mRNAs were detected in the hypothalamus of Gpr54$^{-/-}$ or Gpr54$^{-/-}$Tg mice of both sexes fed HFD (Suppl. Fig. S6).

In contrast to males, glucose tolerance was significantly worsened by HFD but only in global Gpr54$^{-/-}$ female mice, whereas Gpr54$^{-/-}$ Tg rescued females displayed GTT responses that were strictly similar to those of control female mice fed with HFD (Fig. 5E–F). Analogous differences between Gpr54$^{-/-}$ and Gpr54$^{-/-}$ Tg female mice in terms of responses to other obesogenic insult, namely, OVX, were detected for BW gain and glucose tolerance. Thus, while global Gpr54$^{-/-}$ null mice displayed exaggerated BW gain, perturbed glucose tolerance and suppressed dark-phase and accumulated 24-h food intake after OVX, Gpr54$^{-/-}$ Tg mice presented responses to OVX that were similar to those of WT females, except for a moderate suppression of feeding during the dark-phase (Suppl. Fig. S7). Likewise, combination of the two obesogenic insults, namely HFD and OVX, further perturbed glucose tolerance and feeding (during dark phase and the 24-h period) only in global Gpr54$^{-/-}$ female mice, while Gpr54$^{-/-}$ Tg rescued females displayed responses similar to those of WT animals subjected to HFD and OVX (Suppl. Fig. S7).

4. Discussion

Kisspeptins have emerged as master regulators of the reproductive axis, acting primarily upon their canonical receptor, Gpr54, in hypothalamic GnRH neurons to potently activate gonadal maturation and hormone secretion [1,2]. Despite this undisputed reproductive role, mounting evidence strongly suggests that kisspeptins may operate also as metabolic modulators, both at central and peripheral levels. This function might include not only the proven capacity of Kiss1 neurons to (directly or indirectly) sense and transmit metabolic information to the reproductive brain centers [36], but also genuine actions in the control of key facets of metabolism, such as feeding, energy balance and glucose homeostasis [8]. However, the physiological relevance of such role is still under debate, partially due to the fact that most of the approaches used so far to unravel the potential metabolic dimension of kisspeptins fell short in discriminating between their primary actions on metabolic circuits and those derived from their proven capacity to modulate the secretion of gonadal hormones, which conduct important metabolic actions on their own. In this work, we have taken advantage of a mouse model of whole-body genetic inactivation of Gpr54, in which Gpr54 is selectively re-expressed in GnRH neurons, hence allowing a complete rescue of pubertal maturation and function of the gonadal axis, especially during postnatal and young-adult ages, as those explored in our study [26,27]. Analyses using this model complement previous studies using global Gpr54 null mice [23], as the Gpr54$^{-/-}$Tg rescued mouse line shares the ablation of kisspeptin signaling in all relevant metabolic tissues and brain centers (other than GnRH neurons), but in contrast to full KOs, it retains gonadal function within a normal range, thus eliminating a potential confounding factor in metabolic studies.

Previous studies in global Gpr54 null mice had revealed an impact of the lack of kisspeptin signaling on body weight and glucose tolerance, preferentially affecting females [23]. Our current data confirm those

previous observations, but provide additional relevant insights regarding the age-, gonadal- and sex-dependence of such effects. Thus, Gpr54$^{-/-}$ males consistently displayed lower BW than WT controls, while Gpr54$^{-/-}$ females had a greater increase of BW during the adult period, which was associated to enhanced adiposity and leptin levels, as well as decreased gene expression of the thermogenic factor, UCP1 in BAT. The increased adiposity of Gpr54 KO female mice is in line with previous reports [23]. Regarding males, a majority of previous studies had demonstrated a significant suppression of total body weight in global Gpr54 null male mice [37,38], although a lack of net changes in body weight had been also reported [23]; a divergence that is likely attributable to differences among the various genetic models of Gpr54 ablation. In any event, our study is the first to document that during the pre/peripubertal period (≤6-wk of age), when the rate of body growth is maximal, both Gpr54$^{-/-}$ males and females showed lower BW gain than controls. Interestingly, this feature was shared by Gpr54$^{-/-}$Tg rescued mice, which have fully preserved puberty onset [26,27], thus indicating that such defect is genuinely due to impaired kisspeptin signaling in non-GnRH cells. This strongly suggests a putative role of kisspeptins in the control of body weight/growth during this developmental window in both sexes. Whether this might be related with the reported capacity of kisspeptins to modulate growth hormone (GH) secretion, as suggested by fragmentary data in different species [39–41], is yet to be defined; admittedly, evidence supporting a stimulatory role of kisspeptins on GH secretion in mammals remains conflictive and inconclusive [42–44]. Of note, decreased BW in adult Gpr54$^{-/-}$ males was, nonetheless, associated to increased adiposity and leptin levels, but reduced lean mass. Since this was not observed in Gpr54$^{-/-}$ Tg rescued males, it is concluded that such a change in the pattern of body composition is the result of suppressed androgen secretion and not related with primary actions of kisspeptins. Likewise, the catch-up in BW seen in Gpr54$^{-/-}$ females seems to be mostly due to lack of ovarian function, since this was not observed in the Gpr54$^{-/-}$Tg model. Yet, in this rescued model, a partial drop in UCP1 expression was detected in the BAT, therefore suggesting an incipient derangement of BAT thermogenesis in the Gpr54$^{-/-}$ Tg line, which fully manifests in the global KO mice.

In line with previous reports [23], global Gpr54$^{-/-}$ female mice were intolerant to an acute glucose challenge, while males were not. Dissection of the gonadal-dependent vs. -independent contribution of ablated kisspeptin signaling to this phenomenon revealed that Gpr54$^{-/-}$Tg rescued females display an intermediate phenotype in terms of glucose tolerance, with a detectable deterioration of GTT profiles vs. WT controls, which was, nonetheless, less severe than in global KO mice. These observations reveal a split contribution of suppressed ovarian secretion and the lack of kisspeptin signaling to the perturbation of glucose homeostasis in models of Gpr54 ablation. In this sense, estrogens have been reported to promote pancreatic beta-cell mass and insulin secretion [45]. Hence, the state of hypo-gonadism observed in Gpr54$^{-/-}$ null mice is likely a major contributor for their basal hyperglycaemia and glucose intolerance. On the other hand, kisspeptins have been proposed to either stimulate or inhibit insulin secretion, possibly depending on the prevailing glycemic state [20,21]. In our rescued model, elimination of kisspeptin signaling outside GnRH neurons worsened the glycemic profile in GTT, without affecting insulin sensitivity, therefore suggesting a positive action of kisspeptins in terms of insulin secretion. Admittedly, these observations challenge the proposed role of liver-born kisspeptin as diabetogenic factor, due to its capacity to eventually suppress insulin secretion in mice [22], but are in line with very recent evidence showing that kisspeptin administration increased insulin secretion following an intravenous glucose load in healthy humans [46]. Differences among experimental models and approaches may, at least partially, explain the above differences. In this sense, it must be noted that our Gpr54$^{-/-}$ Tg model does not allow discrimination of pancreatic vs. extra-pancreatic actions of kisspeptins.

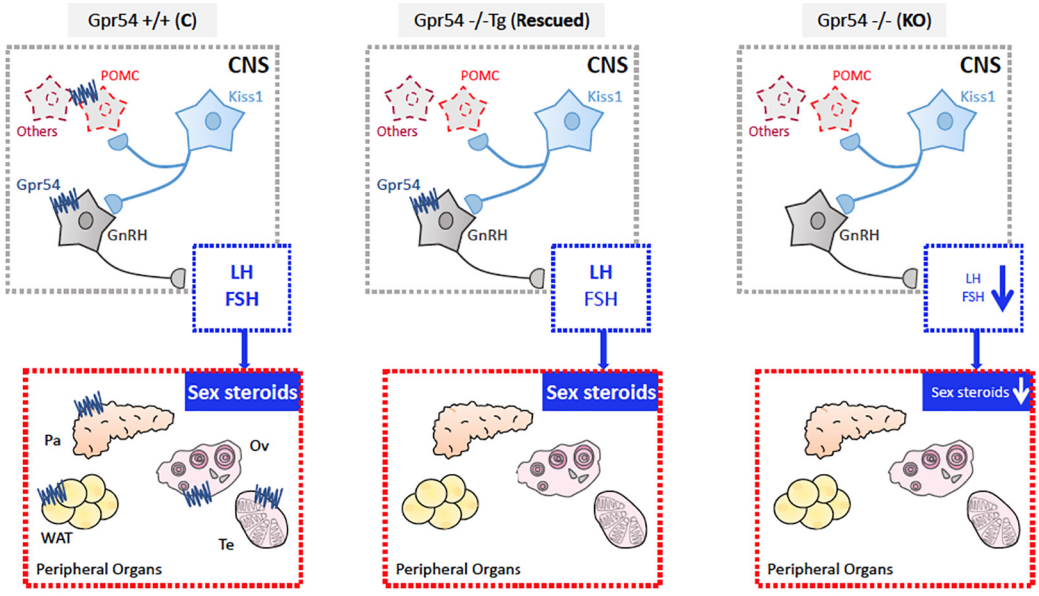
Initial pharmacological studies suggested that kisspeptins may operate as anorexigenic signals, with capacity to induce the electrical firing of POMC neurons [10], and cause a short-term suppression of food intake in mice [12]; an effect that has been confirmed also in male rats [47] and the wild rodent, jerboa [48]. This effect, however, is subtle in magnitude and appears to occur only at pharmacological doses and/or in fasted animals. In contrast to that putative anorectic action, our study documents that mice with global ablation of kisspeptin signaling (Gpr54$^{-/-}$) display suppressed food intake, both during the light and dark phases, thus resulting in a consistent reduction of feeding over a 24-h period. This is associated with an increase in the hypothalamic gene expression of the anorectic factor, POMC, especially in Gpr54$^{-/-}$ female mice. These findings fully confirm previous data showing reduced feeding and increased POMC expression in global Gpr54 KO mice [23,49]. However, analysis of feeding patterns in Gpr54$^{-/-}$Tg rescued mice revealed interesting sex differences. While Gpr54$^{-/-}$Tg rescued males behaved as WT controls in terms of food intake, Gpr54$^{-/-}$Tg females displayed an intermediate phenotype, with a detectable suppression of feeding both during the light and dark phase, as well as accumulated food intake over 24-h, vs. control animals. Moreover, in males, feeding responses to the orexigenic signal, ghrelin, were identical between WT and Gpr54$^{-/-}$Tg rescued mice, whereas they were clearly deferred in global Gpr54$^{-/-}$ animals. This suggest that while suppressed feeding in Gpr54 null male mice is likely due to the absence of testicular hormones, and possibly reflect a compensatory response to a state of increased adiposity (which is detected in Gpr54$^{-/-}$ but not in Gpr54$^{-/-}$Tg rescued mice), in females, part of the feeding suppression is not merely due to the hypogonadal state (or adaptation thereof), but rather stems from the genuine lack of kisspeptin signaling. This is compatible with a potential orexigenic effect of the endogenous peptide in females, whose site(s) of action and physiological relevance warrants further investigation.

Our study is the first to explore the impact of manipulations of kisspeptin signaling on BW and metabolic responses to obesogenic insults, such as HFD and, in females, OVX. Our data document that whole-body absence of kisspeptin signaling causes an exaggerated deterioration of key metabolic parameters after exposure to these obesogenic conditions, with enhanced BW gain and fat mass, decreased lean mass and worsened glucose tolerance. Interestingly, even if Gpr54$^{-/-}$ females displayed significantly lower sex steroid levels, removal of residual ovarian function was capable to evoke exaggerated BW gain responses in global Gpr54 null mice. However, comparison of responses to HFD between the Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg genotypes revealed that, whereas in the case of females, deterioration of the metabolic profile was mainly caused by the lack of ovarian function (as this was not detected in Gpr54$^{-/-}$Tg mice), in males, a discernible worsening of responses to HFD in terms of BW gain and glucose tolerance was also observed in Gpr54$^{-/-}$Tg rescued mice, despite preserved gonadal function. This suggests that the lack of kisspeptin signaling, independent of testicular hormones, might enhance the vulnerability to the metabolic damage caused by HFD in males. This is especially evident for glucose tolerance, which was equally deteriorated in Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg male mice after HFD exposure.

Recent evidence, obtained using sophisticated optogenetic approaches for neuronal activation or inactivation, has suggested that, in addition to the actions of kisspeptins themselves, Kiss1 neurons in the ARC may play a relevant role in the control of BW and metabolic homeostasis via other co-transmitters, such as glutamate signaling to POMC neurons [13,15], whose actions would not be captured by our current approach. In addition, very recent data have proposed that ARC Kiss1 neurons might operate as metabolic controllers due to their ability to modify daily body rhythms, including feeding patterns [16]. Interestingly, in our study, a switch in the orexigenic actions of ghrelin was detected between Gpr54$^{-/-}$ and control mice, with global KO animals showing a deferred orexigenic response to ghrelin. However, the fact that Gpr54$^{-/-}$Tg mice behaved as WT controls suggests a major contribution of testicular hormone secretion in this phenomenon; an influence that cannot be excluded in optogenetic approaches either. In addition, interpretation of our current data must take into account the

congenital nature of the genetic manipulation, which might have driven compensatory events, as well as the fact that in the long term, subtle but detectable gonadal alterations are also observed in the Gpr54$^{-/-}$Tg rescued model [27]. Notwithstanding, our systematic comparison of metabolic parameters between Gpr54$^{-/-}$ and Gpr54$^{-/-}$Tg rescued mice, schematically depicted in Fig. 6, not only supports a relevant contribution of gonadal hormone-dependent actions in the global role of kisspeptin signaling in the control of body weight and metabolic homeostasis, but unveils also discernible primary effects of kisspeptin signaling in the regulation of body weight gain, feeding and metabolic responses to obesogenic insults, which occur in a sexually-dimorphic manner and whose pathophysiological relevance warrants further investigation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.06.007.



MALES	Gpr54 +/+	Gpr54 -/-Tg	Gpr54 -/-
Final BW	Control	↓	↓↓
%Fat mass	Control	=	↑
Food Intake	Control	=	↓
Glucose Intolerance	Control	=	=

FEMALES	Gpr54 +/+	Gpr54 -/-Tg	Gpr54 -/-
Final BW	Control	≤	↑
%Fat mass	Control	≥	↑
Food Intake	Control	↓	↓↓
Glucose Intolerance	Control	↑	↑↑

Fig. 6. Dissection of gonadal-dependent vs. independent kisspeptin actions on metabolism. In the *upper panels*, a schematic representation of the three genotypes analyzed in our study is shown. While control (Gpr54$^{+/+}$) mice have fully preserved kisspeptin signaling in central and peripheral organs, as well as normal function of the gonadotropic axis (denoted by normal LH, FSH and sex steroid levels) due to the integrity of Gpr54 signaling mainly in GnRH neurons, global Gpr54$^{-/-}$ KO mice lack kisspeptin signaling all through the body and display profoundly suppressed gonadotropin and sex steroid secretion. In turn, Gpr54$^{-/-}$Tg rescued mice retain Gpr54 signaling only in GnRH; due to such rescue they show preserved gonadotropin secretion and gonadal function (denoted by normal sex steroid levels), therefore allowing to discriminate gonadal-dependent vs. -independent actions of kisspeptins on metabolism and other body functions. In the *lower panel*, a summary of the main phenotypic manifestations, regarding changes in body weight, % fat mass, food intake and glucose intolerance, observed in Gpr54$^{-/-}$ KO and Gpr54$^{-/-}$ Tg rescued mice is presented, for both males and females. CNS: central nervous system; POMC: Proopiomelanocortin; WAT: white adipose tissue; Pa: Pancreas; Te: Testis; Ov: Ovary.

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Declaration of Competing Interest

The authors have nothing to disclose in relation to the contents of this work.

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