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Kisspeptin impacts on circadian and ultradian rhythms of core body temperature: Evidence in kisspeptin receptor knockout and kisspeptin knockdown mice

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Abstract

Kisspeptin is vital for the regulation of both fertility and metabolism. Kisspeptin receptor (Kiss1r) knockout (KO) mice exhibit increased adiposity and reduced energy expenditure in adulthood. *Kiss1r* mRNA is expressed in brown adipose tissue (BAT) and *Kiss1r* KO mice exhibit reduced *Ucp1* mRNA in BAT and impaired thermogenesis. We hypothesised that mice with diminished kisspeptin signalling would exhibit reduced core body temperature (Tc) and altered dynamics of circadian and ultradian rhythms of Tc. Tc was recorded every 15-min over 14-days in gonadectomised wild-type (WT), *Kiss1r* KO, and also *Kiss1*-Cre (95% reduction in *Kiss1* transcription) mice. Female *Kiss1r* KOs had higher adiposity and lower *Ucp1* mRNA in BAT than WTs. No change was detected in *Kiss1*-Cre mice. Mean Tc during the dark phase was lower in female *Kiss1r* KOs versus WTs, but not *Kiss1*-Cre mice. Female *Kiss1r* KOs had a lower mesor and amplitude of the circadian rhythm of Tc than did WTs. In WT mice, there were more episodic ultradian events (EUEs) of Tc during the dark phase than the light phase, but this measure was similar between dark and light phases in *Kiss1r* KO and *Kiss1*-Cre mice. The amplitude of EUEs was higher in the dark phase in female *Kiss1r* KO and male *Kiss1*-Cre mice. Given the lack of clear metabolic phenotype in *Kiss1*-Cre mice, 5% of *Kiss1* transcription may be sufficient for proper metabolic control, as was shown for fertility. Moreover, the observed alterations in Tc suggest that kisspeptin has a role in circadian and ultradian rhythm-driven pathways.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2021.111530.

Keywords

Kisspeptin; Metabolism; GPR54; Kiss1; Leptin

1. Introduction

In the last twenty years, the prevalence of obesity has increased significantly, and in Australia is expected to reach 75% of the population by 2025 (Walls et al., 2012; Welfare, 2020). Increased adiposity is associated with an increased risk of many diseases, including hypertension, cardiovascular disease, diabetes type II, and some cancers (Boles et al., 2017; Calle and Kaaks, 2004; Hubert et al., 1983). At a fundamental level, adipose stores increase when there is an excess of energy intake relative to energy expenditure (Spiegelman and Flier, 2001). There are, however, a multitude of factors - genetic, physiological, and environmental - that contribute to an individual's susceptibility to weight gain. An understanding of the pathology of obesity and related metabolic disorders is critical for the development of strategies for both prevention and treatment. The hypothalamus is a critical regulator of energy balance, with neurons in the arcuate nucleus (ARC) integral in the pathways that control food intake and energy expenditure. More specifically, arcuate neurons that co-express neuropeptide Y (NPY) and agouti-related peptide (AgRP), and those that express proopiomelanocortin (POMC), are 'first order' neurons involved in the control of food intake. NPY/AgRP neurons and POMC neurons are considered orexigenic and anorexigenic, respectively stimulating and inhibiting food intake (Atasoy et al., 2012; Barsh and Schwartz, 2002). These two neuron populations act in opposition in response to changes in the concentration of circulating metabolic hormones, such as leptin, ghrelin, and insulin.

Kisspeptin neurons, also located in the ARC, have recently emerged as novel players in the control of energy balance (De Bond and Smith, 2014). These neurons synthesise the neuropeptide kisspeptin, encoded by the *Kiss1* gene. Kisspeptin stimulates the secretion of gonadotropin-releasing hormone (GnRH) by binding to the receptor (Kiss1r) found on GnRH neurons (Han et al., 2005). Consistent with the well-characterised phenomenon of reproductive suppression in states of altered energy balance, kisspeptin neurons are sensitive to changes in metabolic status, with altered kisspeptin expression being reported in states of both under- and over-nutrition (Castellano et al., 2005; Quennell et al., 2011; Smith et al., 2006). Information regarding metabolism and available energy stores is relayed to ARC kisspeptin neurons either directly via receptors for metabolic hormones, or indirectly via innervation of NPY/AgRP and POMC neurons (Backholer et al., 2009, 2010; Padilla et al., 2017; Patel and Smith, 2020). Thus, ARC kisspeptin neurons participate as key players in both metabolic and reproductive circuits.

Mice lacking Kiss1r, in addition to being infertile, are obese in adulthood and have impaired glucose tolerance (Tolson et al., 2014), suggesting that kisspeptin signalling plays a crucial role in the regulation of metabolism. Importantly, this obese phenotype is not accompanied by increases in food intake or changes in the expression of *Npy/Agrp* or *Pomc* mRNA (De Bond et al., 2016; Tolson et al., 2014, 2016, 2019), suggesting that kisspeptin signalling has a role in the control of energy expenditure and adiposity independent from effects on ARC

feeding neural circuits. Indeed, *Kiss1r* mRNA expression has also been identified in several peripheral tissues, including brown adipose tissue (BAT), white adipose tissue (WAT), liver, and the pancreas (Brown et al., 2008; Hauge-Evans et al., 2006; Kotani et al., 2001; Song et al., 2014; Tolson et al., 2020) giving rise to the idea that kisspeptin can act directly on peripheral metabolic pathways, potentially independent of hypothalamic signalling. We have shown previously that adult female mice globally lacking Kiss1r (*Kiss1r* KOs) have lower expression of uncoupling protein 1 (*Ucp1*) mRNA in their BAT than wild-type (WT) controls (Halvorson et al., 2020; Tolson et al., 2020). The UCP1 protein acts as a channel that facilitates proton leak in mitochondria, and contributes to non-shivering thermogenesis, a significant contributor to body temperature and energy expenditure maintenance – especially in mice when they are housed below their thermoneutral zone (Maloney et al., 2014). Indeed, matching their lower *Ucp1* expression, core body temperature (Tc) is also lower in female *Kiss1r* KO than in WT (Tolson et al., 2020). Moreover, within the brain, tetanus-toxin silencing of ARC kisspeptin neurons in mice impairs the circadian nature of their feeding behaviour, and also results in lower Tc (Padilla et al., 2019), implicating the disruption of circadian rhythms as a possible mediator of some of the metabolic or thermogenic effects seen in *Kiss1r* KO mice. Finally, episodic ultradian events (EUE) in mice are fundamental biological rhythms (including temperature) with periods that range from 20 min to 6 h (Daan and Aschoff, 1981) and coordinate behaviour (locomotion, food intake) with physiology (heart rate, blood pressure, and Tc) (Blessing and Ootsuka, 2016; Goh et al., 2019). EUEs of Tc are generated by BAT (Ootsuka et al., 2009). Therefore, we also analysed the characteristics of EUEs of Tc in mouse models of altered kisspeptin signalling.

The present study aimed to further characterise the effects of reduced kisspeptin signalling on temperature regulation. We studied both *Kiss1r* KO mice (no kisspeptin signalling) and *Kiss*-Cre *knock-in* transgenic mice. Previous studies have extensively studied metabolic consequences of the absence of kisspeptin receptor (Kiss1r) signalling on obesity, metabolism, and thermoregulation. However, prior studies have not also determined whether a similar phenotype occurs in the absence of the ligand, kisspeptin protein itself. Thus, in the present study, *Kiss1*-Cre mice were included as a novel model to study, for the first time, the metabolic effect of greatly reduced, though not completely absent, kisspeptin synthesis. We hypothesised that *Kiss1r* KO mice will display lower Tc, and also have alterations in the circadian and ultradian rhythm of Tc. We also hypothesised that *Kiss1*-Cre mice would display a similar metabolic phenotype to that of *Kiss1r* KO mice.

2. Materials and methods

2.1. Animals

We used male and female WT, *Kiss1r* KO (Tolson et al., 2014), and *Kiss1*-Cre transgenic mice that have been described previously (Gottsch et al., 2011; Popa et al., 2013). In the *Kiss1*-Cre mouse ($Kiss1^{Cre/Cre}$), the Cre-GFP cassette contains a polyadenine (poly-A) tail that derails RNA polymerase from the DNA template before *Kiss1* gene transcription, resulting in very low, but not absent, *Kiss1* transcript levels (Popa et al., 2013). Matching their large reduction in kisspeptin levels, these mice have a “sub-fertile phenotype” but

are still able to reproduce (Popa et al., 2013). We determined that these *Kiss1*-Cre mice displayed reduced kisspeptin peptide expression in the hypothalamus along with reduced ovarian and testicular mass, but unchanged concentrations of luteinising hormone and follicle stimulating hormone (Supplementary material 1), consistent with the previously described phenotype (Popa et al., 2013). All mice were individually housed in standard temperature (22 °C) and lighting (12-h light-dark cycle, lights on 0700) conditions. Standard rodent chow (3.35 kcal/g, 20% protein, 4.8% fat, 4.8% crude fibre, 0.8% calcium, 0.7% phosphorous, 0.36% salt) and water was available *adlibitum*. All procedures were approved by the Animal Ethics Committee of The University of Western Australia (UWA) in accordance with guidelines for animal experimentation set by the National Health and Medical Research Council (approval number RA/3/100/1450) and the University of California, San Diego Institutional Animal Care and Use Committee.

2.2. Surgery and experimental design

In young adulthood, at eight weeks of age, all mice were anaesthetised using isoflurane inhalation anaesthesia and gonadectomised (GDX) to remove the effects of circulating gonadal sex steroids, and thereby control for the fact that the *Kiss1r* KOs are hypogonadal, and the *Kiss1*-Cre mouse is sub-fertile. Tc was recorded using DST nano-T temperature loggers (17 mm × 6 mm, 1 g; STAR ODDI Gardabaer, Iceland) that were implanted into the peritoneal cavity of each mouse during gonadectomy surgery. The loggers were programmed to record Tc at 15-min intervals and remained in place for two weeks. Prior to implantation, and following removal from mice, all of the loggers were placed in a water bath and calibrated against a high-accuracy mercury-in-glass thermometer (WIKA Australia, Rydalmere, Australia) certified by the National Association of Testing Authorities, Australia.

At 10 weeks of age, mice were again anaesthetised with isoflurane inhalation anaesthesia to allow for tissue collection and the removal of temperature loggers. A separate set of adult WT and *Kiss1*-Cre mice were also collected later at 20 weeks of age. A 1 mL blood sample was drawn into a syringe containing 20 μl of 0.5M-ethylenediaminetertracetic acid (EDTA) and protease inhibitor (Complete; Roche Applied Science, Mannheim, Germany) and placed immediately on ice, and the plasma separated by centrifugation and stored at −20 °C. Mice were then decapitated, their brains removed, and the hypothalami dissected (Quennell et al., 2011) and frozen in liquid nitrogen. Interscapular BAT and WAT (female, ovarian fat pad; male, epididymal fat pad) were dissected (de Jong et al., 2015), frozen in liquid nitrogen and then stored at −80 °C.

2.3. Plasma analysis

Plasma samples were analysed using a Luminex Corporation (Austin TX, USA) MILLIPLEX®Mouse Metabolic Hormone Assay (Catalogue number: MMHMAG-44K) to determine the concentrations of the plasma metabolic hormones ghrelin, leptin, and insulin. Blood glucose was also measured using an Accu-Check Performa Glucose Monitor (Catalogue number: 046804464003) at the time of collection. The minimum detectable concentration (Min) and intra-assay coefficient of variation (CV) for each hormone was as

follows; ghrelin (Min, 3 pg/ml; CV, 1.47%), insulin (Min, 14 pg/ml; CV, 0.83%), leptin (Min, 19 pg/ml; CV, 1.97%).

2.4. Analysis of metabolic gene expression

The expression of *Npy* and *Pomc* mRNA in the hypothalamus, and *Ucp1* mRNA expression in BAT, were determined using quantitative real-time polymerase chain reaction (PCR). The phenol/chloroform method was used to isolate RNA, which then underwent DNAse treatment, was converted to cDNA via reverse transcription (Promega Corporation, WI, USA), and cleaned up using the UltraClean® PCR Clean-Up Kit (Mo Bio Laboratories, CA, USA). Samples were then used for qRT-PCR using QuantiTect Primer Assays to determine mRNA expression (see Table 1). Relative gene expression was normalised against the reference genes, peptidylpropyl isomerase A (*Ppia*), succinate hydrogenase (*Sdha*), and TATA box binding protein (*Tbp*) using the GENorm algorithm (Vandesompele et al., 2002) as previously described (Halvorson et al., 2020).

2.5. Statistical analysis

All results are expressed as mean ± SEM. Cosinor analysis (Genstat, version 18.1; VSN International Ltd.) was used to analyse the daily body temperature rhythms of the mice using a nonlinear regression model to determine the mesor (circadian rhythm-adjusted mean), amplitude, and acrophase (time of the peak of a rhythm), as well as the goodness of fit of the model via cosinor r^2 (Wharfe et al., 2016). EUEs of body temperature were analysed using wavelet analysis software that includes data reconstruction (to eliminate the circadian rhythm) and allows discrete wavelet transformation, which can detect EUEs (Miyata et al., 2016; Ootsuka et al., 2009). The software was developed through a collaborative effort with the UWA Department of Engineering and Computers Sciences. It allowed the testing of different wavelets (the wavelet selected for analysis was ‘Coif3’), the analysis of profiles to detect EUEs, and the generation of reports that included the timing and characteristics of the shapes of EUEs. The parameters of TC, body mass, food intake, WAT mass, metabolic hormone levels, and metabolic gene mRNA expression were compared between groups of mice one-way ANOVA’s with Fisher’s LSD post-hoc test. The same variables were compared between 20 week old *Kiss1*-Cre and WT males and females using unpaired t-tests. All statistical tests were performed using PRISM version 5 (GraphPad Software Inc., San Diego, CA, USA) with statistical significance set at $P < 0.05$.

3. Results

3.1. Female Kiss1r KO, but not Kiss1-Cre, mice have greater WAT mass at 10 weeks of age

All of the mice in this study were gonadectomised (GDX) to remove the effects of circulating gonadal sex steroids, and thereby to control for the fact that the *Kiss1r* KOs are hypogonadal, and that the *Kiss1*-Cre mouse is sub-fertile. At 10 weeks of age, the body mass of female *Kiss1*-Cre and *Kiss1r* KO mice was similar to that of WT females (Fig. 1A). Although the *Kiss1r* KO mice consumed significantly less food per day (14% lower, $P < 0.05$, Fig. 1B), the WAT mass was 2-fold greater in female *Kiss1r* KO mice compared to WT ($P < 0.01$, Fig. 1C), as was the plasma leptin concentration (3-fold greater, $P < 0.01$,

Fig. 1D) consistent with our earlier publications of an obese phenotype in *Kiss1r* KO mice (Halvorson et al., 2020; Tolson et al., 2020).

In male mice, body mass at 10 weeks of age was significantly lower in both the *Kiss1*-Cre and *Kiss1r* KO mice compared to WT males (11% and 12% respectively, both $P < 0.01$, Fig. 1E) and food intake was 14% lower in the *Kiss1r* KO male mice ($P < 0.05$, Fig. 1F), but not the *Kiss1*-Cre mice, compared to WT. No genotype difference was seen in WAT mass in male mice, but the plasma leptin concentration was 58% higher in male *Kiss1r* KO mice compared to the *Kiss1*-Cre ($P < 0.01$, Fig. 1G). No significant genotype difference was seen in plasma ghrelin, insulin, or blood glucose between groups in either the females or males at 10 weeks of age (Supplementary material 2).

The relative expression of *Ucp1* mRNA was significantly lower in the BAT of female *Kiss1r* KO mice than in the WT at 10 weeks of age (26%, $P < 0.05$, Fig. 2A), consistent with our earlier publications (Halvorson et al., 2020; Tolson et al., 2020). There was no significant difference in BAT *Ucp1* mRNA between 10 week old female *Kiss1*-Cre and WT mice (Fig. 2A) or male mice of any genotype (Fig. 2B). Hypothalamic expression of *Npy* and *Pomc* mRNA did not differ with genotype in either 10 weeks old females or males (Supplementary material 2).

3.2. Core body temperature (Tc) is lower in female Kiss1r KO, but not Kiss1-Cre mice

The mean Tc was significantly higher during the dark phase compared to the light phase ($P < 0.001$; Fig. 3) and also differed with genotype ($P < 0.05$). Specifically during the dark phase (when mice are most active), the mean Tc was lower in *Kiss1r* KO female mice than in the WT ($P < 0.01$, Fig. 3A), consistent the lower BAT *Ucp1* mRNA expression and with our earlier publication (Tolson et al., 2020), but there was no genotype difference during the light phase. There was a trend for a lower Tc during the light phase in *Kiss1r* KO female mice compared to WT, but the difference did not reach significance ($P = 0.06$). Unlike female *Kiss1r* KO, *Kiss1*-Cre females did not display Tc that differed from those of WT females. In male mice, there was no effect of genotype on mean Tc in either the light or dark phase (Fig. 3B).

3.3. Analysis of the circadian rhythm of core body temperature (Tc) in Kiss1r KO and Kiss1-Cre mice

We hypothesised that Kiss1r KO and *Kiss1*-Cre mice would have alterations in the circadian rhythm of Tc. The circadian rhythm of Tc was examined using a cosine function (Fig. 4A–F). The cosinor r2, a measure of goodness of fit, was 0.15 ($P < 0.001$), 0.18 ($P < 0.001$), and 0.17 ($P < 0.001$), respectively in WT, *Kiss1*-Cre, and *Kiss1r* KO females, and 0.10 ($P < 0.001$), 0.05 ($P < 0.001$), and 0.15 ($P < 0.001$), respectively, in WT, *Kiss1*-Cre, and *Kiss1r* KO males. The cosinor parameters of Tc in the *Kiss1r* KO and *Kiss1*-Cre mice were consistent with the characteristics of mean Tc seen above. Specifically, in female *Kiss1r* KO mice, there was a significantly lower mesor compared to WT females (38% lower, $P < 0.05$, Fig. 4G), indicating an overall lower circadian rhythm set-point of Tc in female *Kiss1r* KO mice. Similarly, the circadian rhythm amplitude was lower in female *Kiss1r* KO mice than the WT females (46% lower, $P < 0.05$, Fig. 4H). Female *Kiss1r* KO mice also displayed

a significantly earlier acrophase compared to female *Kiss1*-Cre mice, but not female WT mice, indicating an approximate 2.5 h delay in the peak of daily rhythm in *Kiss1*-Cre females when compared to the *Kiss1r* KO ($P < 0.05$, Fig. 4I). Female *Kiss1*-Cre mice were similar to WT in mesor, amplitude, and acrophase. Interestingly, unlike in females, there were no genotype differences in any cosinor characteristics the male mice (Fig. 4J-L).

3.4. Analysis of episodic ultradian events (EUE) of core body temperature (Tc) in Kiss1r KO and Kiss1-Cre mice

Individual recordings from a representative female WT and *Kiss1r* KO mouse, along with the reconstructed profiles showing EUEs, are shown in Fig. 5A and B. In females, there were typically four EUEs of Tc during the 12 h light phase. In WT females, there were fewer EUEs during the dark phase than the light phase ($P < 0.001$, Fig. 5C), but there were no significant light phase/dark phase differences in the *Kiss1*-Cre or *Kiss1r* KO female mice ($P = 0.12$ and $P = 0.07$ respectively, Fig. 5C), indicating that the loss of kisspeptin signalling may disrupt these physiological events. The amplitude of EUEs of Tc was similar between the light phase and dark phase in WT and *Kiss1*-Cre female mice, but was significantly higher during the dark phase than the light phase in female Kiss1r KO mice ($P < 0.05$, Fig. 5D). No differences, between genotypes or light versus dark phase, were detected in the duration of EUEs in female mice (Fig. 5E).

In males, there were typically four EUEs during the light phase, similar to females. There were fewer EUEs during the dark phase in the WT male mice ($P < 0.05$, Fig. 5F), but similar EUE numbers in the light and dark phase in *Kiss1r* KO male mice ($P = 0.19$, Fig. 5F). In male *Kiss1*-Cre mice, the number of EUEs in the light and dark phase did not differ significantly but there was a trend for fewer EUEs during the dark phase ($P = 0.06$, Fig. 5F). The amplitude of EUEs of Tc was similar between the light phase and dark phase in both male WT and *Kiss1r* KO mice, but was significantly higher during the dark phase in male *Kiss1*-Cre male mice ($P < 0.05$, Fig. 5G). The duration of each EUE was significantly longer during the dark phase compared to the light phase in male WT male mice ($P < 0.05$, Fig. 5H) but no such phase difference was seen in either male *Kiss1*-Cre or *Kiss1r* KO male mice.

3.5. No clear evidence for obesity in Kiss1-Cre mice at 20 weeks of age

Given that *Kiss1*-Cre mice did not display an obese phenotype at 10 weeks of age, we subsequently examined these mice again at 20 weeks of age, a timepoint when the obese phenotype in *Kiss1r* KO females is fully established (Tolson et al., 2014). At 20 weeks of age, in both females and males, no difference was seen between *Kiss1*-Cre mice and WTs in either body mass (Fig. 6A and E), food intake (Fig. 6B and F), or WAT mass (Fig. 6C and G). There were also no genotype differences in the concentration of plasma leptin, insulin, ghrelin, or blood glucose (Supplementary material 3). Hypothalamic expression of *Npy* and *Pomc* mRNA was also similar between *Kiss1*-Cre and WT mice at 20 weeks of age (Supplementary material 3). However, at this older age, the expression of *Ucp1* mRNA in BAT was 47% lower in *Kiss1*-Cre females than WT females ($P < 0.05$, Fig. 6D), but 2.5-fold higher in *Kiss1*-Cre males than WT males ($P < 0.05$, Fig. 6H).

4. Discussion

In this study, we aimed to further characterise the metabolic phenotype of mouse models with altered kisspeptin signalling. Specifically, we sought to further examine whether thermoregulation is impaired in the *Kiss1r* KO mouse, as we have previously shown (Tolson et al., 2020), and for the first time examine obesity and the pattern of Tc in a kisspeptin "knockdown" *Kiss1*-Cre mouse. Alterations in Tc in the *Kiss1r* KO was consistent with our previous findings, with lower temperature in females, but not males, and a novel examination of the circadian rhythm revealed a lower mesor and amplitude in female *Kiss1r* KO mice compared to WTs. These temperature alterations in female *Kiss1r* KO mice were consistent with increased adiposity in these mice at 10 weeks of age. We did not detect an adiposity (or body weight) phenotype in the *Kiss1*-Cre at either 10 weeks or 20 weeks of age, and this was consistent with no change in their temperature profiles compared to WT. Our analysis of EUE reveled general changes between the light and dark phase across WT mice, with more events present during the light phase.

Kiss1r is expressed in several peripheral tissues that are involved in metabolism, including BAT (Brown et al., 2008; Tolson et al., 2019, 2020). BAT is the site of non-shivering thermogenesis, a process that increases energy expenditure and generates heat in response to the stimulation of cold input to the hypothalamic thermoregulatory centre (Cannon and Nedergaard, 2004; Morrison, 2004). Because the administration of exogenous kisspeptin has been shown to increase Tc (Csabafi et al., 2013), and absent kisspeptin signalling reduces Tc (Padilla et al., 2019; Tolson et al., 2020) we sought to further characterise the pattern of Tc in the *Kiss1r* KO and *Kiss1*-Cre mouse models. The latter model studied for the first time, to complement prior examinations of *Kiss1r* KOs, because it was unclear if the absent ligand would produce the same metabolic/energy balance phenotype as the absent receptor. We hypothesised that these mice would display lower Tc, and also have alterations in the circadian and ultradian rhythm of Tc. The mean daily Tc was lower in the female *Kiss1r* KO, consistent with a reduction in BAT *Ucp1* mRNA expression and our previous studies (Halvorson et al., 2020; Tolson et al., 2020). It is notable that the reduction in Tc was statistically significant only during the dark phase, and not the light phase. Owing to this potential shift in daily rhythm, we examined the temperature profile using cosinor analysis. Not surprisingly, we saw a lower mesor in *Kiss1r* KO female mice compared to WT, but we also saw a smaller amplitude of the circadian rhythm in *Kiss1r* KO females, and a shift in their acrophase, but only compared to the female *Kiss1*-Cre. A decrease in circadian amplitude and shifts in acrophase are associated with weakened circadian entrainment and a number of diseases including cancer (Lee et al., 2010) and metabolic syndrome (Turek et al., 2005). A reduction in circadian amplitude was also observed by Padilla et al. (2019) in a mouse model of silenced ARC kisspeptin neurons. Importantly, the change in circadian amplitude these mice has been shown to not simply be a direct result of decreased locomotion (Padilla et al., 2019). Therefore this Tc change is not simply the consequence of altered activity, and can be considered a true circadian rhythm.

As stated previously, the lower Tc is consistent with the lower *Ucp1* mRNA expression in BAT. *Ucp1* mRNA expression in BAT is controlled by clock genes – particularly *Rev-erba* –and *Ucp1* in BAT is expressed in a circadian manner (Gerhart-Hines et al., 2013). This

temporal association suggests a link between BAT metabolism, thermogenesis, and circadian rhythm. While it is tempting to conclude that kisspeptin in the periphery may be acting directly on BAT (which expresses Kiss1r), to stimulate thermogenesis, our previous work seems to contradict that speculation (Tolson et al., 2020). Using a Cre/lox conditional KO approach, we showed that when kisspeptin signalling is impaired specifically and only in BAT, the mice have a lean phenotype with increased energy expenditure and elevated Tc, suggesting that peripheral kisspeptin signalling in BAT normally inhibits thermogenesis (Tolson et al., 2020). Therefore, we speculate that the present reductions in Tc seen in the global *Kiss1r* KO are centrally mediated, owing to decreased kisspeptin signalling in the hypothalamus. A central role would be consistent with the results in the silenced ARC kisspeptin neuron model (Padilla et al., 2019), where the daily timing of food intake, activity, sleep, and Tc were all disturbed. Importantly, it did not appear that the clock gene machinery was altered in that model, so kisspeptin neurons likely do not directly influence the master clock in the suprachiasmatic nucleus (SCN), but may converge with SCN efferents or impact on downstream targets of the SCN to affect circadian input to other brain areas or tissues.

Ultradian rhythms, or more correctly EUEs, are recently characterised fundamental biological rhythms that link behaviour (in particular food intake) with physiology (including Tc) (Blessing and Ootsuka, 2016; Goh et al., 2019). Importantly, food intake does not appear to be the driver of these physiological changes, because ultradian events in Tc persist in a food deprived state prior to feeding (Blessing et al., 2012), and high fidelity recordings show that the increase in Tc precedes activity and food intake (Blessing and Ootsuka, 2016). Ultradian events are associated with increased hippocampal activity, indicating greater alertness to the external environment (Buzsaki and Moser, 2013; Ootsuka et al., 2009). We saw that in male and female WT mice, the number of EUEs was higher in the light phase compared to the dark phase. Previous studies in voles have indicated that the number of EUEs is higher during the active phase of circadian rhythm (Gerkema et al., 1993) with similar findings in rats (Ootsuka et al., 2009). However, another study in mice showed no significant change in temperature EUEs between the light and dark phases (Miyata et al., 2016). Our results point to more events occurring in the light phase and perhaps indicate an inactive phase preparedness to respond when vulnerable to predation in mice. Interestingly, the number of EUEs did not differ significantly between the dark and light phase in *Kiss1r* KO or *Kiss1*-Cre mice. Conversely, we saw an increase in the amplitude of EUEs in the dark phase of female *Kiss1r* KO and male *Kiss1*-Cre mice, while EUE duration was greater in male WT mice.

Orexin neurons in the hypothalamus have been shown to have EUE coordinated activation and may be involved in the integration of ultradian changes in states of wakefulness with activity, Tc and food intake (Miyata et al., 2016). Our data indicate that kisspeptin signalling could also modulate these events, in particular the different pattern of EUEs between the light and dark phases. However, it should be noted that the effects seen in EUE characteristics due to altered kisspeptin signalling are likely to be subtle given that the overall pattern of EUE number, amplitude and duration appeared similar among WT, *Kiss1*-Cre and *Kiss1r* KO mice. One notable limitation to our study was that our Tc recordings were limited to 15 min intervals, and the optimal sampling frequency for wavelet analysis

is equal or less than 5 min (Goh et al., 2019). Nevertheless, the role that EUEs play in physiology, or in the reduced energy expenditure in the *Kiss1r* KO mouse, is intriguing but yet to be fully determined.

Our data again confirm the increased adiposity and hyperleptinemic phenotype previously reported in female *Kiss1r* KO mice at 10 weeks of age (Tolson et al., 2014, 2019). Importantly, body mass changes in the female *Kiss1r* KO do not differ from WT until approximately 10–12 weeks of age and later (Tolson et al., 2014). Adiposity on the other hand (either measured by fat pad mass or dual-energy X-ray absorptiometry) is already increased at earlier ages than when body mass first becomes larger (Tolson et al., 2014, 2019). We chose to focus on this 10 week age to ascertain effects of the genotype that may *cause* the metabolic phenotype as opposed to being a *consequence* of the phenotype. Also consistent with previous studies was the lack of a clear metabolic phenotype in the male *Kiss1r* KO mice. Importantly, our previous data has shown increased adiposity and leptin concentrations in male *Kiss1r* KO mice (albeit not to the same degree as the female *Kiss1r* KO), but that phenotype seems to develop only at older ages (approximately 20 weeks of age) (Halvorson et al., 2020; Tolson et al., 2014, 2019).

Unlike *Kiss1r* KO females, there was no evidence of obesity in our *Kiss1*-Cre mice (male or female) at 10 or 20 weeks of age. Body mass in the male *Kiss1*-Cre was lower than the male WT at 10 weeks of age – similar to the male *Kiss1r* KO – but otherwise there were no genotype differences in adiposity, leptin, or Tc of *Kiss1*-Cre mice compared to WT. Interestingly, we did see a significant difference in the Tc acrophase of *Kiss1*-Cre mice – but only when compared to the *Kiss1r* KO model. Why such a difference is apparent when there is absent receptor versus “near-absent” kisspeptin is not known, but it is worth clarifying that neither of the two groups presented significant difference in the acrophase compared to the WT. Nevertheless, these data again point to kisspeptin playing a key role in mediating circadian control of Tc (Padilla et al., 2019) and in particular a shift in phase, which may occur due to altered response to circadian zeitgebers such as light/dark cycle. Interestingly, these data also point to diverging roles between kisspeptin and Kiss1r – and this requires further investigation. The *Kiss1*-Cre mice have an approximate 95% reduction in *Kiss1* transcript levels and are “sub--fertile” (Popa et al., 2013), therefore we hypothesised that they would also possess a metabolic phenotype. The lack of obesity in the *Kiss1*-Cre could indicate that the 5% of kisspeptin that remains available is adequate to prevent the obesity phenotype that we see in the global *Kiss1r* KO model. It is also possible that this effect is due to constitutive activity of Kiss1r independent of its ligand. To check whether a phenotype might develop later in life, as with the male *Kiss1r* KO, we examined a cohort of *Kiss1*-Cre and WT mice at 20 weeks of age. Again, these mice showed no difference in body mass, adiposity, or leptin concentrations. At 20 weeks of age, we did find a significantly lower *Ucp1* mRNA expression in BAT of female *Kiss1*-Cre, but an increase in the male *Kiss1*-Cre, compared to their respective WT controls. Therefore, it is possible that a metabolic phenotype may develop as the mice age further. Additional analysis of Tc would be informative in similarly aged 20 week old *Kiss1*-Cre mice and mice aged further to determine if there are any subsequent changes in thermoregulation and energy balance.

Overall, our data show a lower Tc in *Kiss1r* KO females, consistent with lower *Ucp1* mRNA expression in BAT. Moreover, we show alterations in the circadian rhythms and EUEs of Tc, which likely contribute to differences in metabolic heat production and energy expenditure, and therefore the predisposition to obesity in these mice. The observed alterations in thermogenesis in these global *Kiss1r* KOs suggest that endogenous kisspeptin signalling has a role in circadian and ultradian rhythm-driven pathways that ultimately result in the control of energy expenditure and contribute to obesity. Moreover, a lack of metabolic or temperature phenotype in *Kiss1*-Cre mice suggests that only a very small amount of endogenous kisspeptin may be sufficient for achieving metabolic regulation, as seems to be the case for kisspeptin's control of fertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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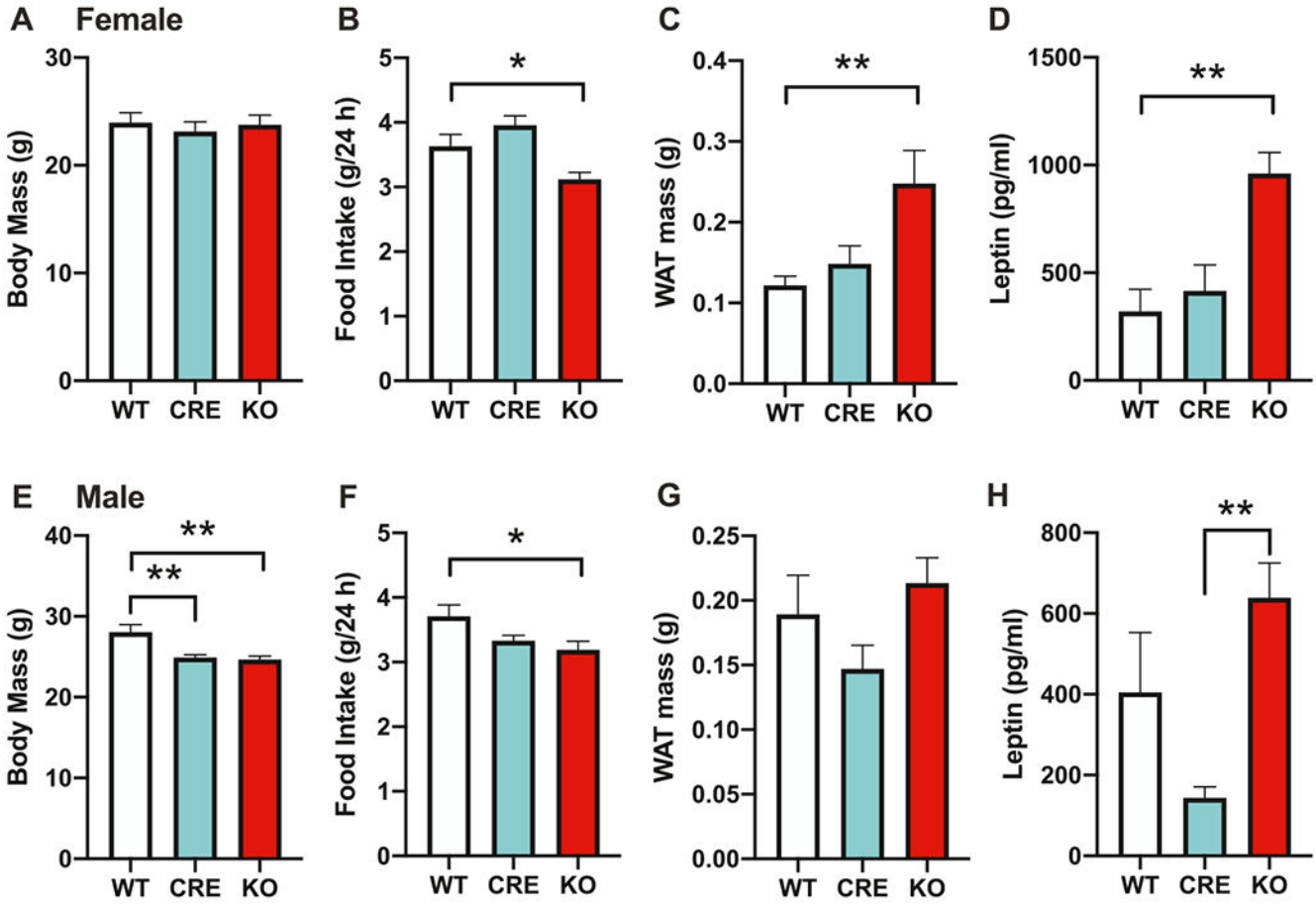

Fig. 1.

Female *Kiss1r* knock out (KO) mice display increase adiposity at 10 weeks of age. Body mass (A, E), 24 h cumulative food intake (B, F), white adipose tissue (WAT) mass (C, G) and plasma leptin concentrations (D, H) in female and male *Kiss1r* KO, *Kiss1*-Cre (CRE) and wild type (WT) mice. Genotype effects are indicated * $P < 0.05$, **$P < 0.01$. Values are displayed as mean ± SEM (Female WT n = 8, CRE n = 7, KO n = 6; Male n = 6 per group).

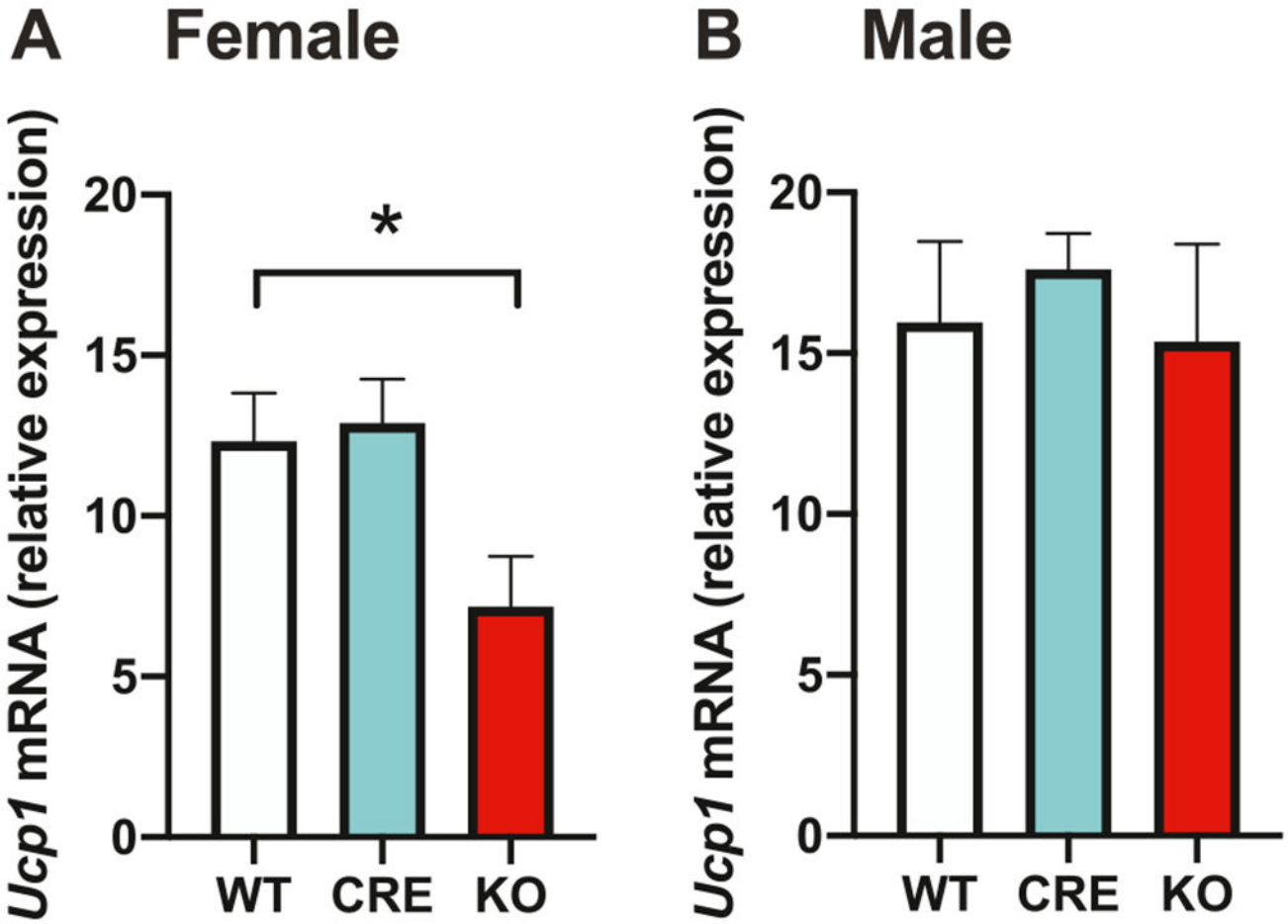

Fig. 2.

Brown adipose tissue (BAT) *Ucp1* mRNA expression in female (A) and male (B) wild type (WT), *Kiss1*-Cre (CRE) *Kiss1r* knock out (KO) and wild type (WT) mice. Genotype effects are indicated * $P < 0.05$. Values are displayed as mean ± SEM (Female WT n = 8, CRE n = 7, KO n = 6; Male n = 6 per group).

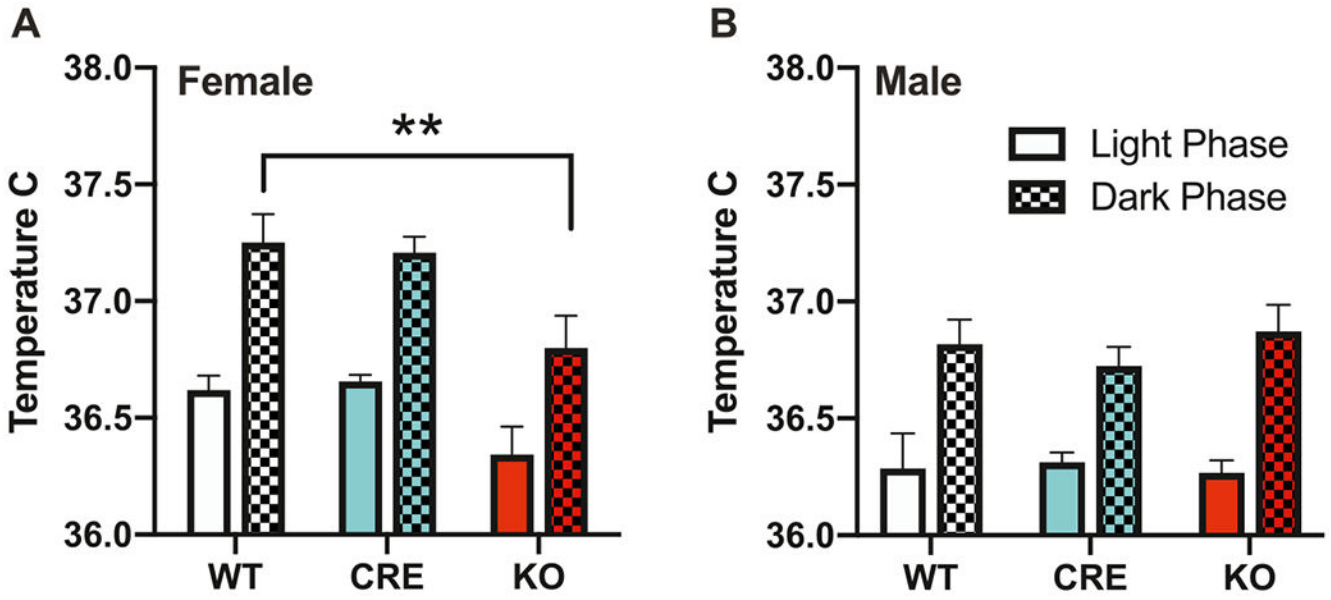

Fig. 3.

Core body temperature is decreased in female *Kiss1r* knock out (KO), but not *Kiss1*-Cre (CRE) mice. *Kiss1r* KO females (A) have lower body temperature compared to WT during the dark phase of the light cycle (cross hatched bars). No change was seen in males (B). Genotype effects are indicated **P < 0.01. Values are displayed as mean ± SEM (Female WT n = 12, CRE n = 5, KO n = 7; Male WT n = 5, CRE n = 5, KO n = 4 per group).

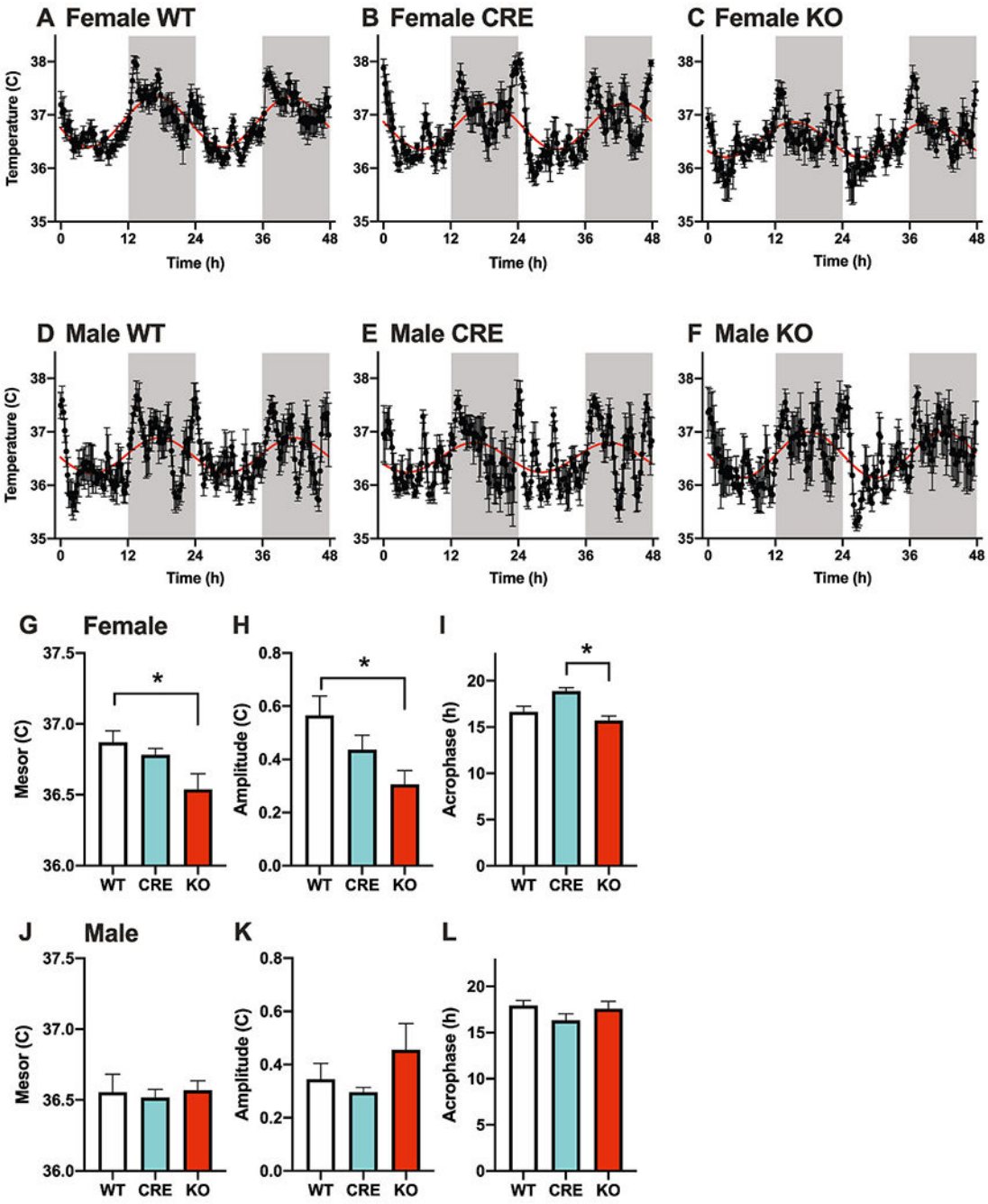

Fig. 4.

Analysis of circadian rhythms of core body temperature (Tc) in *Kiss1r* knock out (KO) and *Kiss1*-Cre (CRE) mice. Mean Tc over 48 h is shown for female (A–C) and male (D–F) wild type (WT), *Kiss1*-Cre (CRE) and *Kiss1r* knock out (KO) mice with cosinor curves superimposed (red). Mean mesor, amplitude and acrophase for cosinor curves are shown for female (G–I) and male (J–L) WT, CRE and KO mice. Genotype effects are indicated * $P < 0.05$. Values are displayed as mean ± SEM (Female WT n = 12, CRE n = 5, KO n = 7; Male WT n = 5, CRE n = 5, KO n = 4 per group).

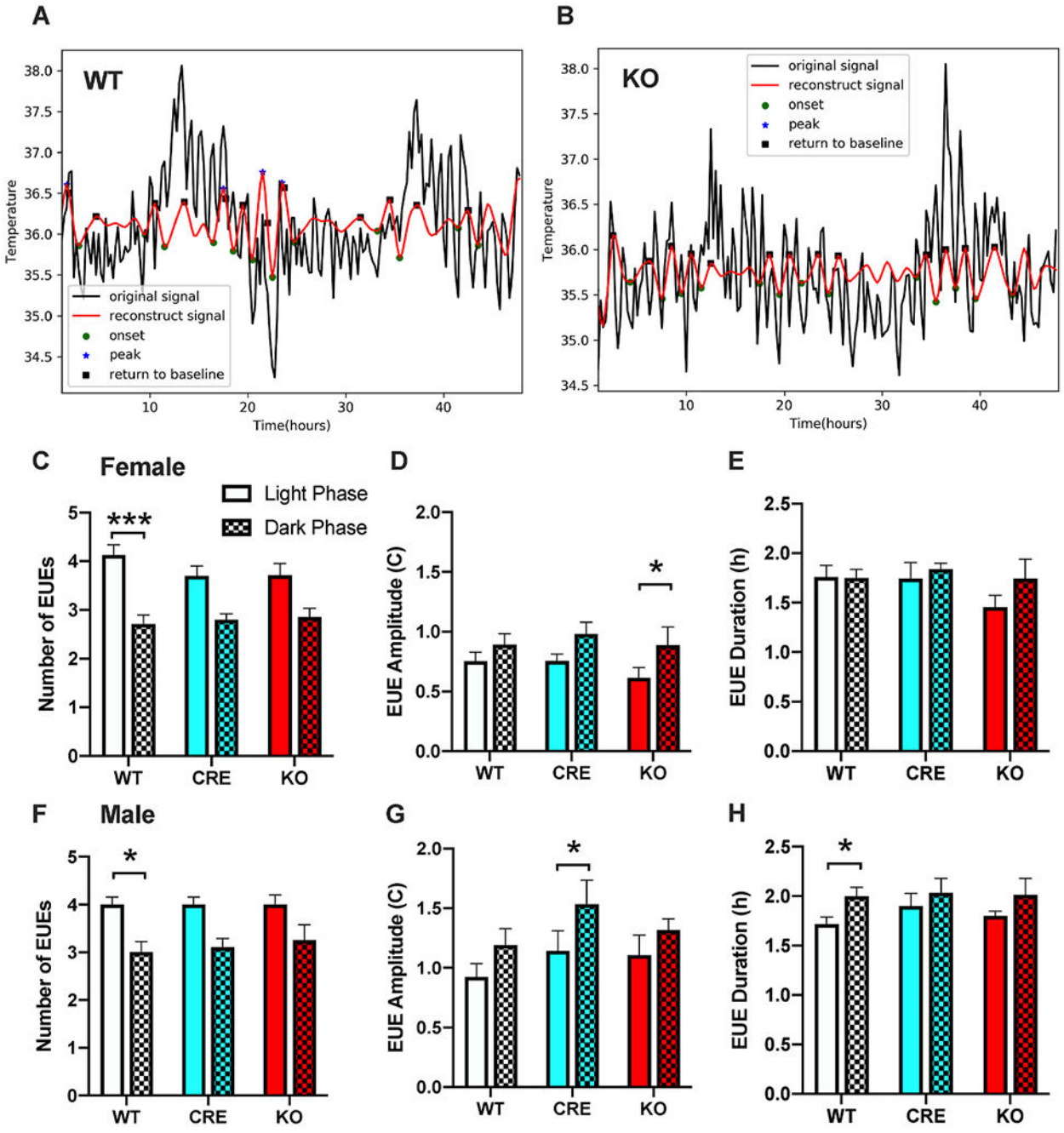

Fig. 5.

Analysis of ultradian rhythms of core body temperature in *Kiss1r* knock out (KO) and *Kiss1*-Cre (CRE) mice. Individual recordings over 48 h from a representative female WT (A) and KO (B) mouse are shown with the reconstructed temperature signals (red) displaying episodic ultradian events (EUE). Mean number of EUEs, EUE amplitude and EUE duration are shown for female (C–E) and male (F–H) WT, CRE and KO mice. Genotype effects are indicated * $P < 0.05$. Values are displayed as mean ± SEM (Female WT n = 12, CRE n = 5, KO n = 7; Male WT n = 5, CRE n = 5, KO n = 4 per group).

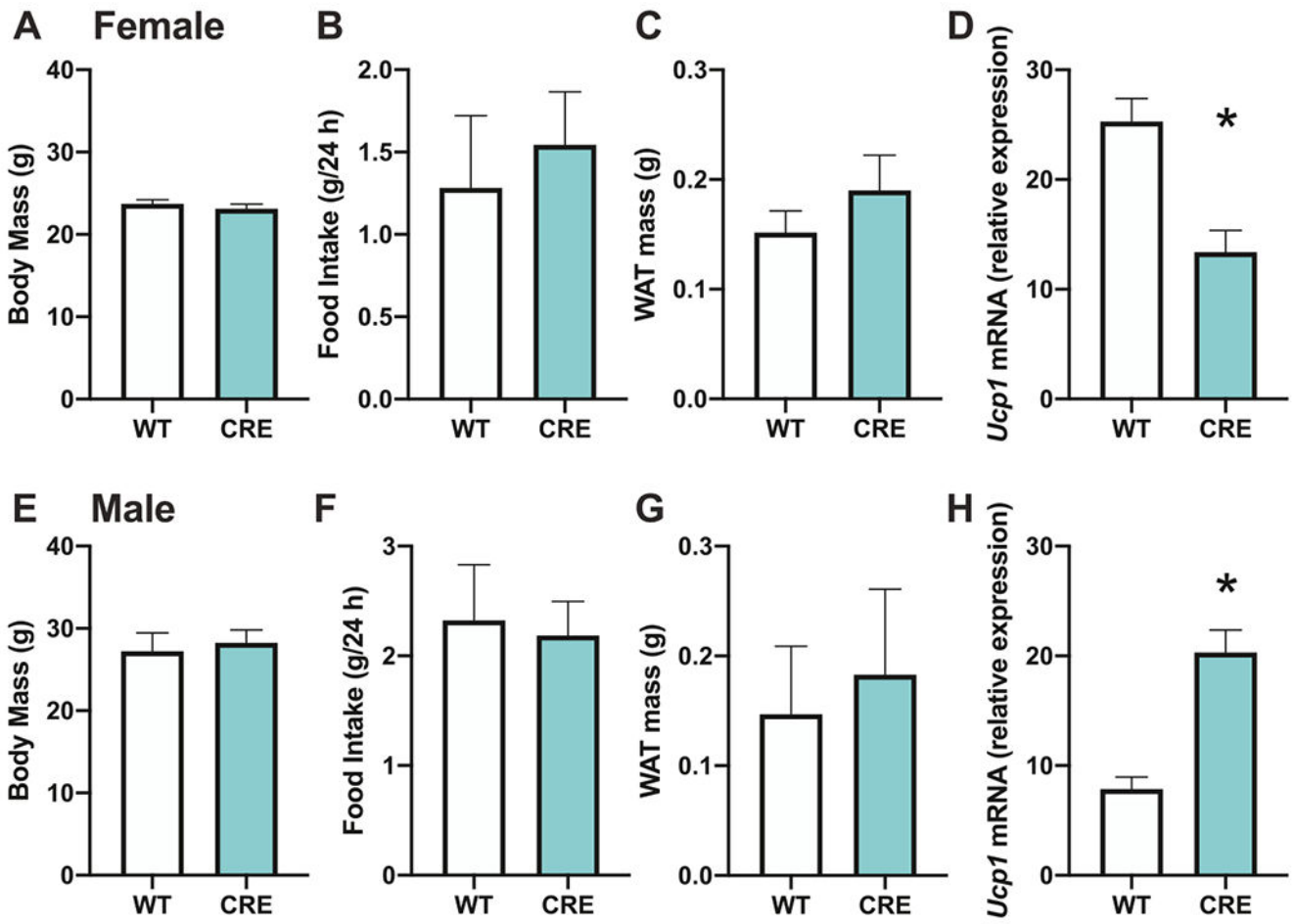

Fig. 6.

No clear evidence for obesity in *Kiss1*-Cre mice at 20 weeks of age. Body mass (A, E), 24 h cumulative food intake (B, F), white adipose tissue (WAT) mass (C, G) and brown adipose tissue (BAT) *Ucp1* mRNA expression (D, H) in female and male *Kiss1*-Cre (CRE) and wild type (WT) mice. Genotype effects in BAT *Ucp1* mRNA expression are indicated * $P < 0.05$, Values are displayed as mean ± SEM (Female WT n = 4, CRE n = 8, n = 6; Male n = 4 per group).

Table 1

Polymerase chain reaction primer details for Neuropeptide Y (*Npy*), Proopiomelanocortin (*Pomc*), and Uncoupling-protein 1 (*Ucp1*) (Qiagen QuantiTect) as well as mouse peptidylprolyl isomerase A (*Ppia*), mouse succinate hydrogenase (*Sdha*) and TATA box binding protein (*Tbp*) (Geneworks).

Gene	QuantiTect Primer Assay Detail	NCBI Reference Sequence	Cat. No	Product size
Npy	Mm_Npy_1_SG	NM_023456	QT00134302	150 bp
Pomc	Mm_Pomc_1_SG	NM_008895	QT00162218	99 bp
Ucp1	Mm_Ucp1_1_SG	NM_009463	QT00097300	70 bp

	Geneworks Forward Primer	Geneworks Reverse Primer	
mPpia	AGCATACAGGTCCTGGCATC	TTCACCTTCCCAAAGACCAC	127 bp
mSdha	TGGGGAGTGCCGTGGTGTCA	CTGTGCCGTCCCCTGTGCTG	149 bp
mTbp	GGGAGAATCATGGACCAGAA	CCGTAAGGCATCATTGGACT	113 bp