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Authors

De Bond, Julie-Ann P
Tolson, Kristen P
Nasamran, Chanond
[et al.](#)

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Unaltered hypothalamic metabolic gene expression in *Kiss1r* KO mice despite obesity and reduced energy expenditure

Julie-Ann P. De Bond^a, Kristen P. Tolson^b, Chanond Nasamran^b, Alexander S. Kauffman^b,
and Jeremy T. Smith^a

^a School of Anatomy, Physiology and Human Biology, University of Western Australia,
Perth, Australia

^b Department of Reproductive Medicine, University of California San Diego, La Jolla, CA,
92093, United States.

Corresponding Author:

Dr Jeremy Smith

Department of Anatomy, Physiology and Human Biology, The University of Western
Australia, The University of Western Australia, Hackett Drive, Nedlands, Perth, WA 6009,
Australia.

jeremy.smith@uwa.edu.au

Phone: +61-3-6488-8688

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Short Title:

Hypothalamic genes in *Kiss1r* knockout mice

Key Words:

Kisspeptin, Metabolism, GPR54, Kiss1, Leptin

Abstract

Kisspeptin controls reproduction by stimulating GnRH neurons via its receptor *Kiss1r*. *Kiss1r* is also expressed other brain areas and in peripheral tissues, suggesting additional non-reproductive roles. We recently determined that *Kiss1r* knockout (KO) mice develop an obese and diabetic phenotype. Here, we investigated whether *Kiss1r* KOs develop this metabolic phenotype due to alterations in the expression of metabolic genes involved in the appetite regulating system of the hypothalamus, including neuropeptide Y (*Npy*) and pro-opiomelanocortin (*Pomc*), as well as leptin receptor (*Lepr*), ghrelin receptor (*Ghsr*), and melanocortin receptor 3 and 4 (*Mc3r*, *Mc4r*). Body weights, leptin levels, and hypothalamic gene expression were measured in both gonad-intact and gonadectomised (GNX) mice at 8 and 20 weeks of age, fed either normal chow or a high-fat diet. We detected significant increases in *Pomc* expression in gonad-intact *Kiss1r* KOs at 8 weeks and 20 weeks, but no alterations in the other metabolic-related genes. However, the *Pomc* increases appeared to reflect genotype differences in circulating sex steroids, because GNX wildtype (WT) and *Kiss1r* KOs exhibited similar *Pomc* levels, along with similar *Npy* levels. The altered *Pomc* gene expression in gonad-intact *Kiss1r* KOs is consistent with previous reports of reduced food intake in these mice and may serve to increase the anorexigenic drive, perhaps compensating for the obese state. However, the surprising overall lack of changes in any of the hypothalamic metabolic genes in GNX KOs suggests that the aetiology of obesity in the

absence of kisspeptin signalling may reflect peripheral rather than central metabolic impairments.

Introduction

Kisspeptin (encoded by the *Kiss1* gene) is a neuropeptide made in the hypothalamus that acts via the kisspeptin receptor, *Kiss1r* (originally known as *Gpr54*), to drive reproduction (1).

Hypothalamic kisspeptin activates the reproductive axis by potently and directly stimulating gonadotropin releasing hormone (GnRH) neurons, which express *Kiss1r* (2-7). In humans and mice, both *Kiss1* and *Kiss1r* mutations lead to hypogonadotropic hypogonadism (1, 4, 8-12), highlighting the vital role for kisspeptin signalling in puberty and the maintenance of fertility.

In addition to being expressed in reproductive brain regions, *Kiss1* and *Kiss1r* mRNA are both also expressed in ‘non-GnRH related’ areas of the brain (13-16). Moreover, in the periphery, *Kiss1r* is expressed in the liver, pancreas, and brown and white adipose tissue (14).

This suggests kisspeptin may have roles outside of reproduction, perhaps pertaining to metabolic function or energy balance. Indeed, we recently identified a metabolic role of kisspeptin signalling (17). We demonstrated that adult *Kiss1r* knockout (KO) females have robustly greater body weights, increased adiposity, and elevated leptin levels compared to wildtype (WT) littermates. This obesity in *Kiss1r* KOs appears to reflect, at least in part, reduced metabolic rate and energy expenditure (17). Interestingly, despite their obese phenotype, *Kiss1r* KO mice exhibit significantly lower food intake, perhaps reflecting the actions of the elevated leptin in these mice. Importantly, the obese phenotype in *Kiss1r* KOs is maintained even after WTs and KOs are both gonadectomised [to equate circulating sex steroid levels, which are known to influence body weight and metabolism (18, 19)],

indicating that the phenotype is not simply due to absent sex steroids in the KO's hypogonadal state. Thus, kisspeptin signalling has a previously unappreciated metabolic role, but the underlying mechanisms causing the altered body weight, food intake, and energy expenditure in *Kiss1r* KO mice are currently unknown and remain to be determined.

It is possible that some aspects of the body weight or feeding phenotype in *Kiss1r* KO mice are due to altered hypothalamic regulation of food intake or energy expenditure.

Interestingly, kisspeptin treatment has been shown to regulate both *Npy* and *Pomc* neuronal activity (20). Specifically, electrophysiological recordings in mouse brain slices demonstrated that kisspeptin administration reduced *Npy* neuronal firing (by indirect means) and increased *Pomc* neuronal firing (by direct actions). Alternatively, kisspeptin treatment increased *Npy* gene expression and decreased *Pomc* expression in ewes (21), though similar examinations have not yet been reported for rodents or other species. Therefore, it appears that kisspeptin is sufficient to alter the appetite regulating systems in the hypothalamus, but whether this actually occurs *in vivo* is unknown. Furthermore, whether the hypothalamic *Pomc* and *Npy* systems are perturbed in *Kiss1r* KO mice, perhaps contributing to their metabolic or feeding phenotype, is similarly not known.

In the present study, we hypothesised that hypothalamic metabolic gene expression in *Kiss1r* KO mice would be altered compared to WT mice, consistent with the former's obese phenotype. We examined key neuropeptide genes in the appetite regulating system of the arcuate nucleus of the hypothalamus, including the primary orexigenic neuropeptide neuropeptide Y (*Npy*) and the main anorexigenic neuropeptide pro-opiomelanocortin (*Pomc*) (22). We also examined the hypothalamic expression of several key metabolic-related receptors present in *Npy* and/or *Pomc* neurons, including leptin receptor (*Lepr*), ghrelin

receptor (*Ghsr*) and melanocortin 3 and melanocortin 4 receptors (*Mc3r* and *Mc4r*). All these genes were examined in mice at both 8 and 20 weeks of age, the former representing an age just prior to the emergence of the elevated body weight and the latter representing an age when the KOs are fully overweight.

Materials and Methods

Animals

Kiss1r KO mice were generated by Omeros Corporation (Seattle, WA) via retroviral mutagenesis (17). WT and KO littermates were generated from heterozygote breeder pairs. For our experiments, only WT and KO littermate offspring were used; heterozygous offspring were not studied. Experiments were performed based on the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation/Australian Animal Commission Code of Practice of Care and the Use of Animals for Experimental Purposes. This study was approved by the Animal Ethics Committee from the University of Western Australia and by the Institutional Animal Care and Use Committee from the University of California San Diego.

Experimental Design

Mice were examined at two ages: 8 weeks, just prior to when the increased body weight is first starting to emerge in female *Kiss1r* KO mice (17), and 20 weeks old, when the obese phenotype is fully present. For each age group, both WTs and KOs were examined either gonad-intact or gonadectomised (GNX), the latter cohort to control for the effects of absent circulating gonadal sex steroids in *Kiss1r* KOs (which are hypogonadal). All GNXs occurred at 6 weeks of age, with mice anaesthetised under isoflurane inhalation, as previously described (23). A subset of mice examined at 20 weeks were also challenged with a high fat diet (HFD)

(23% fat, 4.7% crude fibre and 18.4% protein; 46% total energy from lipids; SFO4-027; Speciality Feeds, Perth, Australia. Standard chow was 4.8% fat, 4.8% crude fibre and 19.4% protein) administered for a duration of 12 weeks, from 8 to 20 weeks of age.

For tissue collection, 8 week or 20 week old mice were deeply anaesthetised under isoflurane inhalation, body weight was recorded, and blood collected via cardiac puncture. Mice were then euthanised by rapid decapitation and brains were subsequently removed and frozen on dry ice. Brains and blood were stored at -80°C until further analysis.

Plasma leptin measurements

Plasma leptin levels were measured in GNX mice of both genotypes at 8 and 20 weeks.

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) and a protease inhibitor cocktail (Complete, Roche Applied Science, Mannheim, Germany), and the plasma immediately harvested and stored at -20 C. For the 8 week GNX mice, leptin concentrations were measured using the Milliplex Map Mouse Metabolic Hormone Magnetic Bead Panel (Millipore, Missouri, USA, cat no. MMHMAG-44K) using Luminex technology and Exponent software in accordance with manufacturer's instructions. The minimum detectable concentration of leptin was 19 pg/mL and the intra-assay CV% was 5%. For 20 week GNX mice, leptin concentrations were determined with a mouse leptin ELISA kit (#90030; Crystal Chem Inc.) in accordance with the manufacturer's instructions. The minimum detectable concentration of leptin was 2 pg/mL and the intra-assay CV% was <10%.

Quantitative real time PCR (qRT-PCR) for metabolic hypothalamic gene expression in the hypothalamus

For qRT-PCR analyses of metabolic genes, medial basal hypothalami were dissected from whole hypothalamic samples as described in (24). Total RNA was isolated using the phenol/chloroform method and converted to cDNA via reverse transcription (Promega, Sydney, Australia), followed by clean up using the PCR Clean Up Kit (Mio Bio Laboratories, Carlsbad, CA, USA). qRT-PCR was performed in 10 μ l reaction volumes and samples were tested in duplicate using a Rotorgene 3000 (Corbett Life Science, US). Reactions used either BioRad IQ SYBR Green Supermix (Biorad, Australia) or Qiagen SYBR Green PCR Kit (Qiagen, Australia). The master mix varied dependant on whether mouse Qiagen QuantiTect primers (*Npy*, *Pomc*, *Ghsr*, *Mc3r* and *Mc4r*) or Geneworks (leptin receptor, *ObRb*) primers were used (Supplementary Table 1 and 2). Samples were compared to a standard curve (10 fold dilution) and relative gene expression was normalised using a GE Norm algorithm (25) of housekeeping genes peptidylpropyl isomerase A (*Ppia*), succinate hydrogenase (*Sdha*) and TATA box binding protein (*Tbp*). No significant variability was noted in each housekeeping gene.

***In situ* hybridisation**

A subset of brains from chow-fed GNX 20 week female mice were analysed for *Npy* or *Pomc* mRNA expression in the arcuate nucleus (ARC) by *in situ* hybridisation (ISH). Briefly, brains were cut into 5 alternating sets of 20 μ m sections. For both *Npy* and *Pomc*, each group consisted of 6 brains, with one full set of slide-mounted brain sections encompassing the entire ARC region assayed using radiolabeled (³³P) antisense *Npy* or *Pomc* riboprobes (0.04 pmol/mL), as previously described (26). *Pomc* was cloned from mouse hypothalamic RNA into pBluescript SK-plasmid and transcribed using T7 polymerase. The *Pomc* probe

corresponds to bases 58-926 of the murine mRNA (NM_008895.4), and the *Npy* probe was previously described (26). ISH slides were analysed blindly with an automated grains imaging processing system (Dr Don Clifton, University of Washington, Seattle, Washington) that counted the number of silver grain clusters representing *Npy* or *Pomc* cells and the number of silver grains in each cell (a semiquantitative index of *Pomc* or *Npy* mRNA content per cell) (27).

Statistical analysis

All data are presented as mean \pm SEM. For 20 week gonad intact and 8 week GNX mice, gene expression was examined in each sex separately by an unpaired t test (due to different standard curves for qRT-PCR analysis). For 8 week gonad intact and 20 week GDX mice, males and females were analysed under the same standard curve and a two-way ANOVA with a Tukey post hoc test was used, followed by student t tests. Unpaired t tests were also used to examine ISH data. For plasma leptin measurements, two-way ANOVAs with a Tukey post hoc test were performed. Body weight in 20 week GNX mice was re-examined using data in our past study (17). All statistical tests were performed with Graph Pad Prism 5 and significance was set at $p < 0.05$.

Results

Normal body weights but elevated plasma leptin levels in 8 week GNX *Kiss1r* KO mice.

As previously reported (17), no significant difference in body weight was seen in 8 week *Kiss1r* KO mice of either sex compared to WT (Figure 1A and B). However, at this age, the concentration of circulating leptin was already significantly elevated in GNX *Kiss1r* KO mice of both sexes: leptin levels were 120% greater in KO males and 37% greater in KO females compared to WT controls (both $p < 0.01$, Figure 1C). When corrected for body

weight, the elevated leptin levels in *Kiss1r* KO mice remained. Specifically, leptin per gram bodyweight was 123% higher in male KOs and 39% higher in female KOs compared to their WT counterparts (both $p < 0.01$, Figure 1D).

Increased *Pomc*, but not *Npy*, gene expression in gonad intact, but not GNX, 8 week *Kiss1r* KO mice.

Hypothalamic *Npy* gene expression did not differ in 8 week old *Kiss1r* KO mice compared to WTs in either gonad intact (Figure 2A) or GNX conditions (Figure 2C). Hypothalamic *Pomc* gene expression was 31% (males) and 78% (females) higher in gonad intact *Kiss1r* KOs (both $p < 0.05$) compared to WTs at 8 weeks of age (Figure 2B). However, in mice that were GNX to equate sex steroid levels, there was no longer a genotype difference in *Pomc* gene expression in either sex (Figure 2D).

No changes in hypothalamic metabolic receptor gene expression in 8-week *Kiss1r* KO mice.

Hypothalamic expression of metabolic receptor genes was also investigated in 8 week old mice (Figure 3). There were no genotype differences in hypothalamic gene expression in leptin receptor (*Lepr*, Figure 3A and 3E), ghrelin receptor (*Ghsr*, Figure 3B and 3F), melanocortin 3 receptor (*Mc3r*, Figure 3C and 3G), or melanocortin 4 receptor (*Mc4r*, Figure 3D and 3H) in either gonad intact or GNX mice of either sex.

Elevated body weights and higher leptin levels in 20 week *Kiss1r* KO mice.

At 20 weeks of age, HFD fed gonad intact *Kiss1r* KO females were 13% heavier compared to WTs ($p < 0.05$, Figure 4A). As previously reported, no difference in body weight was seen in 20 week old gonad intact males (Figure 4A). At this 20 week age, plasma leptin was 166%

higher ($p < 0.05$) in gonad intact *Kiss1r* KO females compared to WT's (Figure 4B), and this genotype difference remained even when correcting for bodyweight: leptin per gram bodyweight was significantly greater in gonad-intact *Kiss1r* KO's for both sexes (60% in males and 145% in females, $P < 0.05$, Figure 4C). Following gonadectomy, the elevated body weight in female *Kiss1r* KO's remained (17% $p < 0.05$, Figure 4D), as previously reported. While no genotype differences in overall leptin levels were seen in GNX *Kiss1r* KO mice of either sex at 20 weeks of age (Figure 4E), when corrected for body weight, leptin per gram bodyweight was 31% higher in *Kiss1r* KO males and 30% higher in *Kiss1r* KO females ($p < 0.05$) compared to their WT controls (Figure 4F).

Increased *Pomc* gene expression in gonad intact, but not GNX, *Kiss1r* KO mice at 20 weeks of age.

At 20 weeks of age, hypothalamic *Pomc* gene expression was 72% higher in gonad intact *Kiss1r* KO males ($p < 0.01$) and 73% higher in gonad intact *Kiss1r* KO females ($p < 0.001$, Figure 5B) compared to gonad intact WT counterparts. As with 8 week old mice, no genotype differences were evident in hypothalamic *Npy* gene expression at 20 weeks of age in gonad intact mice (Figure 5A). Following gonadectomy, no genotype difference was present in hypothalamic *Pomc* or *Npy* gene expression in mice of either sex (Figures 5C, 5D).

Normal ARC *Npy* or *Pomc* gene expression in GNX *Kiss1r* KO mice at 20 weeks of age.

Consistent with our hypothalamic qRT-PCR data, *in situ* hybridisation analyses determined that *Npy* and *Pomc* gene expression in the ARC was similar between chow fed GNX female *Kiss1r* KO's and WT's at 20 weeks of age (Figure 6). No genotype differences were seen in either total *Pomc* or *Npy* cell number (Figure 7A and B), *Pomc* or *Npy* mRNA content per

cell (Figure 7C and D), or the total *Pomc* or *Npy* mRNA content in the ARC region (Figure 7E and F).

Normal hypothalamic metabolic receptor gene expression in GNX *Kiss1r* KO mice at 20 weeks of age.

In 20 week gonad intact and GNX *Kiss1r* KO mice, no significant genotype differences were evident in the hypothalamic expression of *Lepr* (Figure 8A and 8E) and *Mc3r* (Figure 8C and 8G). However, in gonad intact *Kiss1r* KO females, hypothalamic *Ghsr* gene expression was 28% lower compared to WT ($p < 0.05$, Figure 8B). Alternatively, hypothalamic *Mc4r* gene expression was 25% lower in gonad intact *Kiss1r* KO males compared to WT ($p < 0.05$, Figure 8D). However, as with *Pomc* expression, these *Ghsr* and *Mc4r* genotype differences at 20 weeks of age were no longer present following gonadectomy of both WTs and KOs (Figure 8F and H).

Discussion

We previously reported that female, but not male, mice lacking kisspeptin signalling start to become obese between 8-10 weeks of age and subsequently show significantly elevated body weight, adiposity, leptin levels, and impaired glucose homeostasis and metabolic rates (17). However, given that kisspeptin and its receptor are present in multiple parts of the brain and periphery, the reason(s) why *Kiss1r* KO mice develop this metabolic phenotype remain unknown. Here, we examined key gene expression in the hypothalamic appetite regulating system to assess if alteration in neural feeding circuits may underlie, fully or in part, the elevated body weight and adiposity that develops in the absence of kisspeptin signalling. We first examined *Kiss1r* KO mice at 8 weeks of age, just before the point where the metabolic and body weight phenotype first appears, and again at 20 weeks of age, when there is a more

pronounced metabolic phenotype. At both 8 and 20 weeks of age, gonad intact *Kiss1r* KO mice showed higher *Pomc* gene expression compared to WT mice though few other changes in hypothalamic gene expression were evident. This genotype difference in hypothalamic *Pomc* gene expression appears to be sex steroid dependent, as *Pomc* levels were equivalent in GNX WT and KO mice at both 8 and 20 weeks. Likewise, minor genotype differences in *Ghsr* and *Mc4r* gene expression observed in 20 week gonad intact *Kiss1r* KO mice also appeared to be sex steroid dependent, as no genotype differences were present in these genes when mice from both genotypes were GNX. Because the hypothalamic metabolic genes were normal in GNX KO mice, yet these GNX KO mice still display elevated body weights, adiposity, and leptin levels (17), these data suggest that the metabolic phenotype of *Kiss1r* KO mice is more likely driven by peripheral metabolic mechanisms rather than major changes in central feeding circuits (albeit we did not test all possible metabolic genes).

In this study, we confirm our previous report of the sexually dimorphic metabolic phenotype of *Kiss1r* KO mice. Consistent with our previous data, at 20 weeks of age only female mice have increased body weight compared to WT controls (17), though we did note in the original paper greater adiposity and leptin levels in KO males (17). Despite the obese phenotype, *Pomc* gene expression appeared to be elevated in gonad intact *Kiss1r* KO mice of both sexes, which is surprising because *Pomc* is anorexigenic. However, this increase in *Pomc* gene expression in both 8 week and 20 week gonad intact *Kiss1r* KO mice is consistent with sheep data showing reduced *Pomc* gene expression after treatment with kisspeptin (21), which would thereby predict an increase in *Pomc* gene expression in the absence of kisspeptin signalling. Clearly, higher *Pomc* expression does not predicate the increased body weight and adiposity observed in *Kiss1r* KO mice, but it may comprise part of the mechanism underlying the significantly reduced food intake in gonad intact *Kiss1r* KO mice.

(17). Moreover, it is also possible that higher *Pomc* might indicate a compensatory mechanism in these overweight mice, reducing food intake in an attempt to defend bodyweight.

Being hypogonadal, *Kiss1r* KO mice have much lower circulating sex steroids compared to their WT counterparts. Because sex steroids can influence metabolic status (18, 19), we also analysed WTs and KOs that were both GNX, thereby equating the sex steroid status between genotypes. Interestingly, we found that the elevations in *Pomc* gene expression observed in gonad intact *Kiss1r* KOs were no longer apparent when mice of both genotypes were GNX to eliminate genotype differences in sex steroid levels. This suggests that the increased *Pomc* levels in gonad intact KOs reflects the absence of gonadal sex steroids, in particular estradiol, compared to normal sex steroid concentrations in WTs (28). The lack of any genotype differences in *Pomc* in GNX animals, despite these GNX animals still presenting an overweight, hyperleptinemic phenotype, suggests that this metabolic phenotype is more likely driven by peripheral metabolic factors/mechanisms rather than simple changes in central appetite neural circuitry.

Our present data suggest that the obesity and metabolic impairments in the absence of kisspeptin signalling likely arise from peripheral alterations rather than marked changes in primary hypothalamic metabolic genes. Supporting this possibility, both kisspeptin and its receptor are expressed in several peripheral tissues. *Kiss1r* is expressed in the liver, fat, and pancreas (14). *Kiss1* is expressed in liver and adipose tissue and has been reported to be upregulated by fasting and down-regulated by either HFD or a diabetic state (29).

Alternatively, Dudek and colleagues (30) reported an increase in both *Kiss1* and *Kiss1r* mRNA in gonadal fat, pancreas, and liver of streptozotocin-induced type 1 diabetes in rats.

Kisspeptin levels are also reportedly increased in both the liver and serum from humans and mice with diabetes (31). *In vitro*, kisspeptin has been shown to stimulate basal insulin secretion from isolated human and mouse islets (32, 33) whereas another study reported that kisspeptin suppresses glucose-stimulated insulin secretion both *in vitro* and *in vivo* in mice (31) indicative of kisspeptin as a possible regulator of beta cell function. Further exploration of the peripheral activity of kisspeptin signalling and its effects on metabolism is warranted in future studies, possibly through the experimental use of novel *Kiss1r* flox mouse models (34).

Our 20 week old gonad intact and GNX mice examined by qRT-PCR were challenged with a HFD for 12 weeks. This cohort was used initially to investigate whether a HFD would exacerbate the obese phenotype in our previous study (17). HFD has been used in other studies to exacerbate the phenotypic difference in KO models, including *Npy* KO mice, which show increased *Pomc* gene expression and reduced body fat compared to controls only when challenged with a HFD (35). In both our previous report and the present study, the body weight and adiposity differences are still present, though less robust, when mice were on a HFD versus normal chow, reflecting elevated body weight gain in the WT mice on the former diet. Even so, body weights and leptin levels are still significantly higher in the KO mice on HFD. Despite this, we did not detect any change in metabolic gene expression in GNX *Kiss1r* KO mice on a HFD. We further examined this age group on a chow diet using *in situ* hybridisation. This allowed us to both examine any change in *Pomc* or *Npy* gene expression in *Kiss1r* KO mice fed with normal chow, and also observe their neuroanatomical expression specifically in the ARC nucleus (*Npy*, for example, is expressed primarily in the ARC but also to a lesser degree in other hypothalamic areas, such as the DMN). Our findings again

indicated that *Npy* and *Pomc* levels in the ARC are similar in adult GNX WT and KO mice, despite an overweight phenotype in the latter.

Unlike *Pomc*, there were no changes in *Npy* gene expression in gonad intact *Kiss1r* KO mice. *Npy* neurons receive peripheral signals from ghrelin via its receptor, *Ghsr* (also known as the growth hormone secretagogue receptor) (36). Ghrelin is a peptide released from the stomach that stimulates food intake by acting directly on NPY neurons (36-39). Our data demonstrate that 20 week gonad intact *Kiss1r* KO females had mildly reduced *Ghsr* gene expression, potentially suggestive for a reduction in orexigenic ghrelin drive to *Npy* neurons. Reduced ghrelin input may then subsequently have a potential additive effect towards driving the higher *Pomc* gene expression observed in these gonad intact mice.

GNX *Kiss1r* KO mice exhibited higher leptin levels compared to their WT controls at both 8 and 20 weeks. It should be noted that different assays were employed to examine plasma leptin at 8 and 20 weeks, so direct comparison between ages were avoided. The higher leptin in KO mice at 8 weeks occurs prior to any detectable elevation in body weight phenotype (though we have found that underlying adiposity is already moderately elevated by this age, matching the elevated leptin observed here [*Tolson and Kauffman, unpublished observation*]). The higher leptin levels were particularly evident when leptin concentration was corrected for body weight, indicative of the potential for partial leptin resistance (40) are consistent with an obese phenotype. Alternatively, we speculate that the high leptin from the periphery may be signalling in the ARC to induce the lower food intake previously observed in gonad intact *Kiss1r* KO mice, perhaps via increasing *Pomc*.

In conclusion, we have shown that the overweight, hyperleptinemic phenotype in *Kiss1r* KO mice is associated with increased hypothalamic *Pomc* gene expression, though this alteration in *Pomc* expression is no longer present when both genotypes are equalised for their sex steroid levels. Our previous study demonstrated that gonad intact *Kiss1r* KO females eat less (17), and increased *Pomc* synthesis may be at least one causative factor for that phenotype. However, our current study was unable to directly identify the cause of the elevated body weight and adiposity in *Kiss1r* KO mice, because no metabolic gene changes in the hypothalamus were evident in GNX mice, despite their elevated body weight and high leptin levels. Thus, these results suggest that this body weight phenotype may not simply be caused by alterations in hypothalamic feeding circuits. Rather, our findings suggest that non-central metabolic mechanisms, perhaps at the level of the liver, fat, or pancreas, all tissues where *Kiss1r* is also expressed, may be key causative factors for the obesity that emerges in the absence of kisspeptin signalling. Further studies are necessary to investigate impaired kisspeptin signalling outside the appetite regulating system, potentially looking to the periphery where kisspeptin and *Kiss1r* are both expressed.

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Disclosures

The authors have nothing to disclose.

Author contributions

K.P.T., A.S.K. and J.T.S. contributed to the conception and design of the research; J.P.D.B., K.P.T., C.N., A.S.K. and J.T.S. performed the experiments, analysed the data and interpreted the results of the experiments; J.P.D.B. drafted the manuscript and prepared the figures; K.P.T., A.S.K. and J.T.S. edited and revised the manuscript and figures; J.P.D.B., K.P.T., C.N., A.S.K. and J.T.S. approved the final version of the manuscript.

Figure Legends

Figure 1. The effect of impaired kisspeptin signalling on body weight (BW) in 8 week (wk) intact (A) or gonadectomised (GNX) mice (B). (C, D) Leptin concentration and BW corrected leptin concentration in GNX *Kiss1r* KO mice. As indicated, ** $p < 0.01$ compared to wild type (WT – shown as white bars, *Kiss1r* KO – black bars). Values are the mean \pm SEM. $n = 7-9$ per group.

Figure 2. The effect of impaired kisspeptin signalling on neuropeptide Y (*Npy*) and pro-opiomelanocortin (*Pomc*) hypothalamic gene expression in 8 week (wk) gonad intact (A, B) and gonadectomised (GNX) (C, D) mice. As indicated, * $p < 0.05$ compared to wild type (WT

– shown as white bars, *Kiss1r* KO – black bars). Values are the mean \pm SEM. n=3-9 per group (n=3 in the intact female KO group only).

Figure 3. The effect of impaired kisspeptin signalling on metabolic receptor expression in 8 week (wk) gonad intact and gonadectomised (GNX) mice. Leptin receptor (*Lepr*) (A, E) ghrelin receptor (*Ghsr*) (B, F), melanocortin 3 receptor (*Mc3r*) (C, G) and melanocortin 4 receptor (*Mc4r*) (D, H) hypothalamic gene expression in males and females (WT – shown as white bars, *Kiss1r* KO – black bars). Values are the mean \pm SEM. n=3-9 per group (n=3 in the intact female KO group only).

Figure 4. The effect of impaired kisspeptin signalling on body weight (BW) in 20 week (wk) HFD fed intact (A) or gonadectomised (GNX) mice (D). (B, C) Leptin concentration and BW corrected leptin concentration in intact *Kiss1r* KO mice. (E, F) Leptin concentration and BW corrected leptin concentration in GNX *Kiss1r* KO mice. As indicated, * p<0.05 compared to wild type (WT – shown as white bars, *Kiss1r* KO – black bars). Values are the mean \pm SEM. n=5-11 per group. Body weight in 20 week GNX *Kiss1r* KO mice was re-examined using data in our past study (17).

Figure 5. The effect of impaired kisspeptin signalling on neuropeptide Y (*Npy*) and pro-opiomelanocortin (*Pomc*) hypothalamic gene expression in 20 week (wk) HFD fed gonad intact and gonadectomised (GNX) mice. As indicated, ** p<0.01 and *** p<0.001 compared to wild-type (WT – shown as white bars, *Kiss1r* KO – black bars). Values are the mean \pm SEM. n=5-11 per group.

Figure 6. Representative dark-field photomicrographs showing *Npy* and *Pomc* mRNA-expressing cells (as reflected by the presence of *white clusters of silver grains*) in the ARC from 20 week old gonadectomised (GNX) chow fed female WT and *Kiss1r* KO mice. 3V, Third ventricle. *Scale bars*, 100 μ m.

Figure 7. The effect of impaired kisspeptin signalling on *Npy* and *Pomc* expression in the arcuate nucleus of 20 week old gonadectomised (GNX) chow fed female mice. No significant change was evident (WT – shown as white bars, *Kiss1r* KO – black bars) in either number of cells (A, B) mRNA per cell content (grains per cell, C, D) or total (combined) expression (E, F). Values are the mean \pm SEM. n=6 per group.

Figure 8. – The effect of impaired kisspeptin signalling on metabolic receptor expression in 20 week (wk) gonad intact and gonadectomised (GNX) HFD fed mice. Leptin receptor (*Lepr*) (A, E) ghrelin receptor (*Ghsr*) (B, F), melanocortin 3 receptor (*Mc3r*) (C, G) and melanocortin 4 receptor (*Mc4r*) (D, H) hypothalamic gene expression in males and females. As indicated, * $p < 0.05$ compared to wild type (WT – shown as white bars, *Kiss1r* KO – black bars). Values are the mean \pm SEM. n=5-11 per group.

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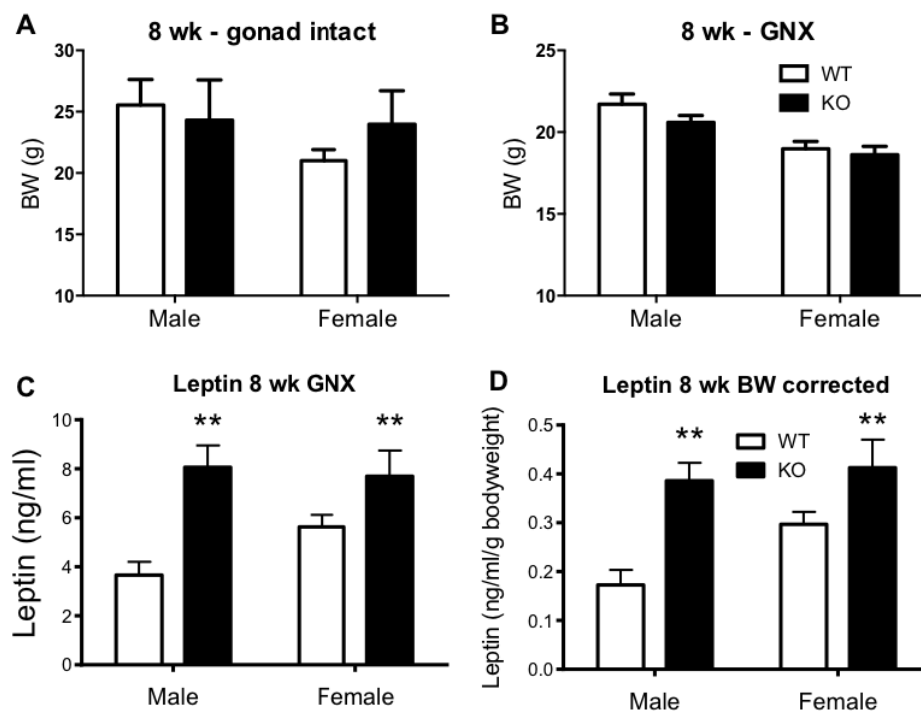
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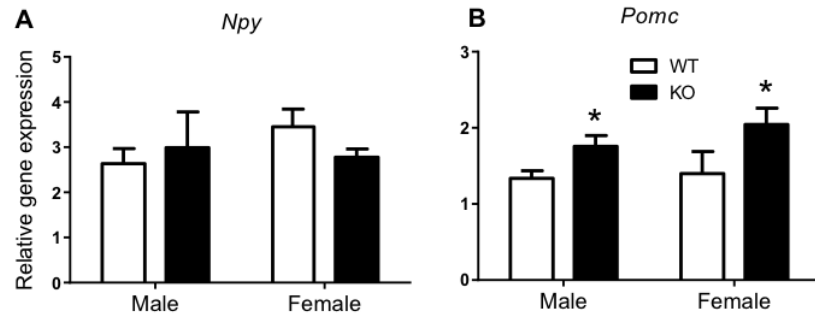
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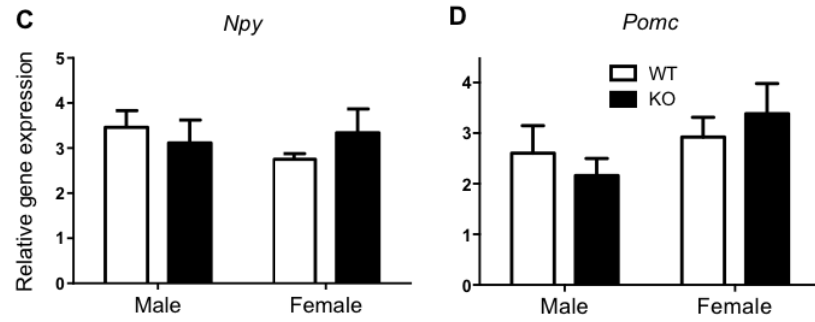
De Bond et al. Figure 1.



8 wk - gonad intact

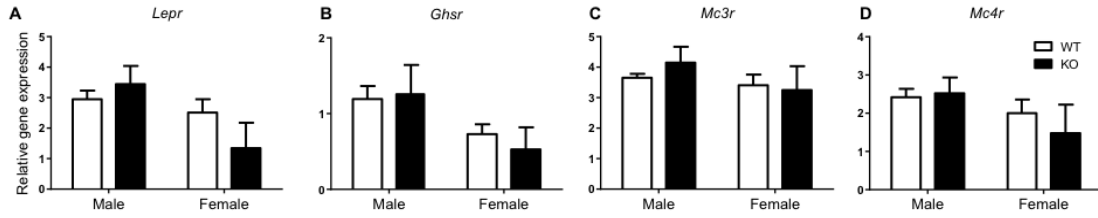


8 wk - GNX

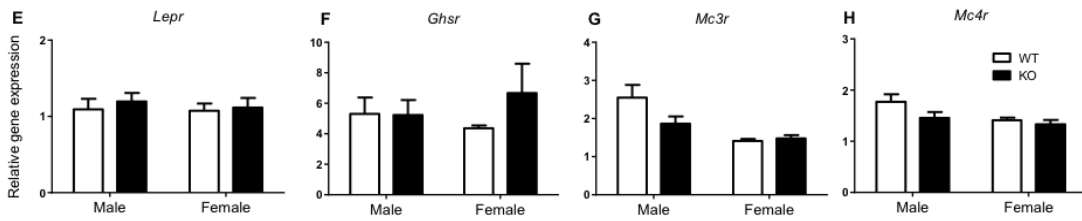


De Bond et al. Figure 3.

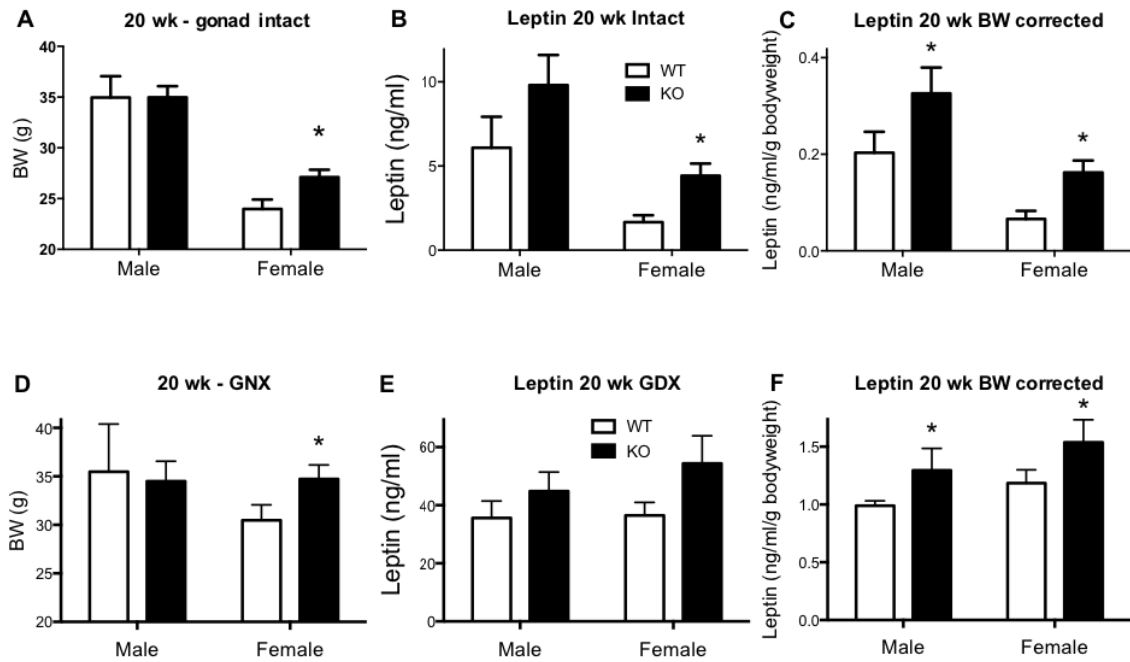
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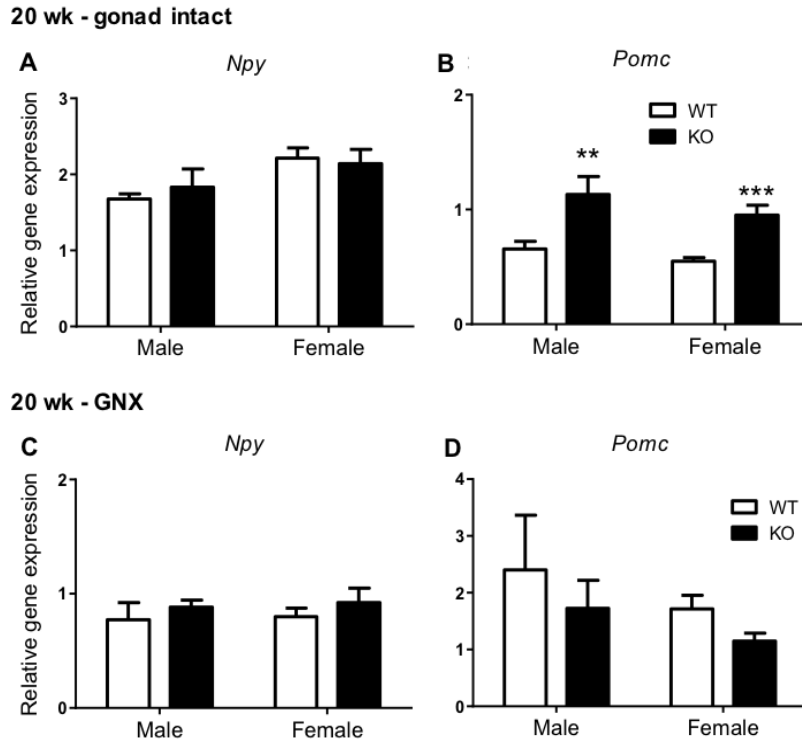
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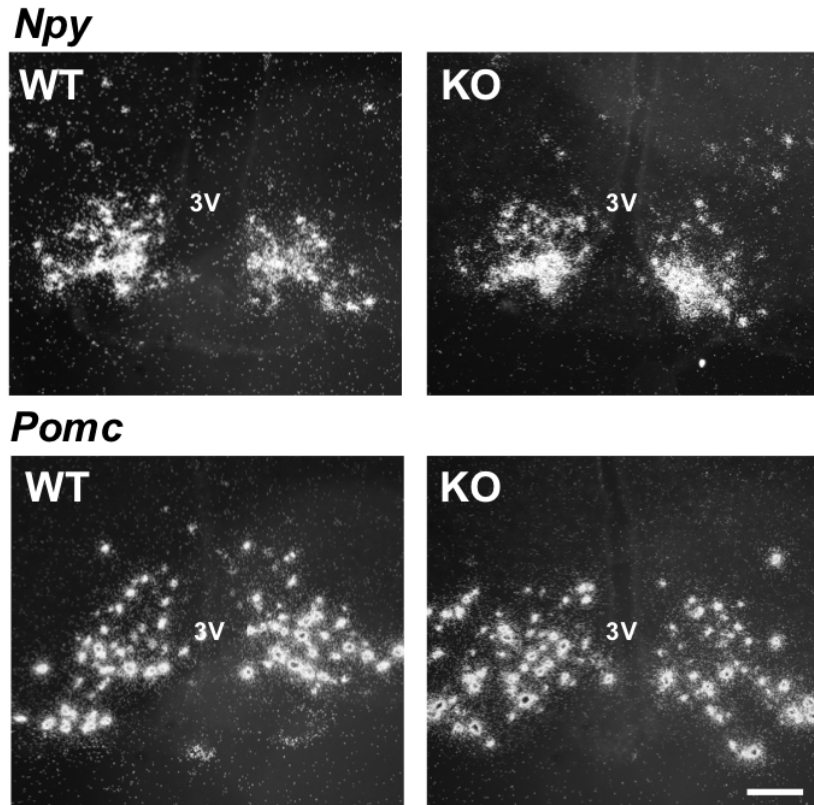
De Bond et al. Figure 4.



De Bond et al. Figure 5.

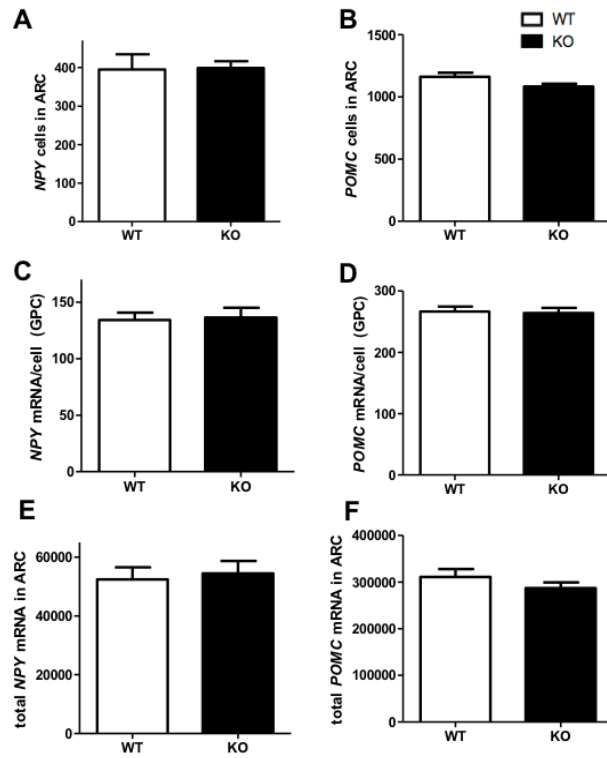


De Bond et al. Figure 6.



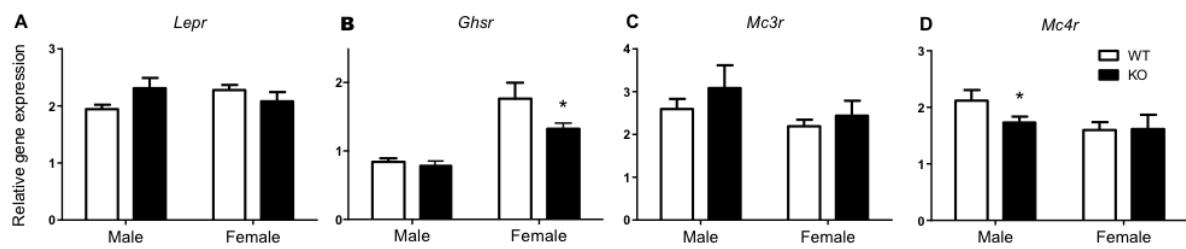
De Bond et al. Figure 7.

20 wk - GNX



De Bond et al. Figure 8.

20 wk - gonad intact



20 wk - GNX

