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# Kinase Suppressor of Ras 2 (KSR2) expression in the brain regulates energy balance and glucose homeostasis

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## ABSTRACT

**Objective:** Kinase Suppressor of Ras 2 (KSR2) is a molecular scaffold coordinating Raf/MEK/ERK signaling that is expressed at high levels in the brain. KSR2 disruption in humans and mice causes obesity and insulin resistance. Understanding the anatomical location and mechanism of KSR2 function should lead to a better understanding of physiological regulation over energy balance.

**Methods:** Mice bearing floxed alleles of KSR2 (KSR2<sup>fl/fl</sup>) were crossed with mice expressing the Cre recombinase expressed by the Nestin promoter (Nes-Cre) to produce Nes-CreKSR2<sup>fl/fl</sup> mice. Growth, body composition, food consumption, cold tolerance, insulin and free fatty acid levels, glucose, and AICAR tolerance were measured in gender and age matched KSR2<sup>-/-</sup> mice

**Results:** Nes-CreKSR2<sup>fl/fl</sup> mice lack detectable levels of KSR2 in the brain. The growth and onset of obesity of Nes-CreKSR2<sup>fl/fl</sup> mice parallel those observed in KSR2<sup>-/-</sup> mice. As in KSR2<sup>-/-</sup> mice, Nes-CreKSR2<sup>fl/fl</sup> are glucose intolerant with elevated fasting and cold intolerance. Male Nes-CreKSR2<sup>fl/fl</sup> mice are hyperphagic, but female Nes-CreKSR2<sup>fl/fl</sup> mice are not. Unlike KSR2<sup>-/-</sup> mice, Nes-CreKSR2<sup>fl/fl</sup> mice respond normally to leptin and AICAR, which may explain why the degree of obesity of adult Nes-CreKSR2<sup>fl/fl</sup> mice is not as severe as that observed in KSR2<sup>-/-</sup> animals.

**Conclusions:** These observations suggest that, in the brain, KSR2 regulates energy balance via control of feeding behavior and adaptive thermogenesis, while a second KSR2-dependent mechanism, functioning through one or more other tissues, modulates sensitivity to leptin and activators of the energy sensor AMPK.

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**Keywords** Obesity; Glucose metabolism; Insulin resistance; KSR2; AMPK

## 1. INTRODUCTION

The brain plays a critical role in sensing energy demands and regulating fuel storage to maintain body weight within a tight range. Extensive analysis has identified key conserved genes and neural pathways critical in regulating energy balance [1,2]. At the core of this homeostatic pathway is the central melanocortin system, which is composed of the melanocortin 4 receptor (Mc4r), its agonist  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH), which is derived from cleavage of the precursor polypeptide proopiomelanocortin (POMC), and the Mc4r inverse agonist, Agouti gene-related peptide (AgRP). Orexigenic Neuropeptide Y (NPY) is co-expressed with AgRP. The anorexigenic peptide leptin feeds back on the melanocortin system, activating POMC neurons to stimulate the generation and release of  $\alpha$ -MSH. Coincident

with this stimulatory action, leptin also limits the inhibitory signals in this pathway by inhibiting NPY/AgRP neurons and suppressing the production and release of NPY and AgRP. Genetic manipulation of these pathways in preclinical models and the identification of melanocortin pathway mutations in humans has led to strategies for therapeutic intervention that may modulate energy balance in humans to ameliorate obesity and its associated co-morbidities [3,4]. However, additional targets that play limited and narrowly defined roles in affecting energy balance may provide therapeutically tractable targets with reduced off-target effects.

Kinase Suppressor of Ras 2 (KSR2) is a molecular scaffold coordinating Raf/MEK/ERK signaling that potently regulates energy consumption and expenditure [5–7]. Like its paralog Kinase Suppressor of Ras 1 (KSR1) [8–10], KSR2 coordinates the interaction of Raf/MEK/ERK

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signaling to facilitate signal transduction and regulate the intensity and duration of ERK signaling [6]. KSR2 also promotes activation of the primary regulator of cellular energy homeostasis, 5'-adenosine monophosphate-activated protein kinase (AMPK) [5,7]. KSR2 was found to interact directly with AMPK [5], and ectopic expression of KSR2 enhanced AMPK activation and signaling in a cell autonomous manner [7]. However, defective AMPK activation was also observed in the adipose tissue of mice even though KSR2 mRNA is not significantly expressed there. These observations suggest that KSR2 may have cell autonomous and cell non-autonomous effects on this key energy sensor.

KSR2<sup>-/-</sup> mice develop normally but grow slowly immediately after birth. Increased adiposity is evident after weaning at 8–9 weeks of age [5]. In the DBA1/LacJ mouse strain, KSR2 disruption causes hyperactivity without hyperphagia, revealing that increased adiposity results from a defect in energy expenditure [5]. Disruption of KSR2 in C57BL/6 mice caused obesity and hyperphagia, which led some to conclude that KSR2-dependent regulation of food consumption was the sole cause of obesity in KSR2<sup>-/-</sup> mice [11]. Doubt was cast on this contention by pair-feeding experiments showing that hyperphagia exacerbates, but does not cause, obesity in C57BL/6 mice KSR2<sup>-/-</sup> mice. KSR2<sup>-/-</sup> mice are also profoundly insulin-resistant in liver and adipose tissue [12]. Insulin resistance appears to be secondary to the obesity, as dietary restriction after weaning prevents obesity and glucose intolerance in KSR2<sup>-/-</sup> mice. Insulin resistance returns when KSR2<sup>-/-</sup> mice are fed *ad libitum* [13].

In KSR2<sup>-/-</sup> mice, decreased AMPK activation may impair the oxidation of fatty acids and increase their storage as triglycerides, contributing to obesity and insulin resistance [5]. Some KSR2 mutations in individuals with early-onset obesity disrupt ERK activation or impair interaction of the scaffold with AMPK [12]. These data identify KSR2 as a key effector in whole-body energy regulation in mice and humans. The recent identification of KSR2 mutations in humans, in combination with the observation that humans bearing these mutations have phenotypic characteristics found in KSR2<sup>-/-</sup> mice [5,11,12] suggests that KSR2 and KSR2-regulated pathways may be potential targets for therapeutic intervention for type 2 diabetes and obesity.

KSR2 is expressed abundantly in many areas of the brain, but in relatively low levels in muscle, liver, and adipose tissue [5,14]. Within the brain, KSR2 expression is highest in the cortex and cerebellum and somewhat lower in the hippocampus, hypothalamus, amygdala, substantia nigra, and various areas of basal ganglia [1A]. We recently reported that growth hormone (GH) signaling is altered in the liver of KSR2<sup>-/-</sup> mice and that some of the phenotypic changes observed in these mice, especially decreased body length, can be rescued by administration of IGF-1 during the neonatal period [15]. Nevertheless, hepatocytes isolated from KSR2<sup>-/-</sup> mice exhibited normal GH-induced signaling under *in vitro* culture conditions. These findings suggested that the systemic effects of KSR2 knockout might be mediated in part by a cell non-autonomous mechanism. We hypothesized that the source of this regulation is the brain. Here we show that brain-specific disruption of KSR2 is sufficient to reduce body temperature, promote cold intolerance, cause obesity, and impair glucose homeostasis, while elevating fasting insulin and free fatty acid levels in blood. Disruption of KSR2 selectively in the brain causes hyperphagia in male, but not female, mice. Though still obese, adiposity in female mice lacking KSR2 in the brain is correspondingly reduced. These data demonstrate that KSR2 functions centrally to regulate energy balance through effects on feeding behavior and adaptive thermogenesis.

## 2. MATERIALS AND METHODS

### 2.1. Animals

KSR2<sup>-/-</sup> mice were generated as previously described [5,13]. Nes-CreKSR2<sup>fl/fl</sup> mice were generated by crossing B6.Cg-Tg(Nes-cre)1Kln/J, (Jackson Labs; hereafter referred to as 'Nes-Cre') to mice in which LoxP sites had been inserted flanking exon 3 of the KSR2 locus (KSR2<sup>fl/fl</sup>, inGenius Targeting Laboratory, Ronkonkoma, NY, Figure 1). The Institutional Animal Care and Use Committee (University of Nebraska Medical Center, Omaha, NE) approved all studies. Animals were maintained on a 12-h light/dark schedule and had free access to laboratory chow (Harlan Teklad LM 485) and water, except as described below.

### 2.2. Dual energy X-ray absorptiometry (DEXA)

Mice were weighed weekly on a digital scale. Lean mass and fat mass were quantified every two weeks by dual-energy X-ray absorptiometry (DEXA) with a Lunar PIXImus™ densitometer (GE Medical-Lunar, Madison, WI). Mice were anesthetized using a mixture of inhaled isoflurane and oxygen (anesthetization using 3% isoflurane and 1 L/min oxygen; maintenance using 1–2% isoflurane and 1 L/min oxygen) and placed prone on the imaging positioning tray. Adipose mass was determined by excising and weighing each fat depot after euthanasia.

### 2.3. Glucose tolerance test (GTT) and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) tolerance test (ATT)

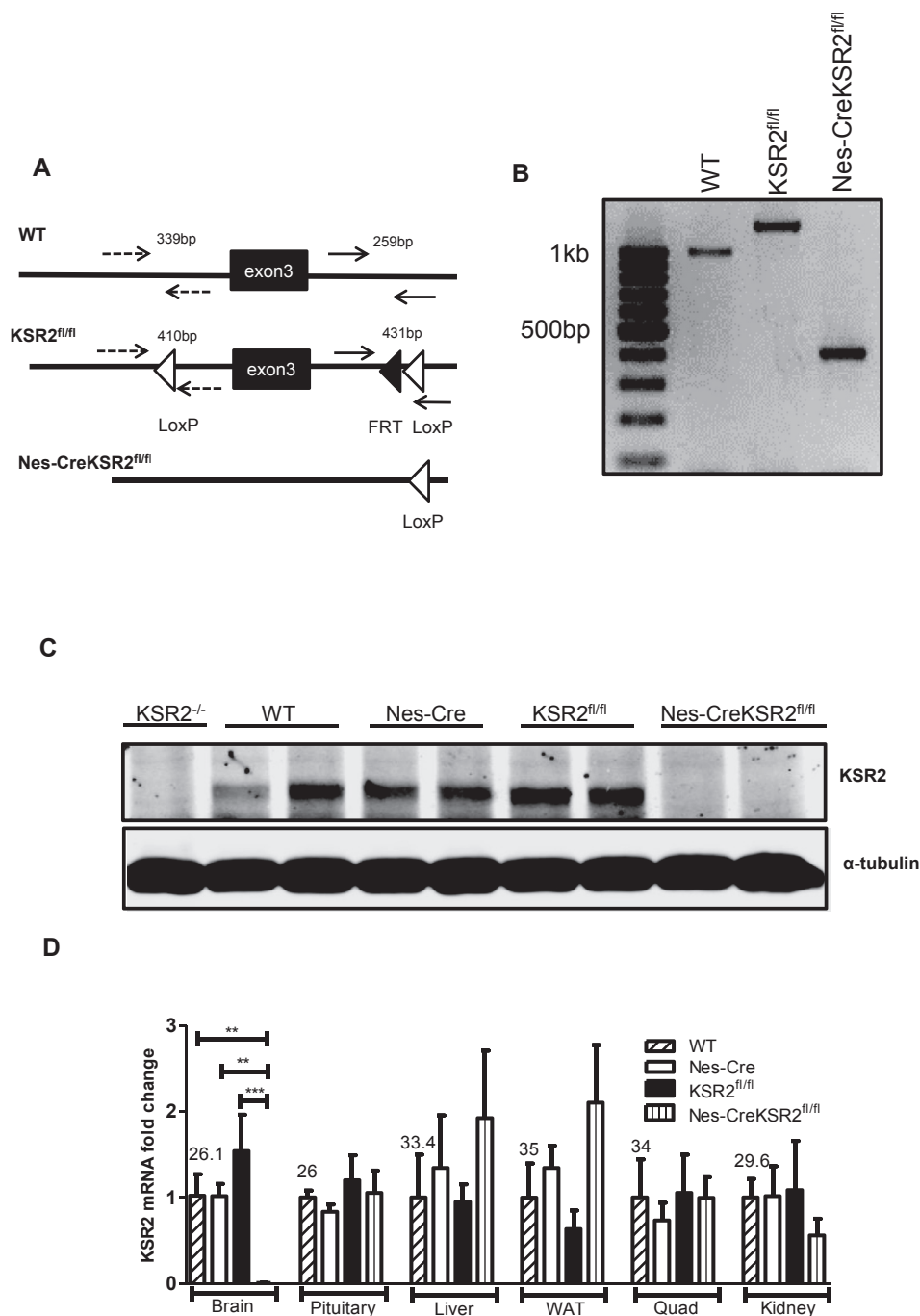
To determine the role KSR2 plays in glucose homeostasis, mice were assessed by GTT and ATT. Each GTT was performed after a 10-h overnight fast; each ATT was performed following a 4-h morning fast. Mice were injected intraperitoneally (IP) with D-glucose (20% solution, 2 g/kg of body weight) for GTT, or with AICAR (0.25 g/kg of body weight) for ATT. Glucose levels were determined in blood collected from the tail vein at the indicated times following injection.

### 2.4. Metabolite assays

Blood was collected by tail bleeds of live animals or via cardiac puncture of euthanized animals. Animals were fasted overnight for 10–12 h prior to collection for blood glucose and serum insulin measurements. Blood glucose was measured with a Bayer Contour Glucometer. For serum analysis, blood was allowed to clot at 4 °C for 8–24 h, and the serum was separated by centrifugation for 10 min at 10,000 rpm. Serum was transferred to a new tube and stored at –80 °C until assayed. Serum insulin was measured with the Ultra Sensitive Mouse Insulin ELISA Kit (Chrysal Chem, Downers Grove, IL) using mouse standards. Serum leptin was measured with a Mouse Leptin ELISA kit (Millipore, Billerica, MA). Serum free fatty acid (FFA) levels were quantified using a Free Fatty Acids Half Micro Test (11383175001, Roche, Indianapolis, IN).

### 2.5. Measurement of food intake

Food intake was calculated by single-housing mice for 4–5 days, and taking a daily average of grams of food consumed during that time period. The effect of chronic leptin treatment on food intake was measured in mice that were allowed to acclimate 2 days prior to starting the experiment. On subsequent days, mice were given phosphate-buffered saline (PBS) or leptin (2.5 mg/kg in PBS) intraperitoneally, 2 h prior to the onset of the dark cycle, and food intake was measured over a 24-hour period (dose 1). The above experiment was repeated twice more (dose 2 and dose 3), allowing for food intake to normalize between doses. All mice served as their own controls, receiving PBS first, followed by leptin.



**Figure 1:** Selective disruption of KSR2 in brain. A: Schematic representation of the WT, KSR2<sup>fl/fl</sup>, and recombined alleles lacking KSR2 exon 3 (Nes-CreKSR2<sup>fl/fl</sup>). LoxP and FRT sites are indicated as open and closed arrowheads, respectively. Solid arrows indicate the relative positions of primers used to determine presence of *Neo*. Dashed arrows represent the primers used to detect the 5' LoxP site. The sizes of PCR products generated from WT, KSR2<sup>fl/fl</sup>, and Nes-CreKSR2<sup>fl/fl</sup> are 1004 bp, 1184 bp and 428 bp respectively. PCR using the primers indicated in panel A to determine the presence or absence of exon 3 of KSR2 in WT, KSR2<sup>fl/fl</sup>, Nes-Cre, or Nes-CreKSR2<sup>fl/fl</sup> mice. C: Western blot of KSR2 in lysates of brain from KSR2<sup>-/-</sup>, WT, Nes-Cre, KSR2<sup>fl/fl</sup>, or Nes-CreKSR2<sup>fl/fl</sup> mice. D: qPCR for KSR2 mRNA in lysates of brain, pituitary, liver, white adipose tissue (WAT), skeletal muscle (Quad), and kidney from WT, Nes-Cre, KSR2<sup>fl/fl</sup>, or Nes-CreKSR2<sup>fl/fl</sup> mice. Ct values for WT samples are shown for comparison of KSR2 mRNA levels between tissues.

## 2.6. Histology

Lipid accumulation in mice was visualized by hematoxylin and eosin staining of sections of white adipose tissue (WAT) and brown adipose tissue (BAT). The tissues were fixed overnight in 4% paraformaldehyde, and transferred to 70% ethanol until paraffin embedding. Sections were 4–6  $\mu$ m.

## 2.7. Rectal temperature and cold tolerance study

Five-month-old mice were individually housed and rectal temperatures were taken using a MicroTherma 2T handheld thermometer (ThermoWorks, Lindon, UT) during resting (2 pm) and active (9 pm) time periods. For the cold tolerance study, six-month-old male mice were fasted overnight for 10 h. Mice were then housed individually and

placed for an additional 2.5 h in micro-isolator cages that had been acclimated to 4 °C. Rectal temperatures were monitored at the times indicated.

### 2.8. Immunoblots

Fifteen minutes after AICAR injection, mice were sacrificed, and subcutaneous white adipose tissue was removed, frozen in liquid N<sub>2</sub>, and stored at -80 °C until use. Whole-cell lysates were made with 50–100 mg of tissue homogenized in 1% NP-40 lysis buffer. Protein was run on SDS-PAGE gels, using antibodies recognizing phospho-5'-AMPK (phospho AMPK Thr172, Cell Signaling #2531), 5'-AMPK subunit alpha (AMPK $\alpha$ , Cell Signaling #2532), phospho acetyl-CoA-carboxylase (phospho ACC Ser79, Cell Signaling #3661), acetyl-CoA-carboxylase (ACC, Cell Signaling #3676) and  $\alpha$ -tubulin (Santa Cruz, 8017).

### 2.9. Quantitative PCR

Tissues were removed from mice after sacrifice and immediately frozen on dry ice or liquid nitrogen and stored at -80 °C. RNA was extracted using TRI Reagent (Molecular Research Center) and RNeasy kit (Qiagen). After treatment with DNase I (Ambion, AM1906), cDNA was synthesized using iScript RT Supermix (BioRad) according to the manufacturer's instructions. Quantitative real-time PCR was performed in a 20- $\mu$ l reaction volume using SsoAdvanced Sybr Green Supermix (BioRad). All reactions were performed in duplicate on a Stratagene MxPro3000p detection system, and relative RNA levels were calculated by using 18S rRNA as internal control. The data were processed using an R script based on the qBase relative quantification framework [16] with the following modifications. The square root of (h-1) replaces (h-1) in Formula 4. The term -1/slope replaces 1/slope as the exponential component in Formula 5. SD should be SE (a presumed typographical error) in Formula 12. Primer sequences were: 18S rRNA: 5'-GTAACCGTTGAACCCATT-3' and 5'-CCATC-CAATCGGTAGTAGCG-3' KSR2: 5'-TGGATGTCCGAAGGAAGTC-3' and 5'-CTTCTCCACGGTCTCACACA-3' UCP1: 5'-TACCAAGCTGTGCGATGT-3' and 5'-AAGCCCAATGATGTTCACT-3'

### 2.10. Statistical analysis

GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA) was used for graphics design and statistical analyses. To determine the extent to which disruption of KSR2 affected the indicated response variable at a single time point, a Student's *t*-test was used, applying a Bonferroni adjustment when multiple pairwise comparisons were made [17]. To determine the effect of KSR2 disruption on the indicated response variable over time or under various treatments, a two-way analysis of variance (ANOVA) was applied with genotype as the independent variable and time/age as the dependent factors [17]. When multiple data points were drawn sequentially from the same animal, pseudoreplication was avoided by performing a repeated measures two-way ANOVA. When results from the two-way ANOVA indicated that genotype had a significant effect, we performed as appropriate Dunnett's post-test or an additional series of Bonferroni-adjusted Student's *t*-tests to identify the time points at which or treatments under which the effect of KSR2 disruption became significant. All data are shown as the mean  $\pm$  standard error of measurement (SEM). Significance was accepted at  $p < 0.05$ . Unless indicated otherwise for clarity, significant comparisons are represented as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

## 3. RESULTS

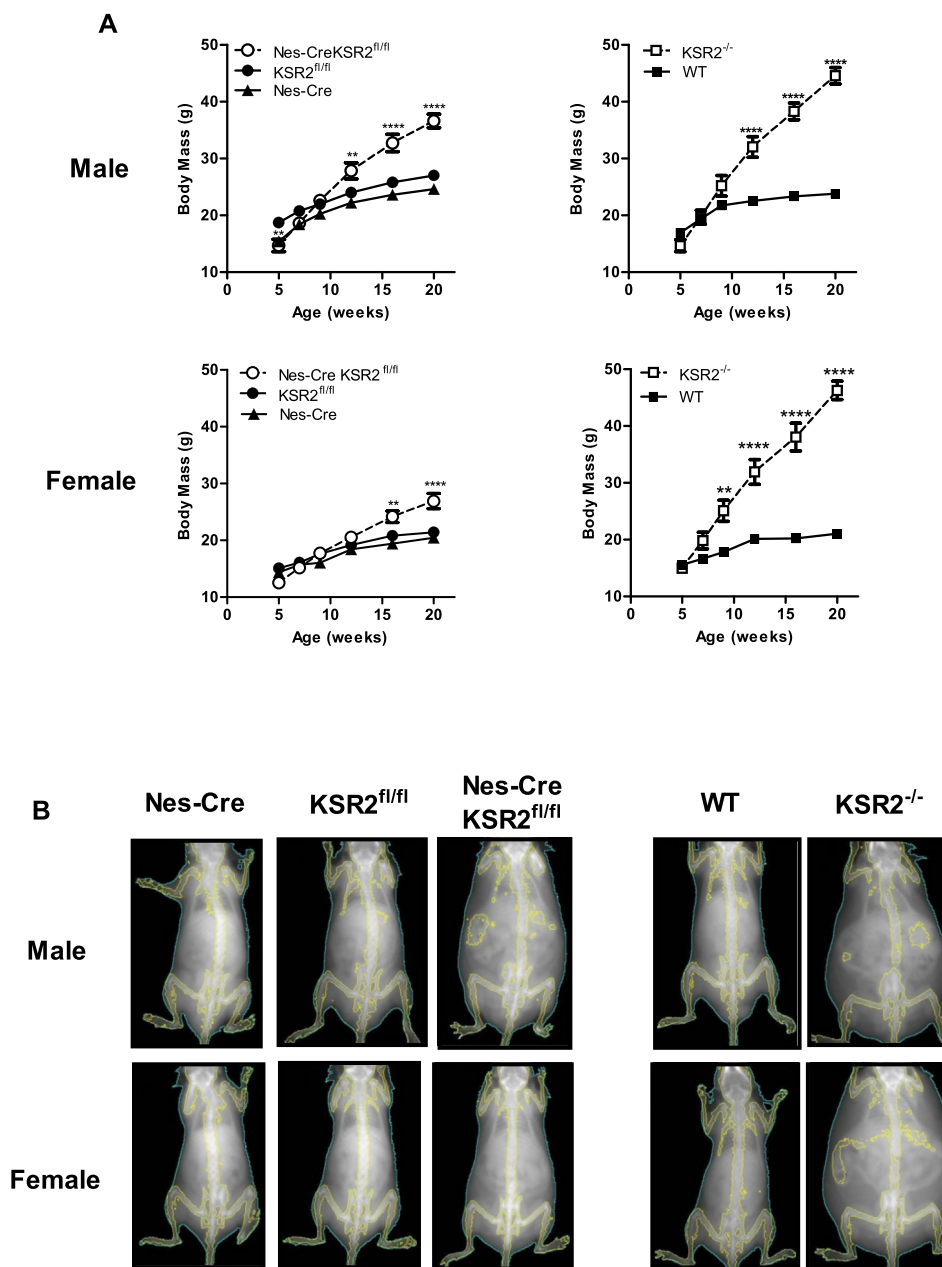
### 3.1. Selective disruption of KSR2 in the brain causes obesity

Nes-Cre mice were crossed to KSR2<sup>fl/fl</sup> mice to generate Nes-CreKSR2<sup>fl/fl</sup> mice deleted in exon 3 of the KSR2 gene (Figure 1, schematic of strategy in panel A and PCR analysis indicating deletion of exon 3 in panel B). Western blotting revealed strong expression of KSR2 in brains from WT, Nes-Cre, and KSR2<sup>fl/fl</sup> mice but no detectable KSR2 in brains from Nes-CreKSR2<sup>fl/fl</sup> mice (Figure 1C). qPCR from selected tissues showed that KSR2 mRNA was undetectable in brains from Nes-CreKSR2<sup>fl/fl</sup> mice; however, KSR2 mRNA was detectable and not significantly changed, relative to control mice, in the pituitary, liver, white adipose tissue (WAT), quadriceps muscle (Quad), and kidney of Nes-CreKSR2<sup>fl/fl</sup> mice (Figure 1D). As we observed previously [5], KSR2 was undetectable by qPCR in brown adipose tissue from mice of any genotype (not shown). The GTex portal indicates that KSR2 expression is high in the human pituitary gland [1A]. Of importance, our data confirm similar high-level expression of KSR2 mRNA in the pituitary of C57BL/6 mice (Figure 1D), and the nestin promoter is clearly not active in the gland, as KSR2 expression was not different between Nes-CreKSR2<sup>fl/fl</sup> mice and WT controls.

Nes-CreKSR2<sup>fl/fl</sup> mice showed a significant increase in body mass relative to control mice beginning at 12 and 16 weeks of age, respectively (Figure 2A). Despite the absence of detectable KSR2 in brain, the rate and degree of growth in Nes-CreKSR2<sup>fl/fl</sup> mice were notably less than those observed in KSR2<sup>-/-</sup> mice (Figure 2A,B; compare right-hand panels to left). To determine the extent to which KSR2 expression in brain contributes to adiposity, lean mass and fat mass were measured in Nes-CreKSR2<sup>fl/fl</sup> mice from 5 to 20 weeks of age (Figure 3). Strikingly, lean mass of KSR2<sup>-/-</sup> mice was elevated relative to WT mice while Nes-CreKSR2<sup>fl/fl</sup> mice exhibited no significant difference in lean mass in comparison to controls (Figure 3B).

Relative to Nes-Cre and KSR2<sup>fl/fl</sup> mice, male Nes-CreKSR2<sup>fl/fl</sup> mice showed a significant increase in total fat mass by 8 weeks of age. In female Nes-CreKSR2<sup>fl/fl</sup> mice, total adipose mass was not significantly different from controls until 16 weeks of age. Fat mass accumulated in female Nes-CreKSR2<sup>fl/fl</sup> mice at about half the rate of male Nes-CreKSR2<sup>fl/fl</sup> mice (Figure 3A), which likely explains why total body mass in female Nes-CreKSR2<sup>fl/fl</sup> mice was not significantly different from control mice until that time (Figure 2A). Female KSR2<sup>-/-</sup> mice were markedly more obese than female Nes-CreKSR2<sup>fl/fl</sup> mice, which still had twice the body fat of control mice at 20 weeks of age. We recently observed that whole-body disruption of KSR2 results in a significant decrease in nose-to-anus length, accompanied by a decrease in bone mineral content and density. The skeletal deficit appears to result from a cell non-autonomous decline of hepatic IGF-1 expression and can be rescued by infection of KSR2<sup>-/-</sup> neonates with an adenovirus encoding an IGF-1 transgene [15]. This decrease in body length is recapitulated in the Nes-CreKSR2<sup>fl/fl</sup> mice. At five months of age, Nes-CreKSR2<sup>fl/fl</sup> males and females are significantly shorter than control KSR2<sup>fl/fl</sup> mice [for males: 9.1  $\pm$  0.05 cm for Nes-CreKSR2<sup>fl/fl</sup> (n = 10) vs 9.6  $\pm$  0.07 cm for KSR2<sup>fl/fl</sup> (n = 11); for females: p < 0.0001; 8.8  $\pm$  0.07 cm for Nes-CreKSR2<sup>fl/fl</sup> (n = 9) vs 9.2  $\pm$  0.08 cm for KSR2<sup>fl/fl</sup> (n = 11), p < 0.001].

As with KSR2<sup>-/-</sup> mice [5], the adiposity of Nes-CreKSR2<sup>fl/fl</sup> mice results from a general increase in the mass of all adipose depots, including brown adipose tissue (Figure 4A). Similar to the enlarged adipocytes observed in KSR2<sup>-/-</sup> mice [5], disruption of KSR2 selectively in the brain also increases adipocyte size (Figure 4B). In contrast,



**Figure 2:** Disruption of KSR2 in the brain causes obesity. A. Body mass measurements of male ( $n = 9-12$ ) and female ( $n = 6-11$ ) mice with the indicated genotypes. Asterisks above open circles indicate significant differences between Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>fl/fl</sup> mice. B. Analysis of body composition by dual energy absorptiometry (DEXA) for representative male and female mice of the indicated genotypes.

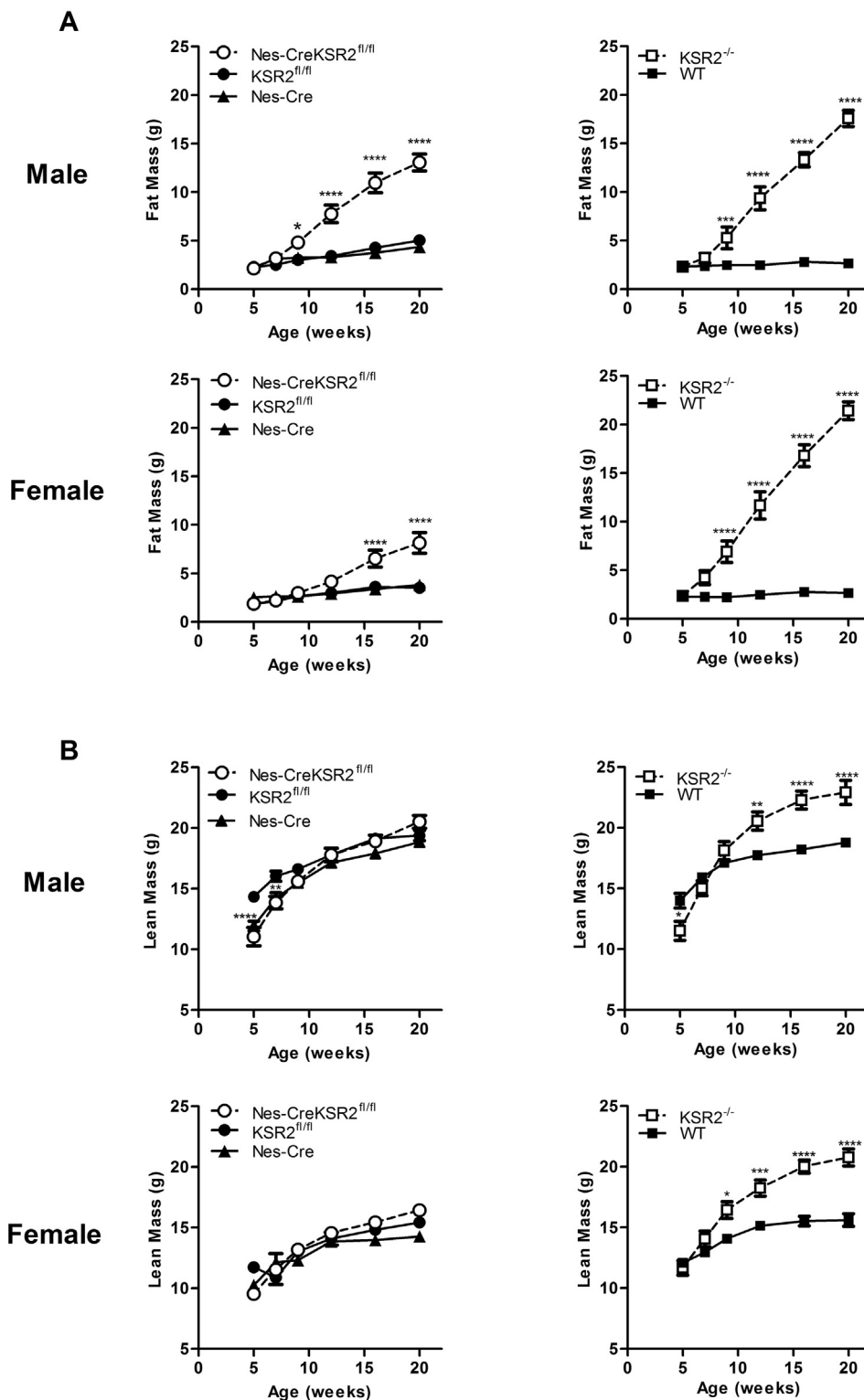
KSR2<sup>-/-</sup> mice show a greater degree of hyperphagia than Nes-CreKSR2<sup>fl/fl</sup> mice, which likely contributes to their greater adiposity. Indeed, female Nes-CreKSR2<sup>fl/fl</sup> mice do not eat significantly more than control Nes-Cre or KSR2<sup>fl/fl</sup> mice (Figure 4C).

KSR2<sup>-/-</sup> mice are reported to be resistant to leptin after being pair-fed for 14 days [11]. However, in KSR2<sup>-/-</sup> mice fed *ad libitum*, leptin inhibits overnight food consumption though not to the degree observed in WT mice [5]. We examined the effect of leptin on Nes-CreKSR2<sup>fl/fl</sup> mice and KSR2<sup>fl/fl</sup> controls. In contrast to the global disruption of KSR2 (Figure 4D, lower panel), which diminished the ability of leptin to inhibit food consumption in mice fed *ad libitum*, leptin inhibited food intake to the same degree in Nes-CreKSR2<sup>fl/fl</sup> mice than it did in control animals.

This was true even when mice were injected successively with multiple leptin doses (Figure 4D, upper panel). Thus, the selective disruption of KSR2 in the brain has no effect on leptin responsiveness, which likely contributes to the more modest obesity observed by disruption of KSR2 in the brain alone.

### 3.2. Brain KSR2 is required for normal metabolism of glucose and lipids

KSR2<sup>-/-</sup> mice show marked defects in glucose metabolism [5,13]. Glucose tolerance tests revealed glucose intolerance in Nes-CreKSR2<sup>fl/fl</sup> mice at five months of age (Figure 5A). Fasting insulin was elevated in male and, to a lesser degree, female, Nes-CreKSR2<sup>fl/fl</sup>

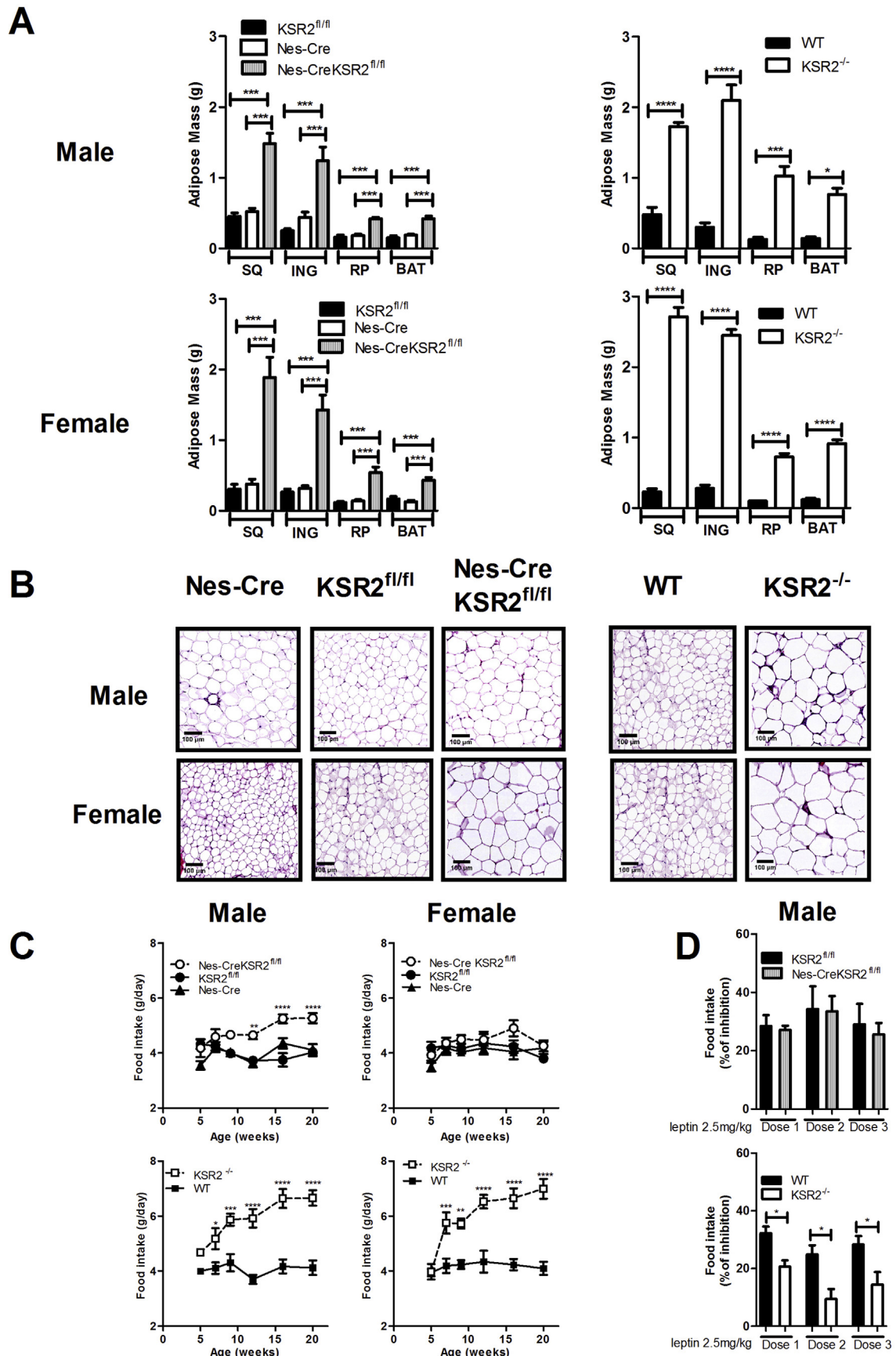


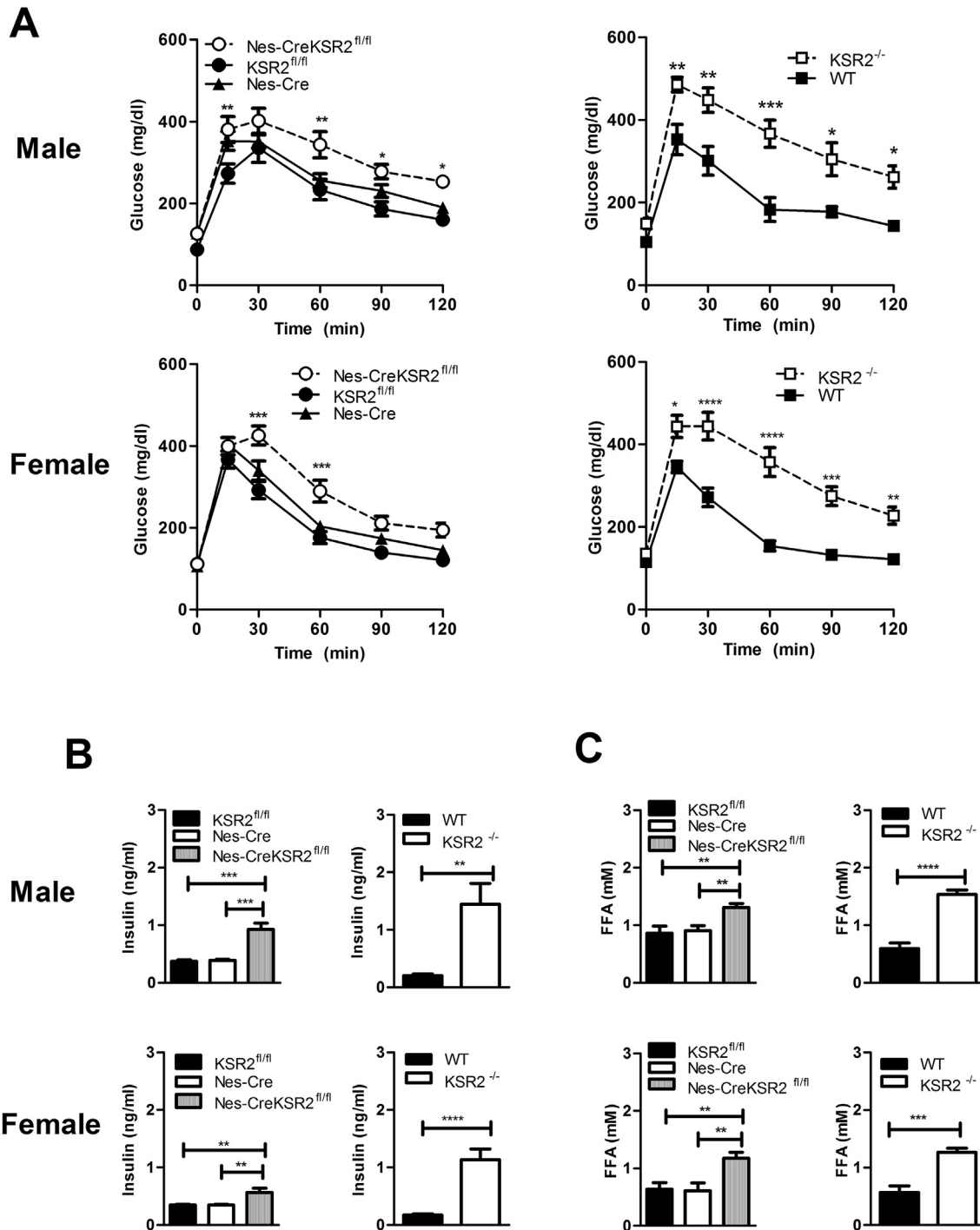
**Figure 3:** —Brain-specific disruption of KSR2 has a less pronounced effect on lean mass and fat mass than whole-body KSR2 knockout. Measurements of lean mass (A) and fat mass (B) for male ( $n = 9-12$ ) and female ( $n = 6-11$ ) mice of the indicated genotypes. Asterisks above open circles show significant differences between Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>fl/fl</sup> mice.

mice (Figure 5B). Insulin levels in male Nes-CreKSR2<sup>fl/fl</sup> mice were increased relative to their controls, which is comparable to the effect seen in KSR2<sup>-/-</sup> mice vs. the WT control. Serum free fatty acids (FFA) were similarly increased in Nes-CreKSR2<sup>fl/fl</sup> mice relative to control

animals (Figure 5C). FFA levels were comparable to that observed in KSR2<sup>-/-</sup> mice, despite a relatively lower level of adiposity. Defects in glucose tolerance, insulin, and FFA were not evident until after the onset of obesity. At 5–7 weeks of age, lean KSR2<sup>-/-</sup> and Nes-







**Figure 5:** Glucose tolerance, fasting serum insulin, and free fatty acid levels in Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>-/-</sup> mice. A: Glucose tolerance test in five-month-old Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>-/-</sup> male (n = 7–14) and female (n = 9–14) mice. Fasting insulin (B) and serum free fatty acid (C) levels in Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>-/-</sup> male (n = 4–10) and female (n = 6–8) mice.

**Figure 4:** Adiposity, food consumption, and leptin sensitivity in mice with brain-specific disruption of KSR2. A: Wet weights of subcutaneous (SQ), inguinal (ING), retroperitoneal (RP), and brown (BAT) adipose tissue in male (n = 4–7) and female (n = 5–8) mice of the indicated genotypes. B: Hematoxylin and eosin staining of SQ adipose tissue from mice of the indicated genotypes. C: Average daily food intake in males (left, n = 5–12) and females (right, n = 8–11). D: Chronic leptin treatment of 6–7 month-old male (n = 4–6) mice of the indicated genotypes.

CreKSR2<sup>fl/fl</sup> mice showed no difference in their ability to handle a glucose load and no significant elevation in fasting insulin or FFA (Figure S1). These data suggest that defects in glucose homeostasis may be secondary to the obesity caused by disruption of KSR2 in the brain.

KSR2<sup>-/-</sup> mice show a marked decrease in response to AICAR treatment, a measure of whole-body response to the activation of AMPK after injected AICAR is metabolized into the allosteric activator, ZMP [5,13]. Selective disruption of KSR2 in the brains of Nes-CreKSR2<sup>fl/fl</sup> mice did not affect AICAR tolerance before or after the onset of obesity (Figure. S2A, Figure 6), suggesting that KSR2 functions in a tissue other than the brain to alter AMPK effects on whole-body glucose metabolism.

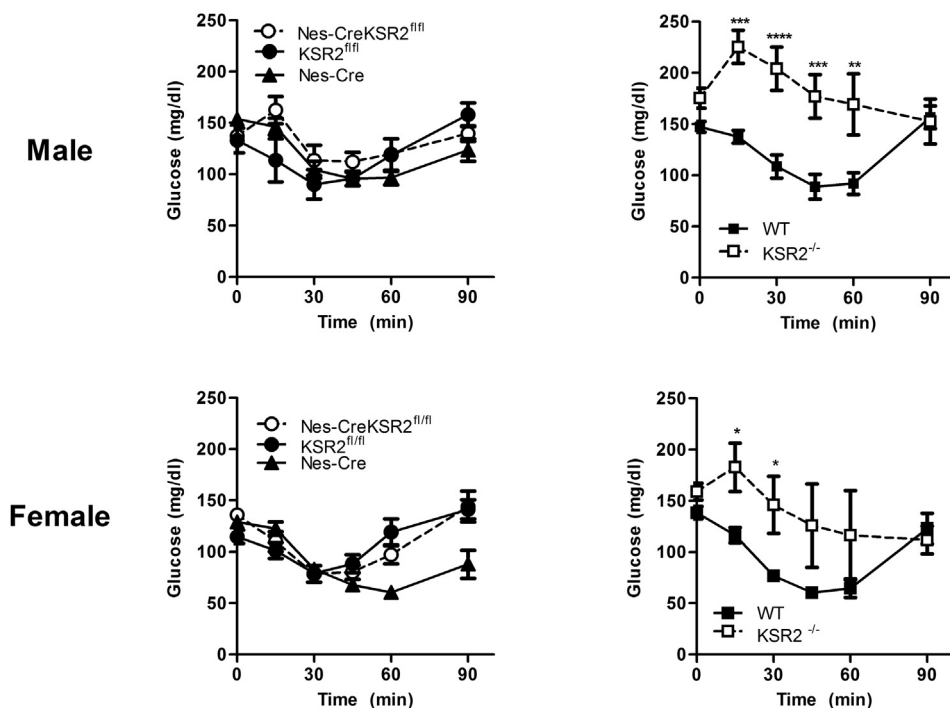
### 3.3. KSR2 in the brain affects thermogenesis

In the DBA1/LacJ background, global disruption of KSR2 reduces rectal and core body temperatures during both quiescent (day) and active (night) periods relative to WT mice. This difference is evident even at thermoneutrality [5]. In contrast, in the C57BL6/J background, no difference in body temperature is evident during the active period in KSR2<sup>-/-</sup> mice compared to WT mice or between mice with brain-specific disruption of KSR2 and their controls (Figure 7A). However, the typical drop in temperature that occurs during the quiescent period (2 pm) is exacerbated by the disruption of KSR2 throughout the body, or selectively in the brain (Figure 7A). This reduced thermogenic capacity is apparent at 5–7 weeks of age and may be a key contributor to the obesity and insulin-resistant phenotype seen in mature animals. While UCP1 mRNA is significantly decreased in brown adipose tissue (BAT) of KSR2<sup>-/-</sup> mice, it only trends lower, but not significantly so, in Nes-CreKSR2<sup>fl/fl</sup> mice (Figure 7B). However, the marked intolerance of the mice to acute cold exposure at 4 °C when fasted (Figure 7C) and the overt accumulation of lipid in the BAT of Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>-/-</sup> mice (Figure 7D) indicate a distinct defect in energy metabolism resulting from the loss of KSR2 in brain that compromises thermogenesis.

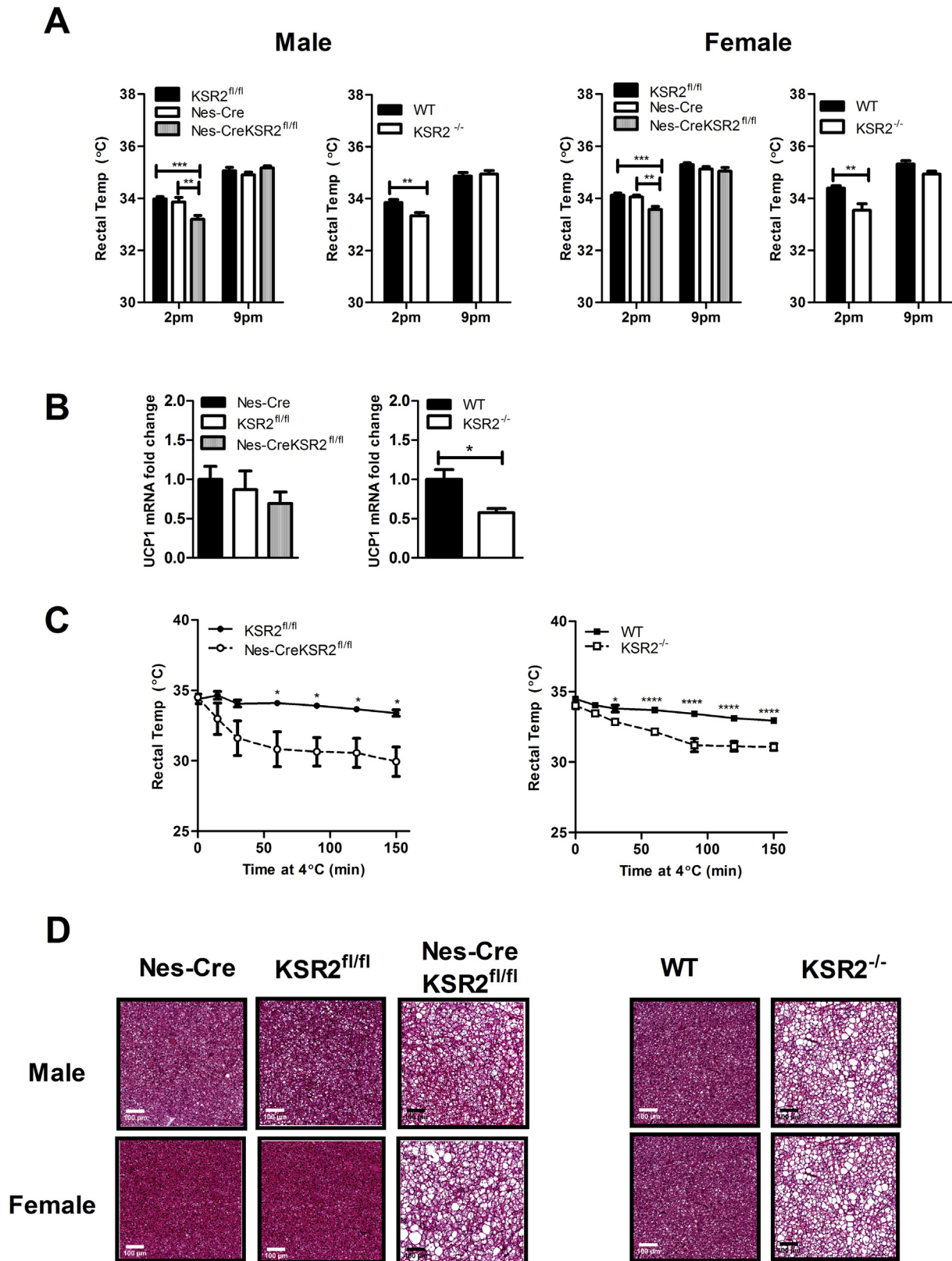
## 4. DISCUSSION

Here we show that brain-specific disruption of the molecular scaffold KSR2 phenocopies the obesity and glucose intolerance of whole-body KSR2 knockout, although the degree of adiposity and glucose intolerance and the increased circulating insulin in fasting Nes-CreKSR2<sup>fl/fl</sup> mice is less than that observed with whole-body knockout of KSR2. KSR2<sup>-/-</sup> and Nes-CreKSR2<sup>fl/fl</sup> brain-specific knockout mice show comparable defects in body temperature maintenance and cold tolerance. However, there are also deficits in the KSR2<sup>-/-</sup> mice that are not present when KSR2 is selectively disrupted in the brain. Though KSR2<sup>-/-</sup> mice are insensitive to AICAR treatment, Nes-CreKSR2<sup>fl/fl</sup> mice do not differ from their controls in sensitivity to AICAR (Figure 6). Further, leptin demonstrates its full acute anorexigenic effect in Nes-CreKSR2<sup>fl/fl</sup> mice, while its actions are blunted in KSR2<sup>-/-</sup> mice (Figure 4D). Thus, although a large component of the effects of KSR2 to regulate energy intake and expenditure leading to obesity operates through KSR2-modulated signals from the brain, at least one other cell non-autonomous mechanism must contribute significantly to KSR2-dependent energy balance.

The precise brain region(s) in which KSR2 functions have yet to be identified, but circumstantial evidence supports the notion that KSR2 expression within discrete nuclei of the hypothalamus is critical for normal energy balance. Mice lacking KSR2 in the brain and whole body share notable similarities to, but also striking differences from, mice lacking the Mc4r receptor. Similar to the Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>-/-</sup> mice, mice mutant or nullizygous for expression of Mc4r become obese around 5–7 weeks of age and exhibit hyperphagia [18,19]. Like KSR2 [5,12], Mc4r also regulates energy balance, at least in part by promoting energy expenditure [18]. However, the hyperphagic, obese and glucose-intolerant phenotype of KSR2<sup>-/-</sup> mice on the C57BL/6 background is markedly more severe than that of the Mc4r<sup>-/-</sup> mice [11]. Treatment with the Mc4r agonist MTII attenuated the hyperphagia



**Figure 6:** AICAR tolerance is impaired in KSR2<sup>-/-</sup> mice but not in Nes-CreKSR2<sup>fl/fl</sup> mice. AICAR tolerance was tested in five-month-old male (n = 7–14) and female (n = 9–14) Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>-/-</sup> mice and analyzed relative to controls.



**Figure 7:** Nes-CreKSR2<sup>fl/fl</sup> and KSR2<sup>-/-</sup> male mice have decreased rectal temperature and cold intolerance. A: Rectal temperatures from 5-month-old male (left, n = 5–11) and female (right, n = 8–12) mice. B: qPCR of UCP1 mRNA from BAT. C: Cold tolerance in 6-month-old KSR2<sup>-/-</sup> and Nes-CreKSR2<sup>fl/fl</sup> fasted mice. D: Hematoxylin and eosin staining of BAT from mice of the indicated genotypes.

of KSR2<sup>-/-</sup> mice [11], suggesting that KSR2 functions upstream of Mc4r. Opposing effects on body length also distinguish the physiology of KSR2 from Mc4r. Mc4r disruption causes an increase in nose to anus length [18,19]. In contrast, mice lacking KSR2 have a significantly decreased nose to anus length that can be rescued by correcting the neonatal defect in hepatic IGF-1 expression with adenovirus-mediated introduction of a KSR2 transgene [15]. These data indicate that KSR2 does not mediate the actions of Mc4r on energy balance.

α-MSH stimulation of Mc4r within the paraventricular nucleus (PVN) of the hypothalamus suppresses food intake and increases energy expenditure [20]. In contrast, the Mc4r inverse agonist, Agouti gene-related peptide (AgRP) is produced by an opposing set of neurons in the arcuate, which are activated during caloric insufficiency. The orexigenic neuropeptide Y (NPY) is co-expressed with AgRP, and, when activated, these anabolic neurons promote feeding behavior, energy storage, and weight gain. Leptin impinges upon the melanocortin system, activating POMC neurons and inhibiting NPY/AgRP neurons [3]. The possibility that KSR2 is involved in Mc4r signaling is supported by reports that AgRP, NPY and POMC are significantly reduced in 6-week-old KSR2<sup>-/-</sup> mice [11]. However, by 8–9 months of age, no differences in these neuropeptides were observed between WT and KSR2<sup>-/-</sup> mice, on the DBA1Lac/J background [5]. These seemingly conflicting data may reveal a role for KSR2 in regulating the rate at which neuronal connections are formed in the brain, which can have a potent impact on the development of neural control over energy balance [21].

At least some connections between hypothalamic nuclei controlling energy balance appear to form developmentally in response to a transient, postnatal surge in leptin [22]. KSR2 may play a critical role in their formation affecting the ability of these nuclei to sense and respond to nutrient status. KSR2<sup>-/-</sup> mice on the C57BL/6 background [11–13], but not the DBA1Lac/J background [5], develop hyperphagic behavior leading to obesity. These data reveal the presence of strain-specific genetic modifiers that affect the expression of KSR2-dependent phenotype. Food restriction of C57BL/6 KSR2<sup>-/-</sup> mice after weaning prevents them from becoming obese and alleviates defects in lipid and glucose metabolism [13]. However, this effect is temporary; once KSR2<sup>-/-</sup> mice resume *ad libitum* feeding, hyperphagia and obesity return. Insensitivity to the anorexigenic adipokine leptin may account for the hyperphagia. Since Nes-CreKSR2<sup>fl/fl</sup> do not display insensitivity to leptin shown by KSR2<sup>-/-</sup> mice, what is the molecular basis for this insensitivity? One possibility is that poor response to leptin observed in KSR2<sup>-/-</sup> mice reflects hypothalamic inflammation. Hypothalamic inflammation can occur in response to a high-fat diet (HFD)-induced downregulation of PGC1α-mediated expression of ERRα [23]. Leptin insensitivity is evident in both male and female KSR2<sup>-/-</sup> mice (Figure 4D and unpublished data). However, HFD-induced effects are evident only in male C57BL/6 mice [23]. Thus, if KSR2 expression regulates hypothalamic inflammation, it likely does so via a mechanism that is not gender-specific.

In our initial report of an effect of KSR2 on energy balance [5], we proposed that the reduced responsiveness to AICAR observed in KSR2<sup>-/-</sup> mice reflected an increase in triglyceride storage and a deficit in mitochondrial import and oxidation of fatty acids resulting from the diminished ability of AMPK to inhibit acetyl CoA carboxylase and impede malonyl CoA synthesis [5]. *In vitro* studies with KSR2 and the closely related protein KSR1 show that these molecular scaffolds can interact directly with AMPK and promote phosphorylation of AMPK substrates [5,7,24]. Furthermore, while global KSR2 disruption markedly reduces AMPK activation in white adipose tissue, little, if any,

KSR2 is expressed in fat (Figure 1D), and no KSR2 is detected in BAT [5]. These data strongly suggest that the KSR2-dependent effects on AMPK-regulated fatty acid metabolism can be both cell autonomous and cell non-autonomous. These observations in combination with the effect of KSR2 on body temperature suggest that BAT may be a key target of KSR2 action. Cell non-autonomous regulation of AMPK and its substrate acetyl CoA carboxylase (ACC) in BAT by KSR2 may be essential for normal fatty acid metabolism in BAT. KSR2 disruption may affect body weight by interrupting AMPK-mediated inhibition of ACC activity in BAT, impairing fat metabolism and heat generation while promoting fatty acid storage as triglycerides.

Cell autonomous effects of KSR2 on AMPK function may occur in the brain and contribute to altered energy balance. Disruption of KSR2 enhances the phosphorylation and activation of mTOR [11], and sustained mTOR activation in the hypothalamus promotes obesity [25]. Phosphorylation of TSC2 and Raptor by AMPK inhibits mTOR anabolic activity [26–30]. Thus, it is possible that KSR2 disruption promotes mTOR-mediated effects on energy balance via loss of AMPK-mediated phosphorylation of key mTOR regulators. However, it should be noted that hypothalamic mTOR-mediated obesity was obtained by disruption of TSC1 and was accompanied by hyperphagia [25], which is only evident in Nes-male CreKSR2<sup>fl/fl</sup> mice (Figure 4C). Thus, extensive analysis is still required to understand the role(s) of these effectors in the action of brain KSR2.

The normal response to AICAR observed in Nes-CreKSR2<sup>fl/fl</sup> mice indicates that this cell non-autonomous effect of KSR2 on AMPK is not mediated by the brain. Based on tissue distribution (Figure 1D and ref. 1A), the pituitary is a strong candidate for mediating KSR2-dependent control of AMPK. The maintenance of robust KSR2 expression in the pituitary of Nes-CreKSR2<sup>fl/fl</sup> mice may explain why their obesity is not as severe as that observed in KSR2<sup>-/-</sup> mice. If correct, this may indicate that KSR2 regulates the timing or extent of secretion of specific hormones from the anterior pituitary that affect the daily control of tissue responsiveness to AMPK-dependent energy sensing or influence the acquisition, during development, of normal AMPK responsiveness to energy stress. In this regard, thyroid hormones are well-established regulators of energy expenditure and thermogenesis [31], and a reduction in TSH secretion might account for some of the effects of KSR2 knockout. However, thyroxine levels in KSR2<sup>-/-</sup> mice are not significantly different from controls [5]. KSR2<sup>-/-</sup> mice are reduced fertility (unpublished data). Initial analysis of reproductive capability indicates that male and female Nes-CreKSR2<sup>fl/fl</sup> mice have reduced fertility relative to controls, suggesting the possibility that gonadotropin secretion or responsiveness may require KSR2. Given the fact that obese humans with KSR2 mutations phenocopy the disruption of KSR2 in mice [12], future work detailing the mechanism of KSR2 function in the brain and identifying other KSR2-expressing tissues that control energy balance is likely to reveal novel mechanisms controlling peripheral metabolism.

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## AUTHOR CONTRIBUTIONS

L.G., D.L.C.-G, D.R.S., and R.E.L. planned the project and designed the experiments. L.G., D.L.C.-G., and D.R.S. performed the experiments. B.K.C. analyzed the indirect calorimetry. L.G., D.L.C.-G,

D.R.S., B.K.C., R.G.M, and R.E.L. wrote and edited the manuscript. R.E.L. is the guarantor of the work, had access to all the data, and takes responsibility for the integrity of the data and the accuracy of its analysis.

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## CONFLICT OF INTEREST

No potential conflicts of interest relevant to this article were reported.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molmet.2016.12.004>.

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