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RESEARCH ARTICLE

Conditional knockout of kisspeptin signaling in brown adipose tissue increases metabolic rate and body temperature and lowers body weight

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Abstract

The peptide kisspeptin and its receptor, Kiss1r, act centrally to stimulate reproduction. Evidence indicates that kisspeptin signaling is also important for body weight (BW) and metabolism. We recently reported that *Kiss1r* KO mice develop obesity, along with reduced metabolism and energy expenditure, independent of estradiol levels. Outside the brain, *Kiss1r* is expressed in several metabolic tissues, including brown adipose tissue (BAT), but it is unknown which specific tissue is responsible for the metabolic phenotype in *Kiss1r* KOs. We first determined that global *Kiss1r* KO mice have significant alterations in body temperature and BAT thermogenic gene expression, perhaps contributing to their obesity. Next, to test whether kisspeptin signaling specifically in BAT influences BW, metabolism, or body temperature, we used Cre/lox technology to generate conditional *Kiss1r* knockout exclusively in BAT (BAT-*Kiss1r* KO). Unlike global *Kiss1r* KOs, BAT-*Kiss1r* KOs (lacking *Kiss1r* in just BAT) were not hypogonadal, as expected. Surprisingly, however, BAT-*Kiss1r* KOs of both sexes displayed significantly lower BW and adiposity than controls. This novel BAT-*Kiss1r* KO phenotype was of greater magnitude in females and was associated with improved glucose tolerance, increased metabolism, energy expenditure, and locomotor activity, along with increased body temperature and BAT gene expression, specifically *Cox8b*. Our findings suggest that the previously observed obesity and decreased metabolism in global *Kiss1r* KOs reflect impaired kisspeptin signaling in non-BAT tissues. However, the novel finding of increased metabolism and body temperature and lower BW in BAT-*Kiss1r* KOs reveal a previously unidentified role for endogenous kisspeptin signaling in BAT in modulating metabolic and thermogenic physiology.

KEYWORDS

adipose, BAT, fat, GPR54, Kiss1, Kiss1r, kisspeptin, metabolism, obesity, temperature

1 | INTRODUCTION

Kisspeptin (encoded by the gene *Kiss1*) is a neuropeptide that binds the receptor, Kiss1r (previously termed GPR54). In mammals, including humans, kisspeptin and its receptor regulate reproduction by acting in the brain to directly stimulate gonadotropin-releasing hormone (GnRH) secretion, supported by the fact that humans and rodents with disrupted kisspeptin signaling are infertile, have undeveloped gonads, and have diminished reproductive hormone secretion (reviewed in [1,2]). In addition to being expressed in GnRH neurons in the brain,[6-8] Kiss1r is also located in several peripheral tissues, such as adipose tissue, pancreas, and gonads.[9-14] Likewise, kisspeptin is expressed in several peripheral tissues, including liver, pancreas, gonad, and placenta.[12-14] This peripheral expression of both kisspeptin and its receptor suggests that kisspeptin signaling likely has additional roles outside of reproductive regulation. However, until recently, non-reproductive roles of kisspeptin have been largely overlooked.

We recently reported that, in addition to stimulating the reproductive axis, the kisspeptin system is also an important player in regulating body weight (BW), energy balance, and glucose homeostasis. Specifically, compared with WT littermates, global *Kiss1r* knockout (KO) female mice—lacking kisspeptin signaling in all tissues—displayed dramatically higher BWs beginning in early adulthood, weighing as much as 30% more than control females.[15,16] In addition to becoming obese, adult *Kiss1r* KO females also had increased adiposity, higher leptin levels, and substantially impaired glucose tolerance, both on standard chow and high-fat diets.[15,16] This obesity phenotype was sexually dimorphic, as *Kiss1r* KO males had normal BW and glucose regulation out to 6 months of age. Despite their obesity, adult *Kiss1r* KO females did not have increased food intake, but they did display significantly reduced metabolic parameters, including lower respiratory rates and energy expenditure, suggesting that their obesity reflects, at least in part, lower daily energy expenditure.[15,16] Importantly, the BW and metabolic phenotype in *Kiss1r* KO females were not solely reflective of absent gonadal sex steroids, in particular ovarian estradiol (E_2), as long-term ovariectomized *Kiss1r* KO females still developed obesity, hyperleptinemia, reduced metabolism, and glucose intolerance versus similarly ovariectomized WT females (ovaries removed before puberty to permanently eliminate ovarian-derived E_2 in both genotypes) .[15,16]

The findings above in *Kiss1r* KOs demonstrated that globally absent kisspeptin signaling can be an important factor in BW, adiposity, glucose tolerance, and metabolism, particularly in females. However, because Kiss1r was absent from all cells in the body in those mice, it remains unknown which specific tissues and cell types are responsible for kisspeptin's influence on the various metabolic phenotypes. Indeed, peripheral Kiss1r is present in several metabolic-related peripheral tissues, suggesting a number of potential candidate target sites for its ability to alter metabolism and energy balance. Furthermore, it is possible that different phenotypic alterations observed in the global *Kiss1*r KOs may reflect absent kisspeptin signaling in different tissues. For example, glucose intolerance might reflect absent Kiss1r in the pancreas, whereas altered adiposity or energy expenditure could hypothetically reflect absent Kiss1r in white or brown adipose tissue (BAT), with the net effect of these tissue-specific changes resulting in the overall obese phenotype.

The present study had two primary goals. First, we tested whether body temperature and thermogenic gene expression in BAT are altered in global *Kiss1r* KO mice, correlating with their previously reported changed BW and metabolism. Second, we sought to use a *Kiss1r* flox mouse line to selectively ablate *Kiss1r* from just one target cell type, thereby isolating potential effects of kisspeptin signaling on BW and metabolism. We chose BAT because we found that our global *Kiss1r* KOs displayed altered body temperature and expression levels of uncoupling protein-1 (*Ucp1*), a thermogenic protein in the mitochondria of BAT. Thus, we used Cre/lox technology to generate specific deletion of *Kiss1r* in just BAT to test the hypothesis that endogenous kisspeptin may normally modulate metabolism or body temperature by acting directly in BAT.

2 | MATERIALS AND METHODS

2.1 | Animals

Experiments used either "global" (whole-body) *Kiss1r* KO mice, the same mouse line as in our previous reports,[15-18] or a new conditional KO mouse line generated with Cre/lox technology: a BAT-specific *Kiss1r* KO line (termed BAT cKO). In all cases, littermate sibling controls (WTs or Cre⁻) were used to compare directly to the KO animals.

To study the role of endogenous Kiss1r specifically in BAT, we generated mice lacking Kiss1r exclusively in cells expressing *Ucp-1*, which are highly localized to BAT. Female *Ucp1*-Cre mice (Jackson Labs) were crossed with our recently created male *Kiss1r*^fl/fl mice[13,19] to generate *Ucp1*-Cre^−/−/*Kiss1r*^fl/wt female progeny, which were then backcrossed with male *Kiss1r*^fl/fl mice to generate BAT Kiss1r KOs (*Ucp1*-Cre^−/−/*Kiss1r*^fl/fl; termed "BAT cKO") and control littermates (*Ucp1*-Cre^−/−/*Kiss1r*^fl/wt and *Ucp1*-Cre^−/−/*Kiss1r*^fl/fl). For confirmation of proper Cre/flox removal of *Kiss1r*, multiple tissues were dissected from cKO mice. DNA was extracted from each tissue and tested for recombination using PCR. To further confirm the expression pattern of *Ucp1*-Cre, which was previously reported to be in BAT only, a cohort of cKO mice were crossed with

Cre-dependent tdTomato reporter mice to generate offspring that express the fluorescent tdTomato reporter gene wherever Cre recombination has occurred. Sections of BAT and brain were then collected and fixed in 4% paraformaldehyde for microscopy analysis of tdTomato reporter expression.

Mice from all lines were genotyped and sexed using PCR of DNA obtained from toe samples at postnatal day 7 (PND7) or tail DNA at weaning. All mice were weaned at PND21 and housed 2-3 per cage with mixed genotype in a 12-hour light/12-hour dark cycle, with *ad libitum* water and standard rodent chow containing 3.5 kcal/g, 45.2% carbohydrates, 11.4% fat, and 17.2% crude protein. All experiments were approved by the Institutional Animal Care and Use Committee from the University of California San Diego and by the Animal Ethics Committee from the University of Western Australia.

2.2 | Ovariectomies

In the global *Kiss1r* KO studies, some mice of each genotype (detailed in the results) were ovariectomized (OVX) to remove any influence of circulating sex steroids (E_2) on the metabolic measures. Mice were briefly anesthetized with isoflurane, the ventral skin shaved and sterilized, and a single midline incision made in the skin and abdominal musculature. Both left and right ovaries were identified and cut out, and the muscle wall sutured with chromic gut. The skin cut was then closed with surgical wound clips. Mice were given analgesic (buprenorphine) via s.c. injection.

2.3 | Reproductive characteristics

Global *Kiss1r* KO mice are well known to have extremely underdeveloped gonads, reduced anogenital distance (AGD; an indirect measure of testosterone exposure) in males, and correspondingly low circulating estrogen and testosterone levels. For the newly generated conditional KO mouse line (BAT cKO), reproductive developmental status for females and males were quantified by measuring AGD at week 12, and gonadal weights at sacrifice. For the former, mice were briefly anesthetized with isoflurane and distance measurement was taken from the anus opening to the base of the reproductive organ. In addition, fertility was assessed in the BAT cKO mouse line by pairing adult female cKOs with control males.

2.4 | Body weight assessment and body composition analyses

Mice of both sexes from the BAT cKO line were analyzed for their BW at multiple ages. Control and experimental littermates were weighed once every 2 weeks, starting at 4 weeks of age and ending around 4-5 months of age. Lean mass and fat mass of BAT cKO and control mice were analyzed using the EchoMRI-3 instrument (EchoMRI LLC, Houston, TX), a quantitative nuclear magnetic resonance (qNMR) imaging system for whole-body composition analysis of unanesthetized small animals, at 19- to 22-weeks old. qNMR body composition analysis with EchoMRI instrumentation has been proposed to be "gold standard" methodology for metabolic studies in the mouse. After calibration of the EchoMRI 3-in-1 instrument, each mouse was positioned into the EchoMRI Chamber. The instrument calculated the lean mass and fat mass.

2.5 | Metabolic and energy expenditure analyses

To measure metabolic rates, adult BAT cKO and control littermates were analyzed at 20-24 weeks of age with indirect calorimetry using equal flow Comprehensive Laboratory Animal Monitoring System (CLAMS) calorimeter system (CLAMS; Columbus Instruments, Columbus, OH). The mice were individually housed 2-3 days prior to the experiment for habituation to being single housed. Food in the CLAMS cages was Tekland diet 2018 (18% protein, 6% fat, and 42% carbohydrate). CLAMS data were collected in clear respiratory chambers ($20 \times 10 \times 12.5$ cm) equipped with a sipper tube delivering water and a food tray connected to a balance. The consumption of O_2 and production of CO_2 was measured by having sample air sequentially passed through O_2 and CO_2 sensors (Columbus Instruments) for determination of O_2 and CO_2 content, from which measures of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. Room air was passed through chambers at a flow rate of 0.5 L/min. Exhaust air from each chamber was sampled at 18-min intervals for 1 minutes. Outdoor air reference values were sampled after every eight measurements. Gas sensors were calibrated prior to the onset of experiments with primary gas standards containing known concentrations of O_2, CO_2, and N_2 (Airgas Puritan Medical, Ontario, CA). Respiratory exchange ratio (RER) was calculated as the ratio of carbon dioxide production (VCO_2) to oxygen consumption (VO_2). Per standard convention, energy expenditure (EE; heat formation) was corrected for each mouse's lean mass. Total locomotor activity was measured by photosensor beam breaks. Data were recorded under ambient room temperature (~24°C) for up to 7 days.

2.6 | Body temperature assessment

Telemeters were implanted into the abdominal cavity of adult mice to measure their core body temperature every 18 min. The sterile transmitters (Emitter 15.5×6.5 mm, 1.1 grams,

sterilized in Spor-Klenz Cold Sterilant) were implanted into the peritoneal cavity and sutured by dissolvable suture thread to the cavity wall. VetBond surgical glue was used to close the external skin cut. A 1-week recovery period was given to the mice before starting measurement under ambient room temperature (~24°C) for several days. In a separate study, global *Kiss1r* KO and WT female mice with telemeters for core body temperature recording were exposed to a cold challenge where they were transferred to a cold room (4°C) for 6 hours, after which they were returned to ~22°C for 1 hour. In that experiment, body temperature was recorded every 30 minutes to track changes over time in the cold.

2.7 | Glucose tolerance tests

At 18-20 weeks of age, BAT cKO mice and their control littermates of both sexes underwent glucose tolerance testing (GTT). Mice of both genotypes and sexes were fasted for 6 hours before starting the glucose measurements, with free access to water throughout the experiment. Blood glucose was measured just before glucose injection using a handheld glucometer. Then, at time 0, mice were given an IP glucose injection; 2 g/kg BW dissolved in saline. Blood glucose levels were then re-measured at 15, 30, 45, 60, 90, and 120 minutes after glucose injection.

2.8 | qPCR of Kiss1r in multiple tissues and thermogenic genes in BAT

Adult female *Kiss1r* KOs show an obese and metabolically impaired phenotype. To determine which tissues express *Kiss1r* in normal adult females, we used RT-PCR to assess presence in multiple metabolic and non-metabolic tissues. To verify these initial results and provide more quantitative analysis, we next performed qPCR to detect *Kiss1r* mRNA in various tissues from normal adult (8-weeks old) females. For the *Kiss1r* gene, we used the QuantiTect Primer Assay using Mm_Kiss1r_1_SG (catalog number QT00140427).

To determine whether thermogenic-related genes were altered in the BAT of *Kiss1r* KO and BAT cKO females, BAT tissue was collected and tested with real-time qPCR for expression of several genes of interest including *Ucp1* (fwd AGGC TTCCAGTACCATTAGGT, rev CTGAGTGAGGCAAAGCT GATTT,) *Coxb8* (fwd TGTGGGGATCTCAGCCATAGT; rev AGTGGGCTAAGACCCATCCTG, and *PRDM16* (fwd CCAAGGCAAGGGCGAAGAA,rev CCAAGGCAAGGGCG AAGAA). For all assays, total RNA was isolated using the phenol/chloroform method and converted to cDNA via reverse transcription (Promega, Sydney, Australia). qRT-PCR was performed in 10 µl 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). Samples were compared to a standard curve (10-fold dilution) and relative gene expression was normalized using a GE Norm algorithm of reference genes peptidylprolyl isomerase A (*Ppia*), succinate hydrogenase (*Sdha*), and TATA box binding protein (*Tbp*).[17]

2.9 | Statistical analyses

All data are presented as mean ± SEM. For data at single points (non-repeated measures), single comparisons were made using one- or two-tailed t tests, as appropriate, and multiple comparisons were performed using one-way ANOVA with Tukey's post-hoc test. For repeated measures (BWs, GTTs, and body temperature during cold exposure), repeated measures ANOVA was performed, with Bonferroni post-hoc tests directly comparing genotypes at specific points for three groups or *t* tests for two groups. Statistical significance was $P < .05$.

3 | RESULTS

3.1 | Body temperature is decreased in global Kiss1r KO female mice

Given the decreased metabolism and energy expenditure of global *Kiss1r* KOs and *Kiss1r* is expressed in BAT, we asked whether absent kisspeptin signaling might alter body temperature, and if so, whether this correlated with changes in BAT size or gene expression. We first determined that core body temperature is significantly lower in global *Kiss1r* KO females compared to WT littermates, especially during the dark phase of the light-dark cycle when mice are most active ($P < .05$, Figure 1). This lower body temperature was true for both gonad-intact ($P < .05$, Figure 1A) and OVX mice ($P < .05$, Figure 1B), indicating that the genotype difference is not simply due to differences in circulating ovarian steroid (E_2) levels. We further tested whether an acute cold challenge to normal body temperature would be impacted by loss of kisspeptin signaling. In a cold challenge at 4°C, global *Kiss1r* KOs showed a sharper rate of body temperature drop than WTs, with a significantly lower mean body temperature after 6 hours ($P < .05$, Figure 1C). Collectively, these findings suggest that the ability to maintain a normal core body temperature is impaired in the absence of normal kisspeptin signaling.

3.2 | BAT gene expression is significantly altered in global Kiss1r KO female mice

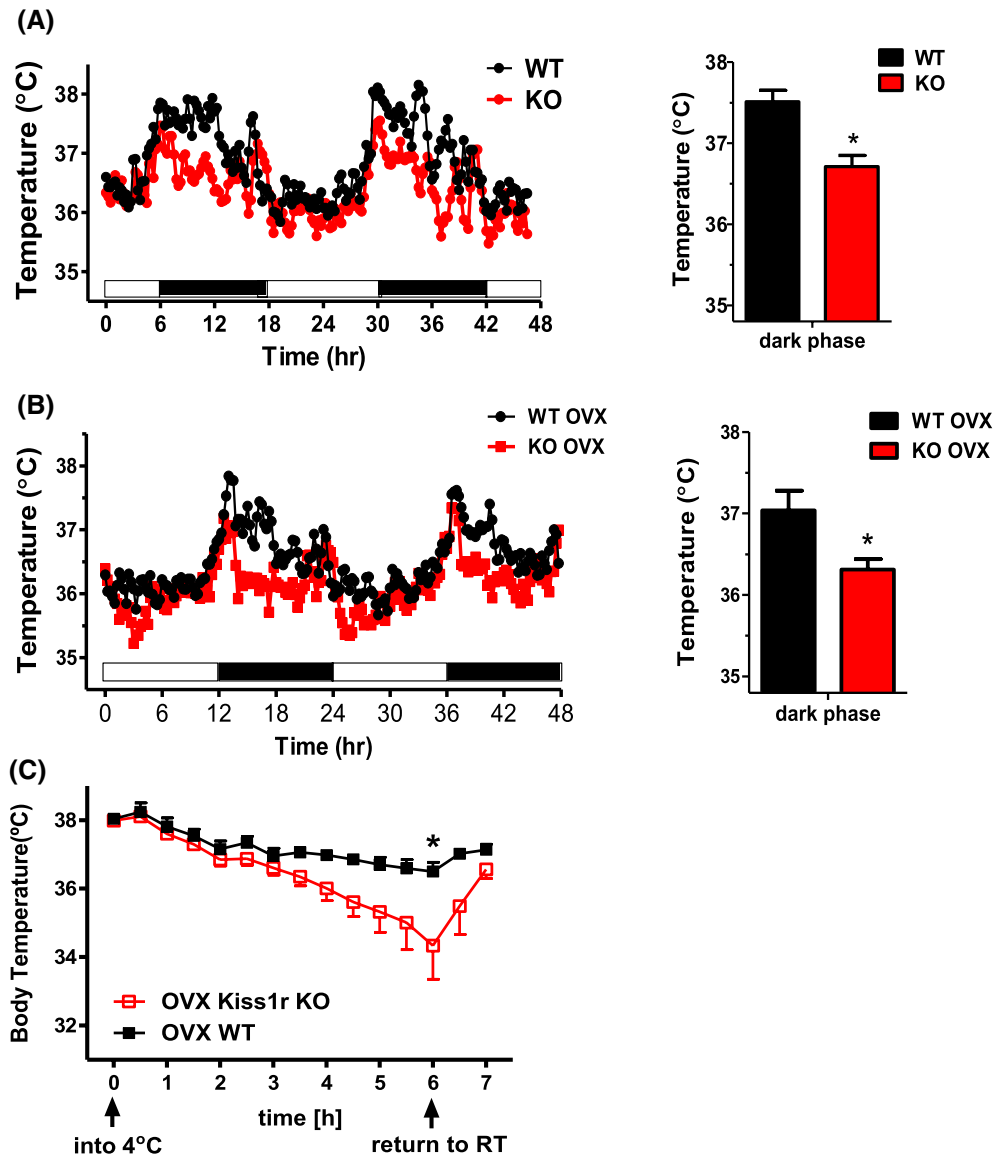Because BAT modulates thermogenesis and metabolic rate, we next tested whether thermogenic-related genes were altered

FIGURE 1 Core body temperature is lower in mice globally lacking kisspeptin signaling. A, In gonad-intact (n = 4/group) and B, chronically OVX mice (n = 5-8/group), global *Kiss1r* KO females have lower body temperature than WT littermates, primarily during the dark phase of the light cycle. C, Cold exposure at 4°C for 6 hours causes a sharper drop in body temperature in OVX global *Kiss1r* KOs than OVX WT controls (n = 6/group). *P < .05

in the BAT of global *Kiss1r* KO females. We found that even though overall BAT weight was not different between genotypes, BAT expression of *Ucp1* (uncoupling protein in the BAT mitochondria, used to generate heat by non-shivering thermogenesis), *Coxb8* (polypeptide of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport)*,* and *Prdm16* (transcriptional coregulator promoting thermogenic processes in BAT) were all significantly lower in gonad-intact global *Kiss1r* KO females versus controls (P < .05, Figure 2), perhaps contributing to the lower body temperature in the KOs. To exclude an influence of E_2 levels on this finding, we also measured BAT gene expression in OVX global *Kiss1r* KO and littermate controls. As with the gonad-intact mice, OVX *Kiss1r*

KOs had significantly lower *Ucp1* and *Cox8b* gene expression in their BAT than OVX WTs (P < .05, Figure 2), correlating with their lower body temperature (Figure 1B).

3.3 | Body temperature and BAT gene expression are normal in global Kiss1r KO males

We previously reported that, unlike global *Kiss1r* KO females, global *Kiss1r* KO males do not exhibit a significant BW phenotype.[16] We therefore tested whether there is also a sex difference in body temperature or BAT thermogenic gene expression
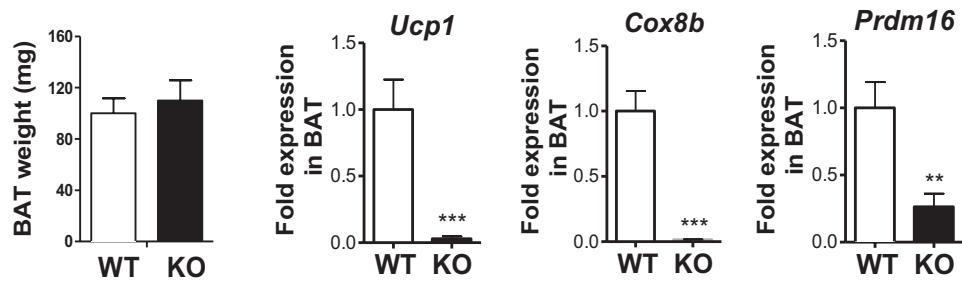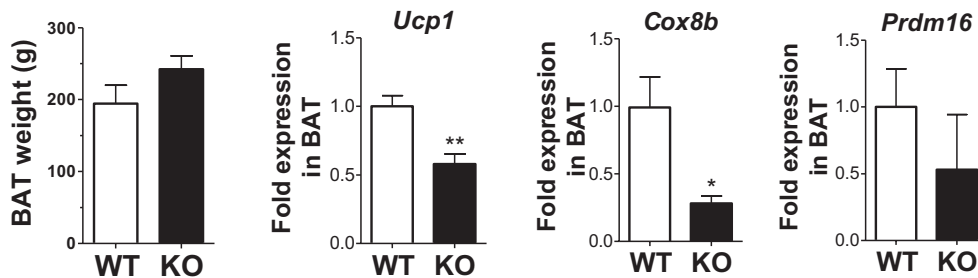
(A) Intact females

(B) OVX females

FIGURE 2   Thermogenic gene expression is decreased in BAT of global *Kiss1r* KO mice. A, In ovary-intact mice, BAT weight is normal but mRNA levels of *Ucp1* (protein in the BAT mitochondria used to generate heat by non-shivering thermogenesis), *Cox8b* (polypeptide of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport), and *Prdm16* (transcriptional coregulator promoting thermogenic processes in BAT) are significantly decreased in global *Kiss1r* KOs compared to WT littermates (n = 10-12/group). B, In chronically OVX females, BAT weight remains normal but *Ucp1* and *Cox8b* expression are still reduced in global *Kiss1r* KOs versus WT controls (n = 6-8/group). *$P < .05$; **$P < .01$, ***$P < .001$

in the global KOs. Indeed, we found that unlike global KO females (Figures 1 and 2), male global KOs have normal body temperature and BAT *Ucp1* levels compared to WT littermates (Figure 3), matching the KO males' normal BW.

## 3.4 | Kiss1r is expressed in various metabolic tissues, including BAT

Given the profound metabolic phenotype in global *Kiss1r* KO females, we assessed where *Kiss1r* is normally expressed in normal adult female mice. RT-PCR analysis detected *Kiss1r* transcript in hypothalamus (as expected) as well as several metabolic-related tissues, including BAT, WAT, adrenal, and liver (Figure 4). *Kiss1r* was also detected in ovary and kidney, and a very faint band was observed in skeletal muscle. We verified this with a more quantitative assessment of *Kiss1r* levels in normal adult females using qPCR. qPCR analysis demonstrated that, in addition to the hypothalamus, *Kiss1r* was expressed in both BAT and WAT, with slightly lower levels detected in liver (Figure 4). Matching the faint band in the RT-PCR assay, very little to no expression was detected in skeletal muscle with qPCR.

## 3.5 | Generation and validation of a BAT-specific Kiss1r KO model

The findings above suggest that impaired kisspeptin signaling somewhere in the body lowers BAT gene expression and core body temperature, likely contributing to the development of the observed obesity and metabolic phenotype. To determine what specific tissues/cell types might be involved in mediating this effect of kisspeptin signaling, we sought to use Cre/lox technology to selectively knock out Kiss1r from specific tissues, starting with BAT. To target BAT cells, we bred *Ucp1*-Cre mice to our *Kiss1r* flox mice, thereby generating selective knockout of *Kiss1r* only in *Ucp1*-expressing cells (Figure 5).

To validate this new conditional KO mouse line, we tested multiple tissues from BAT cKOs for *Kiss1r* recombination. Recombination was strongly detected in BAT but not in WAT, liver, gonads, or tail (Figure 5B). Very faint recombination was also detected in the brain (Figure 5B). To determine exactly where the brain recombination was occurring, we bred our *Ucp1*-Cre line to a Cre-dependent tdTomato reporter mouse line to assess the neuroanatomical location of *Ucp1*-driven Cre in brain, as well as confirm Cre action in BAT.

(A) GDX Males
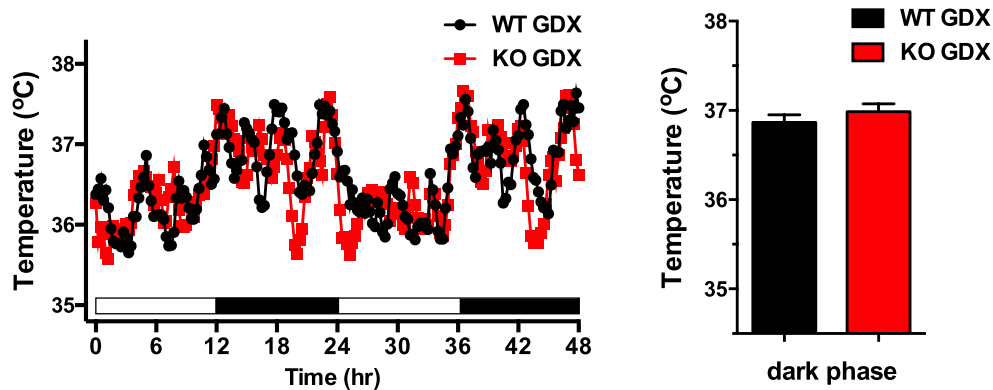
(B) GDX males
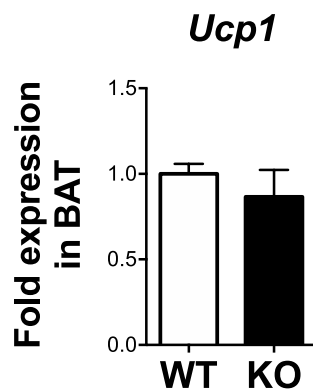
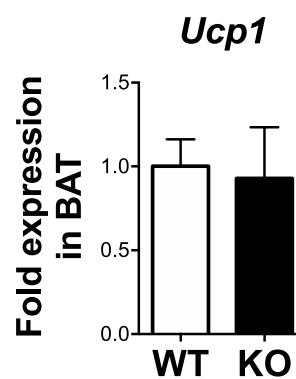### *Ucp1*

C Intact males

Ucp1

FIGURE 3 Core body temperature and *Ucp-1* gene expression levels are normal in adult male mice globally lacking kisspeptin signaling. A, Global *Kiss1r* KO males (n = 4-5/group) have similar body temperature as WT littermate males. B, BAT mRNA levels of *Ucp1* are normal in global *Kiss1r* KO males, regardless of gonadal status (n = 7-9/group)
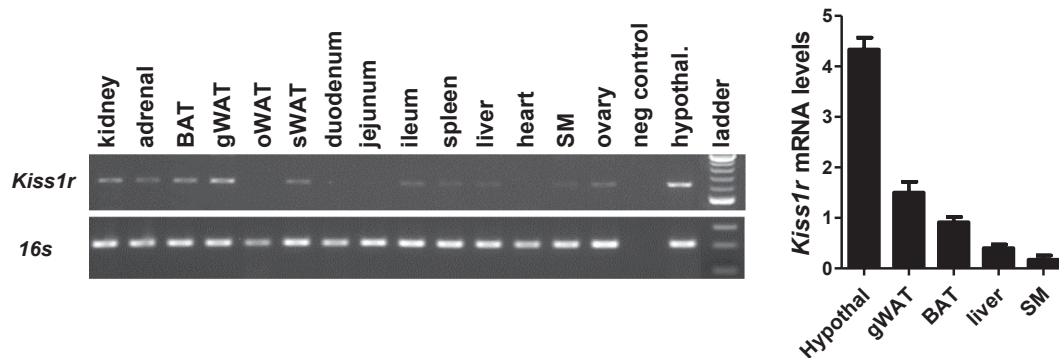

FIGURE 4 *Kiss1r* expression in BAT and other tissues of normal adult female mice. A, Gel image of RT-PCR detection of *Kiss1r* expression in hypothalamus and several metabolic tissues, including BAT, WAT, adrenal, and liver. B, qPCR analysis of *Kiss1r* levels in several metabolic tissues and brain (hypothalamus) of adult WT females (n = 5). *Kiss1r* was expressed in both BAT and WAT, and at slightly lower levels in liver. Very low expression was detected in skeletal muscle (SM)

As expected, Cre expression, identified via tdTomato fluorescence, was present throughout the entire BAT in *Ucp1*-Cre⁺/ tdTomato⁺ mice and completely lacking in the BAT of control *Ucp1*-Cre⁻/ tdTomato⁺ mice (Figure 5C). In the brain,

a few scattered fluorescent cells were present in *Ucp1*-Cre⁺/ tdTomato⁺ mice but not in Cre⁻/tdTomato⁺ controls (Figure 5D). Specifically, some fluorescent cells were observed in the dorsal lateral septum, medial paraventricular thalamus,
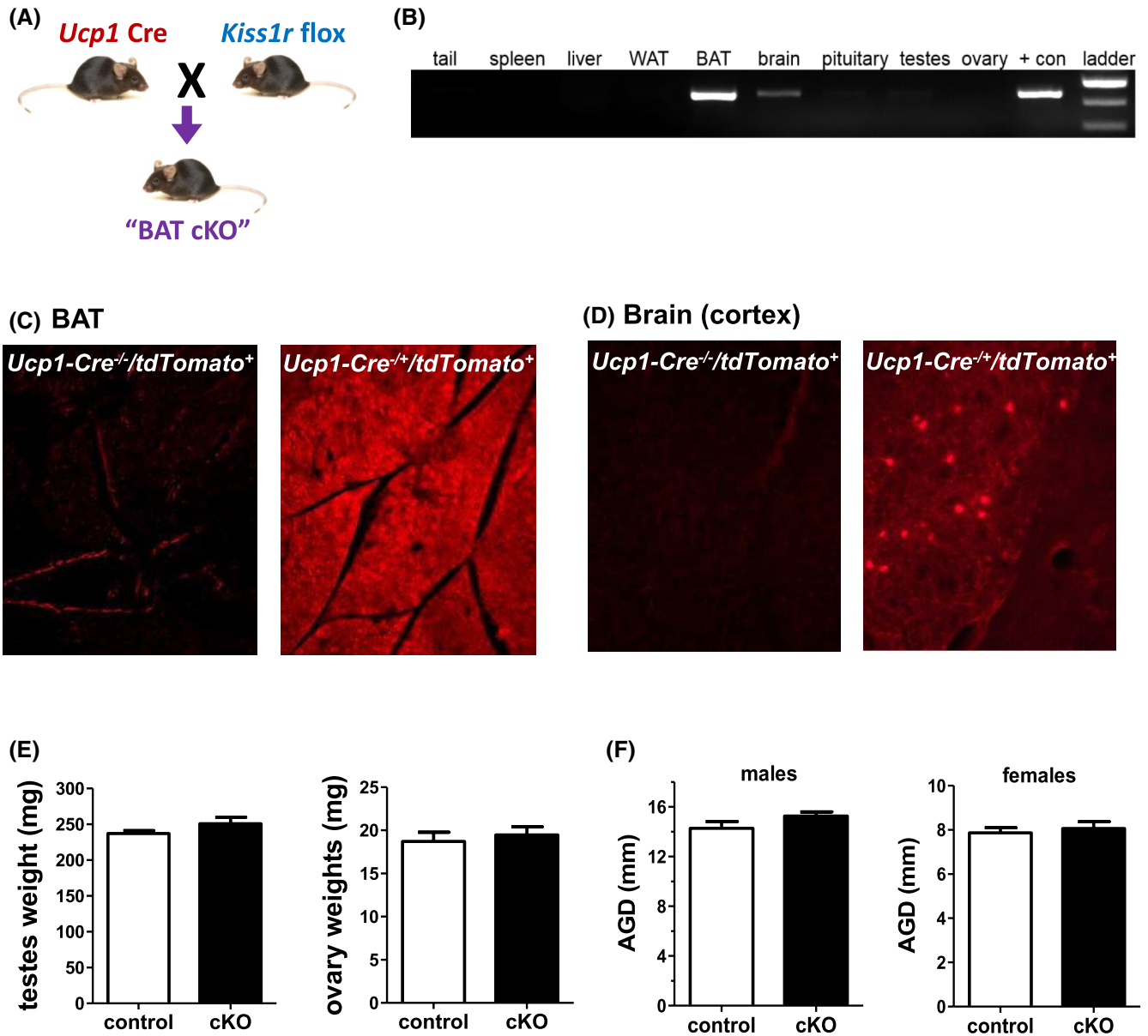
FIGURE 5 Generation and validation of BAT-specific KO of *Kiss1r* mice ("BAT cKO"). A, Strategy for selectively knocking out Kiss1r from BAT tissue by crossing *Ucp1*-Cre and *Kiss1r* floxed mouse lines. B, PCR product of different tissues collected from *Ucp1*-Cre[+]/*Kiss1r*^{fl/fl} (BAT cKO) mice tested for *Kiss1r* gene recombination. C, Cross section of BAT from *Ucp1*-Cre[−]/Tdtomato^{+/−} (control) and *Ucp1*-Cre[+]/TdTomato^{+/−} (recombined) mice demonstrating fluorescent reporter only in the latter. D, Cross section of brain tissue from *Ucp1*-Cre[−]/Tdtomato^{+/−} (control) and *Ucp1*-Cre[+]/Tdtomato^{+/−} (recombined) mice demonstrating a few fluorescent cells in the cortex of the latter (a region of the brain where *Kiss1r* is not expressed). E, Gonad weights from adult BAT cKO males and females (n = 5-8/group for males and n = 11/group for females). F, AGD in adult BAT cKO males and females (n = 7-11/group for males and n = 12-13/group for females)

cortical amygdaloid nucleus, ventromedial hypothalamus, and the cortex, all brain areas in which *Kiss1r* is not expressed in mice.[7] Thus, the brain expression of *Ucp1*-Cre is not expected to remove any neural *Kiss1r* expression in our BAT cKOs.

To assess reproductive characteristics of BAT cKOs, we first measured gonad weights. Unlike the underdeveloped gonads of global KOs, gonad weights of adult BAT cKOs of each sex were normal and not significantly different than control littermates (Figure 5E), as expected.

Additionally, in contrast to the global *Kiss1r* KO models, AGD in male BAT cKOs was normal and comparable to WTs (Figure 5F). This normal gonadal development was supported by completely normal fertility in BAT cKO females (*data not shown*). Thus, as expected, the *Ucp1*-Cre did not target GnRH neurons or other reproductive cell types where *Kiss1r* is normally expressed, ensuring that the selective knockout of endogenous Kiss1r signaling was exclusive to BAT cells.
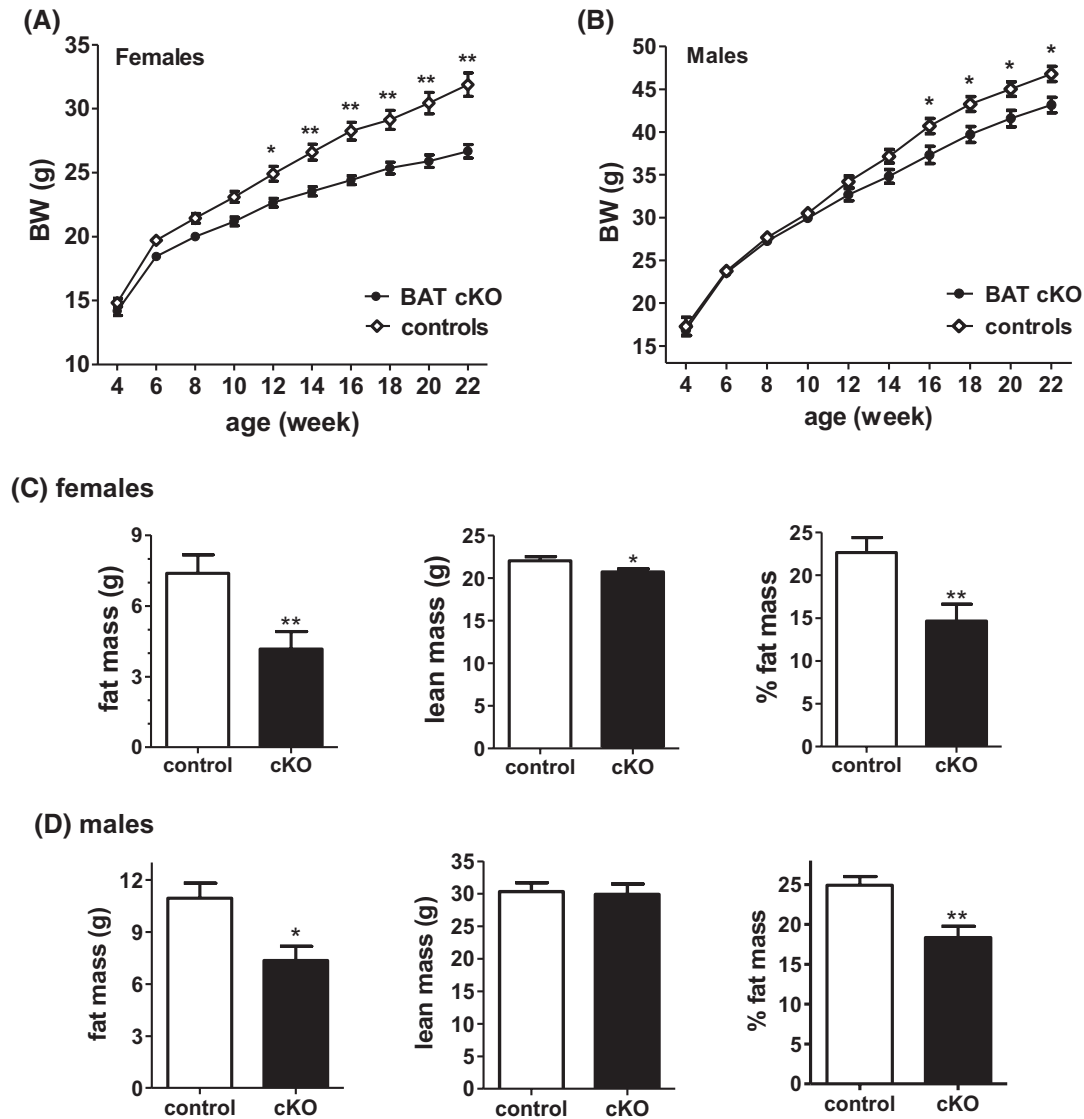
FIGURE 6 BW of adult BAT cKO mice is decreased compared to control littermates in both A, females (n = 31-40/group) and B, males (n = 21-25/group). C and D, Mean body composition (fat mass, lean mass, and % fat mass) is also altered in BAT cKO females (n = 14/group) and males (n = 5-7/group), respectively, as measured by EchoMRI. *$P < .05$; **$P < .01$

3.6 | Body weight and adiposity are lower in BAT-specific Kiss1r KO mice of both sexes

To assess potential metabolic consequences of selective deletion of kisspeptin signaling from BAT, BWs of BAT cKO and control mice were taken every 2 weeks, starting at 4 weeks of age. Surprisingly, unlike global *Kiss1r* KO females which become obese in adulthood, BAT cKO females showed significantly lower BWs compared to control littermates ($P < .05$, Figure 6A). Starting around week 8, cKO females started to gain significantly less weight over time compared to female controls. By 22 weeks of age, cKO females weighed a marked 20% less than their littermate controls ($P < .05$, Figure 6A). Likewise, BAT cKO males showed a similar, though less robust, pattern of decreased BW in adulthood

which started later than BAT cKO females (~ 16 wks vs 8 wks of age) ($P < .05$ vs control males, Figure 6B). By week 22 of age, BAT cKO males weighed 10% less than control males ($P < .05$, Figure 6B). Matching their lower BWs, adult BAT cKOs of both sexes showed significantly lower absolute fat mass and decreased percent fat mass compared to control littermates of the same sex ($P < .01$, Figure 6C and D). Female BAT cKOs also showed a minor but significant decrease in lean mass ($P < .05$, Figure 6C).

Because glucose homeostasis can be influenced by BW, with impaired glucose regulation often observed in heavier or obese animals, ip GTTs were performed in adult BAT cKOs. BAT cKO females showed slightly improved glucose tolerance compared to control females, as evidenced by significantly lower blood glucose levels at 30 minutes after glucose
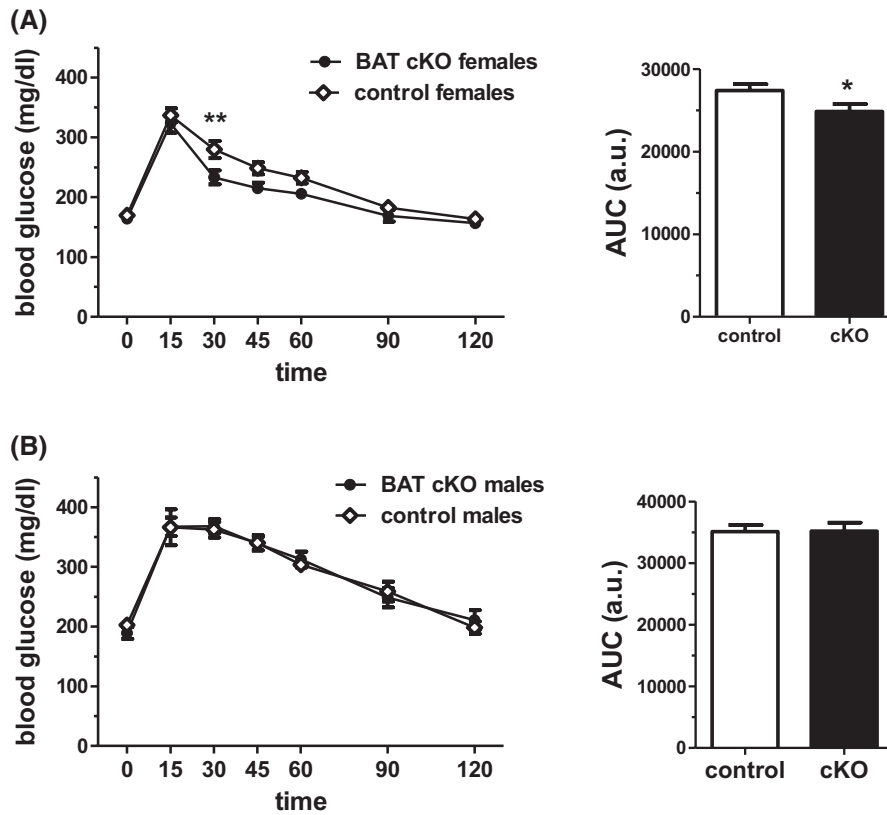
(A)

(B)

FIGURE 7 A, BAT cKO females show moderately improved glucose tolerance in an ip glucose tolerance test (GTT) (n = 14/group). B, Male BAT cKO mice show normal GTT responses compared to control males (n = 6-10/group). *P < .05, **P < .01

injection (P < .01, Figure 7A) as well as significantly lower AUC for the 2-h testing period (P < .05, Figure 7A). Matching their more moderate BW phenotype compared to the females, BAT cKO males displayed no significant difference in glucose tolerance compared to control males (Figure 7B).

3.7 | Metabolic rates and energy expenditure are increased in BAT-specific Kiss1r KO females

Because female BAT cKOs displayed a more pronounced BW, adiposity, and GTT phenotype than males, we next used CLAMS metabolic cages to evaluate if BAT cKO females have increased metabolic rates that might explain their lower BWs. Although BAT cKO females ate the same amount of food as control littermates (*data not shown*), their metabolic rates were significantly elevated. Specifically, BAT cKO females consumed significantly more O_2 (P < .05, Figure 8A) during the dark phase of the light cycle, and showed a near-significant increase (P = .06) in the light phase (when mice are less active). Likewise, BAT cKO females produced more CO_2 relative to control females (P < .05, Figure 8B) and displayed

increased energy expenditure during both the light and dark phases (P < .01, Figure 8C). Matching the respiratory rates, locomotor activity was elevated in BAT cKO females in the dark phase (P < .05, Figure 8E), though this did not reach significance in the light phase (P = .09). RER was not statistically different between genotypes during either phase of the light cycle (Figure 8D).

3.8 | Body Temperature and BAT gene expression are elevated in BAT-specific Kiss1r KO mice

Core body temperature was measured in adult BAT cKO females. Matching their increased metabolic rates and energy expenditure, core body temperature was significantly elevated in BAT cKO females compared to control females (Figure 9A). We also determined whether thermogenic gene expression is altered in the BAT of BAT cKOs. While *Ucp-1* levels were similar between genotypes (Figure 9B), *Cox8b* expression levels in BAT were significantly elevated, by nearly 100%, in BAT cKO females (P < .05, Figure 9C), correlating with their elevated body temperature and metabolic rates. BAT weight was not significantly different between genotypes (Figure 9D);
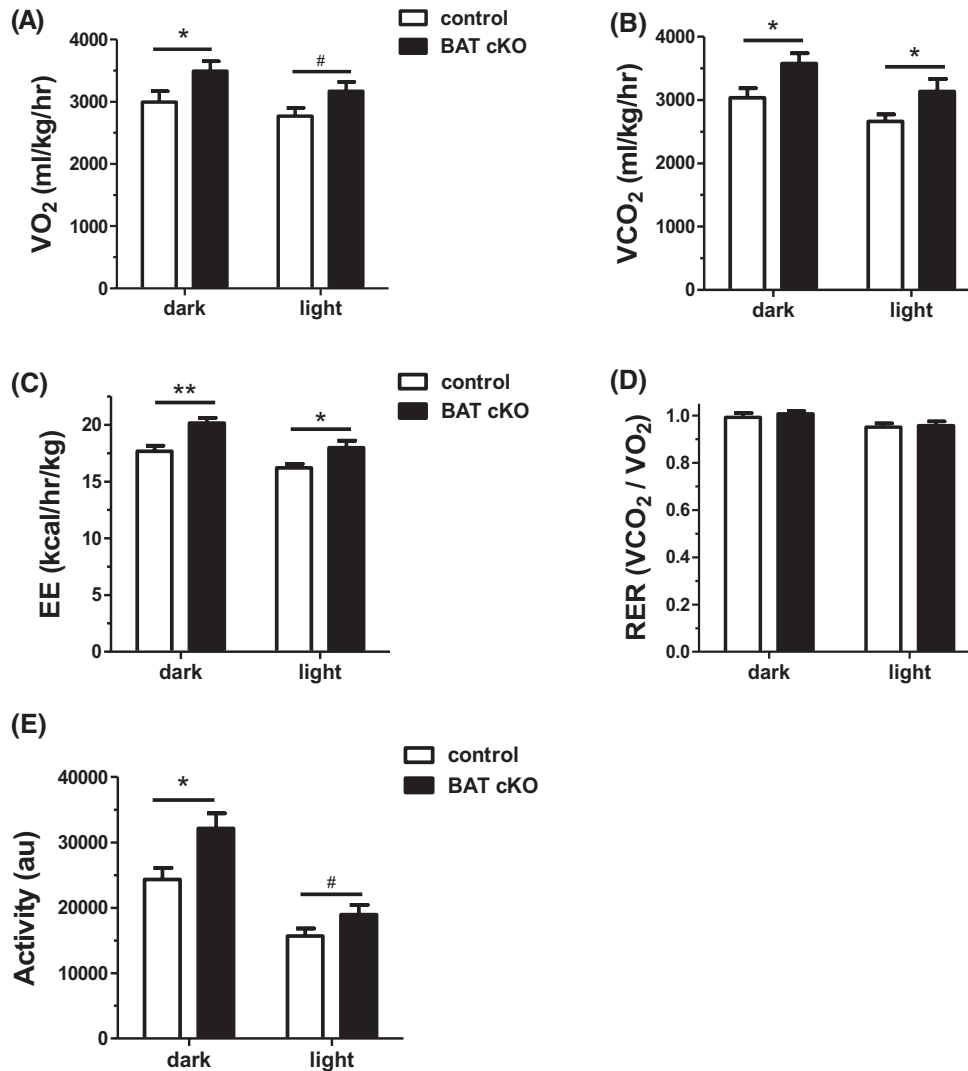
FIGURE 8 CLAMS assessment of metabolism and energy expenditure in adult females demonstrates that BAT cKO mice have A, increased mean oxygen consumption (VO_2) during the dark phase (light phase showed non-significant trend to also be elevated), and increased mean B, carbon dioxide production (VCO_2), and C, energy expenditure (heat divided by kg lean mass) during both the dark and light phases. D, Mean RER did not differ between genotypes, whereas E, mean activity levels were significantly elevated in BAT cKOs during the dark phase. n = 14-15/group. *$P < .05$; **$P < .01$, #$P = .06$

however, eWAT weight was trending lower in BAT cKO females versus WT females ($P = .06$, Figure 9E), matching their lower total adiposity as measured by EchoMRI (Figure 6C).

4 | DISCUSSION

Kisspeptin controls reproductive status via its neural actions, stimulating GnRH neurons, which express Kiss1r. However, recent evidence from our group and others demonstrate that kisspeptin signaling is also important for non-reproductive processes, including metabolism and energy balance. We recently reported that global *Kiss1r* KO females develop obesity, glucose intolerance, and impaired metabolic rates

in adulthood which are not due their absent estrogens.[15-17] Here we further demonstrate that global *Kiss1r* KOs have lower body temperature and decreased BAT thermogenic gene expression, correlating with their lower metabolic rates. However, because Kiss1r is expressed in several peripheral tissues, the specific tissue(s) responsible for the observed obese/metabolic phenotype in the global KOs remains undetermined. Here, we demonstrated that the obese phenotype of the global KOs is likely not due to absent kisspeptin signaling in BAT, as BAT-specific *Kiss1r* KO mice do not become obese or show reduced metabolism. Rather, BAT-specific ablation of kisspeptin signaling *increased* body temperature, metabolic rates, and energy expenditure, correlating with decreased BWs and lowered adiposity in adulthood. Thus, endogenous kisspeptin signaling in BAT cells may be a
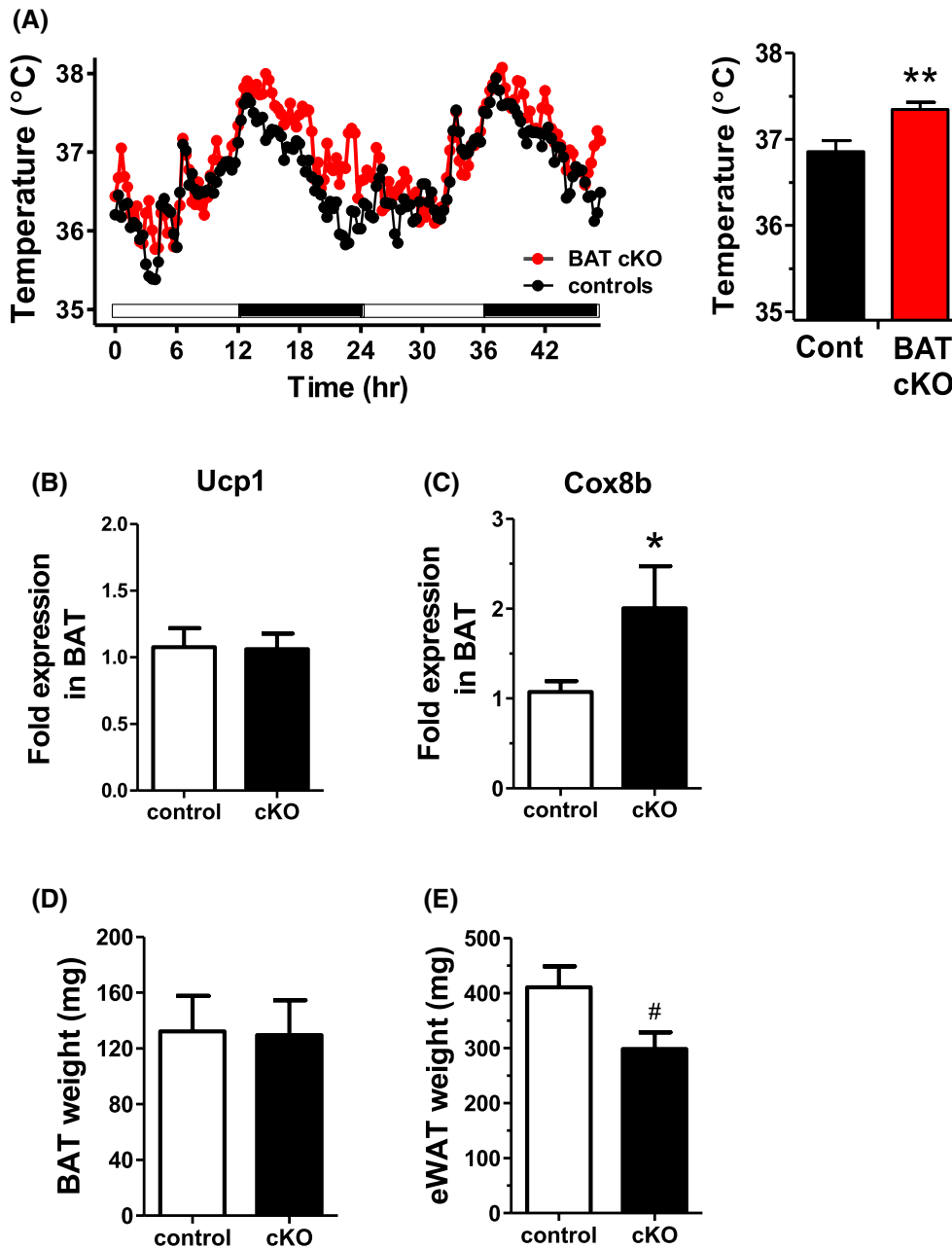
FIGURE 9 A, Core body temperature of adult BAT cKO females is significantly higher than control female littermates, especially during the dark phase when mice are most active (n = 9-12/group). B, C, BAT gene expression, in particular *Cox8b*, in BAT cKO females is increased versus controls (n = 8-10/group). D, E, Mean BAT and eWAT weights in adult BAT cKO females versus WT control littermates (n = 5-6/group). *$P < .05$, **$P < .01$, #$P = .06$

previously unrecognized negative regulator of temperature, energy expenditure, and metabolism.

Given the reported obesity and reduced metabolism of global *Kiss1r* KOs, we analyzed thermogenic gene expression in BAT and found that levels of *Ucp1* and *Cox8b*, two important thermogenic-related BAT genes,[20-23] are strongly reduced in *Kiss1r* KO mice. Moreover, body temperature, which is influenced by UCP1 action and BAT physiology, was significantly lower in global *Kiss1r* KO females than control females. Additionally, the ability to defend core temperature

in global *Kiss1r* KO females was compromised during a 6-h cold challenge, also indicative of deficient UCP1/BAT thermogenic physiology. These novel thermogenic phenotypes may relate to the reduced metabolism and energy expenditure previously observed in these global *Kiss1r* KOs, and suggest that endogenous kisspeptin acting somewhere in the body, directly or indirectly, influences thermoregulation and BAT physiology. We also measured body temperature and BAT *Ucp1* expression in global *Kiss1r* KO males, which have normal BWs and do not become overweight in adulthood.[16]

For both measures, unlike global *Kiss1r* KO females, global *Kiss1r* KO males exhibit normal body temperature and *Ucp1* levels, matching their previously reported normal BW. We recently compared levels of both *Kiss1* and *Kiss1r* in several peripheral tissues, including BAT, WAT, and liver, of normal mice and found no notable sex differences.[24] Thus, at present, the reason for the sex difference in BW, metabolic rates, and body temperature in the global *Kiss1r* KOs is unknown.

The exact tissues and cell types where kisspeptin signaling influences metabolic and thermogenic parameters remains unknown. Recent data indicate that primary neural energy balance populations, like hypothalamic neuropeptide Y (NPY) and pro-opiomelanocortin (POMC), are relatively normal in global *Kiss1r* KO females.[17] Thus, the underlying mechanism(s) causing obesity and reduced metabolism in those KO mice may instead be occurring outside the brain in one or more peripheral systems. Based on the present findings of altered body temperature and BAT gene expression in the global *Kiss1r* KOs, along with Kiss1r expression in BAT of normal mice, we hypothesized that absent Kiss1r signaling specifically in BAT may be responsible, in part, for some of the metabolic impairments in the global *Kiss1r* KOs, perhaps via diminished thermogenesis. To test this possibility, we used Cre/lox technology to conditionally delete *Kiss1r* from just BAT while maintaining intact Kiss1r elsewhere in the body. In our new BAT cKOs, we confirmed successful recombination of the *Kiss1r* flox allele in BAT but not other metabolic tissues, such as WAT and liver, or in gonads or pituitary. A very faint level of recombination was detected in the brain. To evaluate whether this was occurring in brain locations that normally express *Kiss1r*, we also generated *Ucp1*-Cre mice with a fluorescent tdTomato reporter. tdTomato expression, indicative of *Ucp1* Cre-mediated recombination, was highly present throughout BAT, as predicted. In the brain, occasional tdTomato expression was only found in the paraventricular thalamus, cortical amygdaloid nucleus, and ventromedial hypothalamus, and to a lesser extent, the cortex and dorsal lateral septum, which are all brain areas in the mouse that do not express *Kiss1r*.[7] A few previous reports have similarly reported low *Ucp1* expression in mouse cortex,[25,26] though another study failed to find *Ucp1* mRNA or UCP1 protein in the murine brain,[27] perhaps because endogenous levels are only in a few neurons at low levels. Regardless, the non-overlapping anatomical patterns of brain *Ucp1* and *Kiss1r* expression in mice indicates that endogenous Kiss1r signaling within the brain is not hindered in our BAT cKOs. To confirm that our BAT cKO model was not altering GnRH function or the reproductive axis, we assessed if there were any reproductive impairments in BAT cKOs. Unlike global *Kiss1r* KOs, which are hypogonadal and infertile, BAT cKO females and males had normal gonad size and AGD, and the females were completely fertile. Moreover, tdtomato reporter expression of Ucp1 was not present in brain areas containing GnRH neurons. Collectively, these pieces of evidence indicate that Kiss1r signaling in GnRH neurons or other reproductive cell types was not disrupted in the BAT cKOs, as expected if Kiss1r is only functionally deleted from BAT cells.

Our current finding that both body temperature and BAT thermogenic genes were lower in global *Kiss1r* KOs raised the possibility that endogenous kisspeptin signaling in BAT may alter overall metabolism, energy expenditure, thermogenesis, or energy balance. To test this, we studied multiple metabolic and energy balance parameters in the BAT cKOs. To our surprise, BAT cKO females had significantly *lower* BWs in adulthood compared to controls, along with notably *reduced* fat mass as measured by EchoMRI. BAT cKO males showed a similar, though less robust, pattern of decreased BW in adulthood, which started at a later age than in BAT cKO females; the underlying reasons for the stronger phenotype in the females is not currently known.

We used CLAMS cages to determine whether the reduced adiposity of BAT cKO females is related to altered feeding or metabolism. Opposite to global *Kiss1r* KOs, which have reduced metabolism, BAT cKOs demonstrated significantly higher O_2 and CO_2 respiratory rates. Matching this finding, BAT cKO females also had higher energy expenditure than controls, along with a significantly increased core body temperature. Daily food intake was not different between genotypes, suggesting that the observed increases in energy expenditure and metabolism, rather than decreased feeding, contribute to the lower adiposity and BWs. Analysis of BAT gene expression in BAT cKOs found no alteration in *Ucp-1* mRNA levels but detected a significant increase in *Cox8b* levels, which might contribute to the higher body temperature. Whether additional thermogenic or metabolic genes in BAT are similarly altered remains to be determined in future studies. It is also possible that absent kisspeptin signaling alters the functions or actions of these key BAT proteins, in addition to affecting gene expression, but future studies are needed to assess this. Collectively, the present findings suggest that endogenous kisspeptin acting directly in BAT cells normally serves to decrease BAT gene expression and body temperature. Although *Cox8b* seems to be one affected gene in BAT, the specific cellular signaling pathways underlying this newly identified BAT function of kisspeptin remain to be studied. It also remains to be determined where the kisspeptin signal is coming from. Kisspeptin is made in several peripheral tissues, including liver, pancreas, WAT, gonads, and kidney (but not BAT). Thus, the source of the endogenous kisspeptin for BAT *Kiss1r* action is likely circulating from another peripheral tissue, such as WAT, liver, and/or pancreas.

Our data clearly demonstrate that peripheral kisspeptin signaling in BAT does not contribute to the obese phenotype seen in global *Kiss1r* KO mice. On the contrary, BAT cKO females

and males display significant weight loss, reduced fat mass, and increased energy expenditure and body temperature. These findings indicate that[1] the previously observed obesity and metabolic dysfunction in global *Kiss1r* KOs are due to impaired kisspeptin signaling in another tissue(s) besides BAT, such as pancreas, white adipose, adrenal, and/or brain. In this case, it is possible that another Kiss1r-expressing tissue, like WAT, is the main driver of the global KO phenotype, but this masks the less robust—and opposing—effects owing to Kiss1r signaling in BAT; and[2] endogenous kisspeptin signaling in BAT in "normal" animals may serve to lower body temperature, energy expenditure, and metabolic rates, thereby indirectly modulating BW. While surprising that the global and conditional BAT KOs display opposite metabolic phenotypes, this pattern of results is not unprecedented; a previous study found that deletion of *Kiss1r* from just the pancreas improved glucose metabolism, whereas global *Kiss1r* deletion impairs glucose tolerance.[13,16] In fact, it is possible that the specific metabolic actions of kisspeptin in BAT may partially counter the greater summated metabolic actions of kisspeptin elsewhere in multiple other tissues. If so, then restoring *Kiss1r* expression solely in BAT in global *Kiss1r* KOs might be expected to exacerbate their obese phenotype.

In summary, we demonstrate for the first time that whole-body deletion of *Kiss1r* from all tissues/cells results in lower body temperature and decreased BAT gene expression, correlating with increased adiposity and BW. In contrast, selective deletion of kisspeptin signaling in just BAT (*Ucp1*-Cre$^+$/*Kiss1r*$^{fl/fl}$; aka BAT cKO) lowers BW and fat mass, and increases body temperature, energy expenditure, and metabolism. These findings reveal a novel, previously unidentified role for endogenous kisspeptin signaling in BAT relating to metabolic and thermogenic physiology. This highlights the complexity and multi-faceted nature of the kisspeptin system throughout the body and emphasizes the need for more tissue- or cell-specific assessments to compare with global whole-body manipulations. Future studies are needed to determine the underlying mechanisms and sites of kisspeptin action that lead to obesity and metabolic dysfunction in global KOs, as well as to further define the specific thermogenic actions of endogenous kisspeptin signaling in BAT cells.

CONFLICT OF INTEREST

The authors have nothing to disclose.

AUTHOR CONTRIBUTION

KPT, DAO, JTS, and ASK designed research; KPT, NM, JPD, EW, SBS, RBL, RS, and ASK performed research; KPT, JTS, and ASK analyzed data; AW developed and contributed the Kiss1r flox mouse line; KPT, NM, JTS, and ASK wrote the paper; JTS and ASK revised the paper.

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