Original article

MOLECULAR METABOLISM

Ablation of intact hypothalamic and/or hindbrain TrkB signaling leads to perturbations in energy balance

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ABSTRACT

Objective: Brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin receptor kinase B (TrkB), play a paramount role in the central regulation of energy balance. Despite the substantial body of genetic evidence implicating BDNF- or TrkB-deficiency in human obesity, the critical brain region(s) contributing to the endogenous role of BDNF/TrkB signaling in metabolic control remain unknown.

Methods: We assessed the importance of intact hypothalamic or hindbrain TrkB signaling in central regulation of energy balance by generating *Nkx2.1-Ntrk2*−/− and *Phox2b-Ntrk2*+/− mice, respectively, and comparing metabolic parameters (body weight, adiposity, food intake, energy expenditure and glucose homeostasis) under high-fat diet or chow fed conditions.

Results: Our data show that when fed a high-fat diet, male and female *Nkx2.1-Ntrk2*−/− mice have significantly increased body weight and adiposity that is likely driven by reduced locomotor activity and core body temperature. When maintained on a chow diet, female *Nkx2.1-Ntrk2*−/− mice exhibit an increased body weight and adiposity phenotype more robust than in males, which is accompanied by hyperphagia that precedes the onset of a body weight difference. In addition, under both diet conditions, *Nkx2.1-Ntrk2*−/− mice show increased blood glucose, serum insulin and leptin levels. Mice with complete hindbrain TrkB-deficiency (*Phox2b-Ntrk2*−/−) are perinatal lethal, potentially indicating a vital role for TrkB in visceral motor neurons that control cardiovascular, respiratory, and digestive functions during development. *Phox2b-Ntrk2*+/− heterozygous mice are similar in body weight, adiposity and glucose homeostasis parameters compared to wild type littermate controls when maintained on a high-fat or chow diet. Interestingly, despite the absence of a body weight difference, *Phox2b-Ntrk2*+/− heterozygous mice exhibit pronounced hyperphagia.

Conclusion: Taken together, our findings suggest that the hypothalamus is a key brain region involved in endogenous BDNF/TrkB signaling and central metabolic control and that endogenous hindbrain TrkB likely plays a role in modulating food intake and survival of mice. Our findings also show that female mice lacking TrkB in the hypothalamus have a more robust metabolic phenotype.

Keywords TrkB; BDNF; Hypothalamus; Hindbrain; Obesity

1. INTRODUCTION

The alarming increase of obesity worldwide has focused attention on the need for understanding the physiological mechanisms implicated in energy balance regulation [1]. In a recent human genome-wide association study, the role of the central nervous system in body mass regulation was strongly emphasized [2]. A substantial body of evidence has emerged demonstrating that brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin receptor kinase B (TrkB), play a paramount role in the central regulation of energy homeostasis [3,4]. Mutations in either the human *BDNF* or *NTRK2* genes are associated with obesity accompanied by hyperphagia [5,6]. Similarly, mice with central BDNF-deficiency or TrkB deletion [7–11] display increased body weight and hyperphagia. The hypothalamus and the hindbrain are two major regions within the brain that are implicated in BDNF regulation of energy balance although both BDNF and TrkB

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Abbreviations: HFD, high-fat diet; BDNF, brain-derived neurotrophic factor; TrkB, tropomyosin receptor kinase B; VMH, ventromedial nucleus of the hypothalamus; PVH, paraventricular nucleus of the hypothalamus; NTS, nucleus of the solitary tract; DVC, dorsal vagal complex; eWAT, epididymal white adipose tissue; BAT, brown adipose tissue; GTT, glucose tolerance test; HPA axis, hypothalamic-pituitary-adrenal axis; *Cre*, *Cre* recombinase; *Nkx2.1*, Nk2 homeobox 1 protein; *Phox2b*, paired-like homeobox 2b protein; *Pomc*, pro-opiomelanocortin; *Npy*, neuropeptide Y; *Agrp*, agouti-related peptide; *LepR*, leptin receptor; *Mc4R*, melanocortin 4 receptor; *Ucp1*, uncoupling protein 1; *Cidea*, cell death-inducing DFFA-like effector a; *Elovl3*, elongation of very long fatty acids-like 3; *Pgc1α*, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Pparγ, peroxisome proliferator-activated receptor gamma; *Prdm16*, PR domain containing 16

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are broadly distributed throughout the central nervous system [12,13]. Notably, previous studies have shown that intraparenchymal BDNF administration into the ventromedial (VMH) and paraventricular (PVH) nucleus of the hypothalamus [14–19], or dorsal vagal complex (DVC) and nucleus tractus solitarius (NTS) of the hindbrain [20–22], reduces food intake and increases energy expenditure in mice. Whether these two brain regions are critical sites contributing to the endogenous role of BDNF/TrkB signaling in central metabolic control, however, remains largely unknown. We hypothesize that intact endogenous TrkB signaling in the hypothalamus and/or the hindbrain is essential for central metabolic control. In this study, we show that deletion of TrkB in the hypothalamus results in increased body weight, adiposity and impaired glucose homeostasis while a reduction of TrkB in the hindbrain results in hyperphagia without affecting overall body weight.

2. MATERIALS AND METHODS

2.1. Animal care

All animal care protocols and procedures were approved by the University of Pennsylvania Institutional Care and Use Committee. Mice were maintained on a 12-h light/12-h dark cycle in a temperature controlled barrier facility, with *ad libitum* access to water and standard chow (Lab Diet 5010, calories provided by protein (28.7%), fat (12.7%), and carbohydrate (58.5%)) or custom high-fat diet (HFD) (Teklad TD93075, calories provided by protein (21.2%), fat (54.8%), and carbohydrate (24%)) upon weaning (3 weeks of age). Age-matched male and female littermates were used for all experiments.

2.2. Generation of *Nkx2.1-Ntrk2−/−* and *Phox2b-Ntrk2−/−* mice

Nkx2.1-Cre and *Phox2b-Cre* transgenic mice were obtained from The Jackson Laboratory (Stock #008661 and #016223 respectively, Bar Harbor, ME). *Ntrk2fl/fl* mice were obtained from Dr. Robert G. Kalb (Children's Hospital of Pennsylvania) and were originally generated in the lab of Dr. Rüdiger Klein (Max Planck Institute for Neurobiology) [23]. Genotyping primers for *Nkx2.1-Cre, Phox2b-Cre* and the floxed *Ntrk2* allele were previously described [24,25]. Initially, *Nkx2.1-Cre* and *Phox2b-Cre* mice were crossed with *Ntrk2fl/fl* mice to generate *Nkx2.1-Cre+*: *TrkB+/fl* and *Phox2b-Cre+* : *TrkB+/fl* mice which were then crossed with *Ntrk2fl/fl* mice to generate *Nkx2.1-Ntrk2−/−, Nkx2.1-Ntrk2+/−, Phox2b-Ntrk2−/−, Phox2b-Ntrk2+/−* mice and wild type controls. *Cre-*: *Ntrk2fl/fl, Cre-*: *Ntrk2+/fl*, and *Cre*-only mice did not show differences in body weight and were combined to form the "wild type" control group. All mice were on a C57BL/6 background.

2.3. Histological analysis

Mice of the indicated age were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused via transcardial perfusion with 1× Phosphate Buffered Saline (PBS) followed by ice-cold 4% Paraformaldehyde (PFA). Tissues were post-fixed overnight in 4% PFA. 25 μm thick sections through the NTS (bregma −6.95 to −8.15) were collected using a cryostat. Immunofluorescent staining of free-floating brain sections was adapted from a previous protocol [26]. Normal donkey serum (Jackson ImmunoResearch) was used as a blocking solution. Sections were incubated with rabbit polyclonal antibody raised against Phox2b (25276-1-AP, Proteintech), or goat polyclonal antibody against TrkB (C-14) (sc-11-G, Santa Cruz Biotechnology, Inc) as primary antibodies. Cy3-conjugated donkey-anti-rabbit IgG and Alexa-Fluor 488-conjugated donkey-anti-goat IgG (Jackson ImmunoResearch) were used as secondary antibodies. Sections were visualized at the Penn Vet Imaging Core using a Nikon E600 microscope at 20× magnification and Roper Scientific imaging software. Quantification was performed on five sections of unilateral region of interest containing the NTS along the anterior–posterior axis using the multi wavelength cell scoring application within the MetaMorph Image Analysis software.

2.4. Body composition and food intake

At weaning, mice were fed either a standard chow diet or HFD and body weights were assessed weekly. For food intake experiments, mice were singly housed and food intake was measured daily for a period of 5 days at the indicated age. Body length was measured as nose-rump length at the indicated age. Gonadal fat pads were dissected and weighed at the indicated age. Total fat and lean mass was measured using NMR (Echo Medical Systems) at the indicated age at the Penn IDOM Mouse Phenotyping, Physiology and Metabolism Core.

2.5. Energy expenditure measures

Feed efficiency was calculated as grams weight gained/grams food consumed over a period of 5 days. Energy expenditure and infrared locomotor activity monitoring (through beam breaks along the X axis) during a 24 h period were done using comprehensive laboratory animal monitoring system (CLAMS) at the indicated age at the Penn IDOM Mouse Phenotyping, Physiology and Metabolism Core. Core body temperature was measured rectally with a thermistor (Micro-Therma 2T; ThermoWorks) during the light cycle at the indicated age. Serum T4 and T3 levels were measured using a solid phase competitive ELISA (IBL America) in the Penn IDOM Radioimmunoassay and Biomarkers Core. White and brown adipose tissue gene expression and brown adipose tissue protein levels were measured by real-time PCR and immunoblotting, respectively as described below.

2.6. Leptin sensitivity experiment

For *in vivo* leptin sensitivity measurements, recombinant mouse leptin 1 μg/g body weight/injection (A. F. Parlow; National Hormone and Peptide Program) or 0.9% saline was administered i.p. to male mice on a chow diet at 8 weeks of age. Mice were initially injected with saline i.p. every 12 h over the course of 48 h. Leptin was subsequently administered following the same paradigm for 3 days. Mice received both saline and leptin injections using a within subjects design. Body weight and food intake were monitored daily for the 5 experimental days and for 2 additional recovery days. Body weight and food intake measurements for the days before the start of leptin injections were averaged and used to calculate percent change from baseline.

2.7. HPA axis responsivity

HPA axis responsivitiy was performed in singly housed male mice at the indicated age. Plasma corticosterone was measured following an acute 15 min restraint in a 50 mL conical tube. Tail blood was collected in EDTA-serum tubes before and after the restraint (0 and 15 min, respectively) and 15 min and 75 min after the end of the restraint (30 and 90 min, respectively). Serum corticosterone levels were measured as described previously [27]

2.8. Glucose homeostasis and serum analysis

A glucose tolerance test (GTT) was performed in male mice at the indicated age as described previously [28]. Blood glucose was assayed in tail blood using a glucometer (Contour, Bayer). Random-fed or

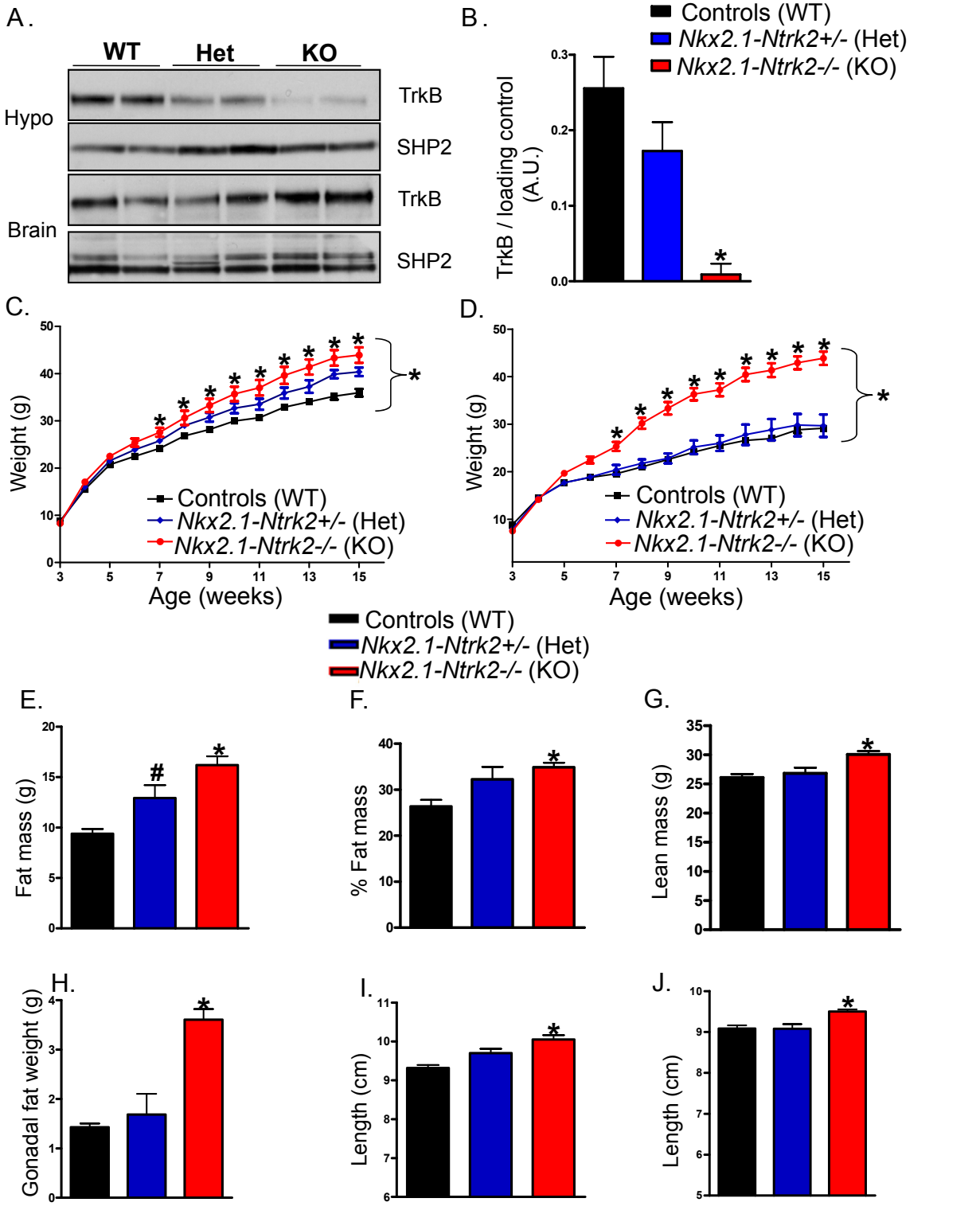

Figure 1: *Nkx2.1-Ntrk2*−/− mice have increased body weight, adiposity and length on HFD. (**A**) TrkB protein levels in the hypothalamus (top 2 blots) and brain (bottom 2 blots) of *Nkx2.1-Ntrk2*−/− (KO) mice compared with *Nkx2.1-Ntrk2*+/− (Het) and *Cre*-controls (WT). SHP2 protein levels are shown as a loading control. (**B**) Blots are quantified using ImageJ software, n = 4 for each genotype. (**C**) Body weights of male *Nkx2.1-Ntrk2*−/− (n = 11), *Nkx2.1-Ntrk2*+/− (n = 10) and wild type controls (n = 19) on HFD. (**D**) Body weights of female *Nkx2.1-Ntrk2*−/− (n = 13), *Nkx2.1-Ntrk2*+/− (n = 5) and wild type controls (n = 26) on HFD. (**E**) Fat mass and (**F**) % fat mass normalized to body weight as determined by NMR of male *Nkx2.1-Ntrk2*−/− (n = 6), *Nkx2.1-Ntrk2*+/− (n = 6), and wild type controls (n = 6). (**G**) Lean mass as determined by NMR of male *Nkx2.1-Ntrk2*−/− (n = 6), *Nkx2.1-Ntrk2*+/− (n = 6), and wild type controls (n = 6). (**H**) Gonadal fat weight of female *Nkx2.1-Ntrk2*−/− (n = 7), *Nkx2.1-Ntrk2*+/− (n = 5), and wild type controls (n = 6). (**I**) Body length for male *Nkx2.1-Ntrk2*−/− (n = 6), *Nkx2.1-Ntrk2*+/− (n = 6), and wild type controls (n = 6). (**J**) Body length for female *Nkx2.1-Ntrk2*−/− (n = 7), *Nkx2.1-Ntrk2*+/− (n = 5), and wild type controls (n = 6). All values are mean ± SEM. Weight curves are analyzed by two-way ANOVA followed by Bonferroni post-hoc pairwise comparison between *Nkx2.1-Ntrk2*−/− mice and wild type controls. Body composition and body length data are analyzed by one-way ANOVA followed by Bonferroni post-hoc pairwise comparison with the wild type controls. *p < 0.05, #p < 0.10 compared to wild type.

overnight fasted serum insulin and leptin levels were measured at the indicated age as described previously [27]. Liver triglyceride and cholesterol analyses were done at the Vanderbilt Hormone Assay and Analytical Services Core as described previously [29].

2.9. Real-time PCR

Total RNA was extracted using TRIzol (Invitrogen) and further purified with the RNeasy kit (Qiagen). cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (RT-PCR) was carried out using RT^2 SYBR Green qPCR Master Mix (SABiosciences) and samples were run using the Eppendorf Mastercycler® ep RealPlex. The relative mRNA expression was calculated using the comparative threshold cycle method as previously described [27]. The housekeeping gene *Hprt1* was used as an internal control. Primers used for real-time PCR were as follows (also see Appendix A):

Mc4r (PPM34139A, SABiosciences),
Pparg (PPM05108C, SABiosciences),
Actinb (PPM02945B, SABiosciences).

2.10. Immunoblotting

Brain tissues were dissected and immediately frozen in isopentane prior to use. NTS-enriched DVC were collected using a cryostat by targeted micropunches (bregma: − 7.76 mm, punch depth 1.0 mm) extending rostrally. Tissues were homogenized in RIPA buffer (10 mM Tris−HCl, pH 7.4, 150 mM sodium chloride, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM sodium fluoride) with protease inhibitor cocktail (Roche, 1:100 dilution), and sodium orthovanadate (2 mM). Protein concentrations were determined using a BCA Protein Assay (Thermo Scientific). Immunoblotting was done as described previously [26]. Antibodies used for immunoblotting were the following: Trk (C-14) (sc-11, Santa Cruz Biotechnology, Inc), SH-PTP2 (C-18) (sc-280, Santa Cruz Biotechnology, Inc), *Ucp1* (ab10983, Abcam), β-actin (4967, Cell Signaling). Blots were quantified using ImageJ software.

2.11. Statistical analysis

2.11.1. Results are expressed as mean ± SEM

Comparisons between groups were made by unpaired 2-tailed Student's *t*-test, 1-way ANOVA or 2-way ANOVA followed by Bonferroni posttest, as appropriate. A *p*-value of less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1. *Nkx2.1-Ntrk2−/−* mice display increased body weight, adiposity and body length on HFD

In order to generate mice with TrkB-deficiency throughout the hypothalamus, *Ntrk2fl/fl* mice were crossed to a line of transgenic *Nkx2.1-Cre* mice which express *Cre* in the ventral forebrain, including the majority of the hypothalamus, but not in caudal brain regions such as the hindbrain [30]. In the *Ntrk2fl/fl* mice, the floxed exon is within the tyrosine kinase domain of the TrkB receptor resulting in deletion of the full length TrkB isoform when recombined [23]. As expected, *Nkx2.1-Ntrk2−/−* mice show significant reduction of total TrkB protein in the hypothalamus but not the rest of the brain (Figure 1A, B). Weekly body weights of male and female *Nkx2.1-Ntrk2−/−* mice were examined and compared with the *Nkx2.1-Ntrk2+/−* heterozygous mice and wild type control littermates upon weaning. When fed a HFD, both male (Figure 1C) and female (Figure 1D) *Nkx2.1-Ntrk2−/−* mice display significantly increased body weight compared to their wild type littermates, with the females showing a greater increase compared to controls. *Nkx2.1-Ntrk2+/−* heterozygous male mice are similar in body weight compared to the *Nkx2.1-Ntrk2−/−* male mice. To determine whether the increase in body weight reflects changes in adiposity, body composition was assessed by NMR in males and gonadal fat was weighed in females. Male *Nkx2.1-Ntrk2−/−* mice display significant increases in both fat mass (Figure 1E, F) and lean mass (Figure 1G). Female *Nkx2.1-Ntrk2−/−* mice also have significantly increased adiposity as measured by gonadal fat weight (Figure 1H). Consistent with the increased adiposity, *Nkx2.1-Ntrk2−/−* mice exhibit increased serum leptin levels under both fed and fasted

Table 1 − Metabolic and neuroendocrine parameters of *Nkx2.1-Ntrk2−/−* mice on HFD. Random fed or overnight fasted *Nkx2.1-Ntrk2−/−*, *Nkx2.1-Ntrk2+/−* and wild type male and female mice are used in this study. Blood glucose, serum insulin and serum leptin measurements are taken on weeks 13 for fed and 15 for fasted conditions. Serum T4 and T3 levels are measured on week 15. Blood glucose, serum insulin and serum leptin are analyzed by one-way ANOVA followed by Bonferroni post-hoc pairwise comparison between *Nkx2.1-Ntrk2−/−* mice and wild type controls. Serum T4 and T3 levels are analyzed by unpaired two tailed Student's *t*-test. *p < 0.05 compared to wild type.

HFD				
	Genotype	Control (WT)	*Nkx2.1-Ntrk2+/−* (Het)	*Nkx2.1-Ntrk2−/−* (KO)
Fed (wk 13)	Blood glucose (mg/dl)	125 ± 4	126 ± 9	145 ± 17
Males	Serum insulin (ng/ml)	6.4 ± 0.9	9.5 ± 2.8	38.1 ± 14.0*
	Serum leptin (ng/ml)	24.4 ± 2.1	35.8 ± 4.9*	37.6 ± 1.8*
	Serum T4 (μg/dl)	2.51 ± 0.19	N.D.	2.81 ± 0.15
	Serum T3 (ng/ml)	0.78 ± 0.07	N.D.	0.82 ± 0.06
Fasted (wk 15)	Blood glucose (mg/dl)	83 ± 7	71 ± 2	83 ± 7
Males	Serum insulin (ng/ml)	1.6 ± 0.2	1.4 ± 0.1	1.8 ± 0.1
	Serum leptin (ng/ml)	7.6 ± 1.9	17.3 ± 5.5	20.3 ± 3.5*
Fed (wk 13)	Blood glucose (mg/dl)	113 ± 7	115 ± 5	163 ± 16*
Females	Serum insulin (ng/ml)	4.5 ± 1.2	8.2 ± 3.8	56.1 ± 10.7*
	Serum leptin (ng/ml)	32.7 ± 3.4	44.1 ± 8.6	78.4 ± 12.5*
	Serum T4 (μg/dl)	3.73 ± 0.22	N.D.	4.35 ± 0.23
	Serum T3 (ng/ml)	0.85 ± 0.14	N.D.	0.98 ± 0.06
Fasted (wk 15)	Blood glucose (mg/dl)	71 ± 2	62 ± 3	99 ± 5*
Females	Serum insulin (ng/ml)	1.0 ± 0.1	1.4 ± 0.1	2.0 ± 0.1*
	Serum leptin (ng/ml)	15.3 ± 8.6	10.7 ± 3.2	46.2 ± 4.3*

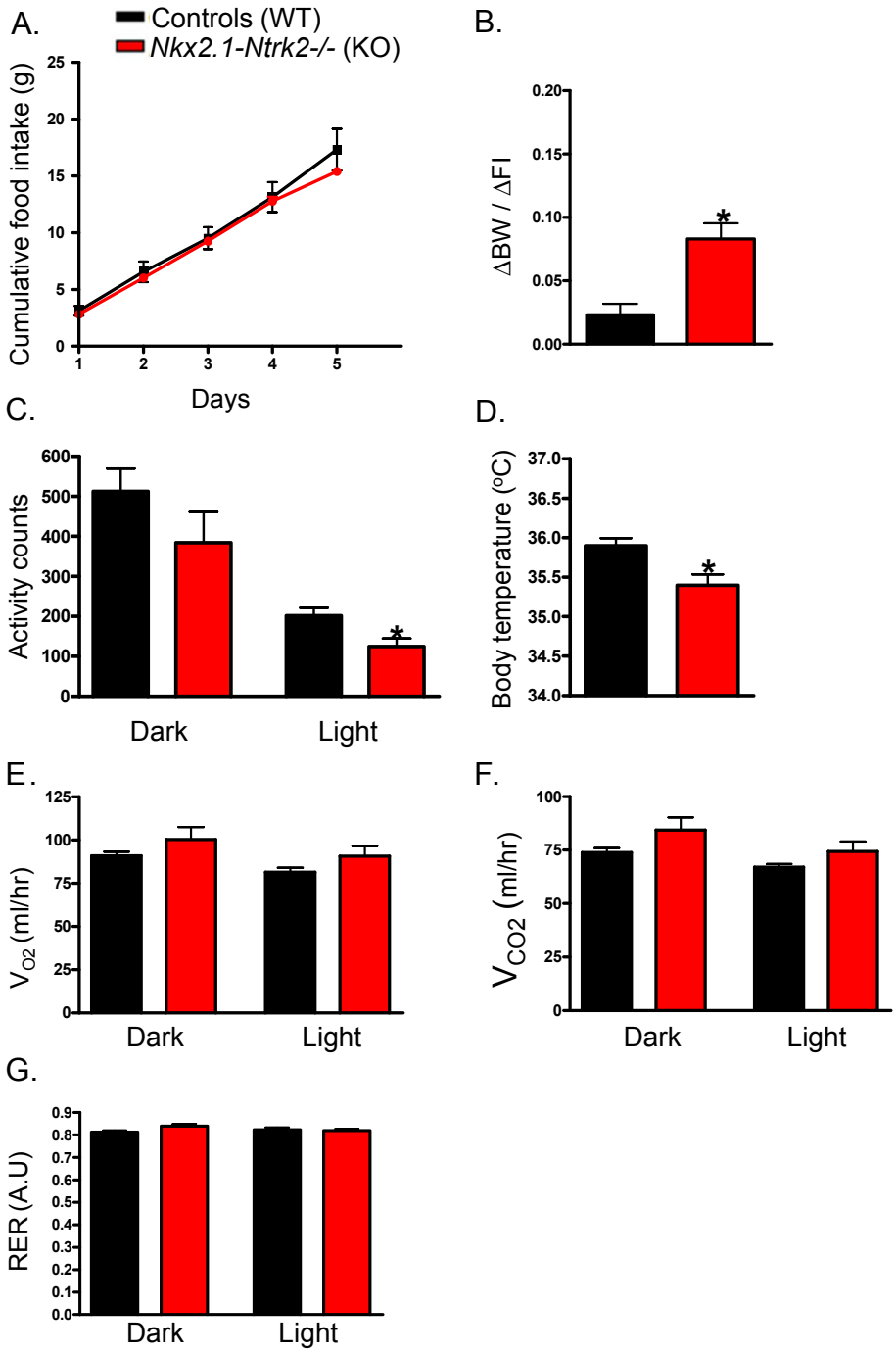

Figure 2: *Nkx2.1-Ntrk2−/−* mice have no difference in food intake on HFD but show decreased activity and core temperature. (**A**) Cumulative food intake of 5–6 week old, female *Nkx2.1-Ntrk2−/−* (n = 6) mice and wild type controls (n = 6) on HFD. (**B**) 5 day feed efficiency of 5–6 week old, female *Nkx2.1-Ntrk2−/−* (n = 6) mice and wild type controls (n = 6). (**C**) Locomotor activity of 5–6 week old, female *Nkx2.1-Ntrk2−/−* (n = 5) mice and wild type controls (n = 5). (**D**) Core temperature of 5–6 week old, female *Nkx2.1-Ntrk2−/−* (n = 6) mice and wild type controls (n = 6). (**E**) Oxygen consumption, (**F**) Carbon dioxide production, (**G**) Respiratory exchange ratio (RER) of 5–6 week old, female *Nkx2.1-Ntrk2−/−* (n = 5) mice and wild type controls (n = 5). All values are mean ± SEM. Cumulative food intake is analyzed by two-way ANOVA followed by Bonferroni post-hoc pairwise comparison between *Nkx2.1-Ntrk2−/−* mice and wild type controls. Feed efficiency and energy expenditure measures are analyzed by unpaired two-tailed Student's *t*-test *p < 0.05 compared to wild type.

conditions (Table 1). Both male (Figure 1I) and female (Figure 1J) *Nkx2.1-Ntrk2−/−* mice show concomitant increases in body length.

3.2. *Nkx2.1-Ntrk2−/−* mice show no difference in food intake on HFD but display decreased activity and core temperature

In order to determine the cause of the increased body weight and adiposity of the *Nkx2.1-Ntrk2−/−* mice on HFD, daily food intake was measured for a period of five days and the mice were also placed in CLAMS metabolic cages to directly measure energy expenditure and locomotor activity. These analyses were performed at 5−6 weeks of age prior to the onset of any body weight difference. *Nkx2.1-Ntrk2−/−* mice do not show changes in high-fat diet intake at this age compared to their wild type littermates (Figure 2A). Interestingly, *Nkx2.1-Ntrk2−/−* mice exhibit increased feed efficiency (Δbody weight/Δfood intake) suggesting that they have energy expenditure impairments (Figure 2B). While V_{02}, V_{CO2} and RER are similar in *Nkx2.1-Ntrk2−/−* and control mice (Figure 2E−G), *Nkx2.1-Ntrk2−/−* mice show reduced locomotor activity (Figure 2C) and reduced core temperature (Figure 2D). No difference in serum T4 or T3 levels is detected (Table 1). Baseline and stress-induced serum corticosterone levels are also similar (Supplemental 1A).

To assess whether there are changes in gene expression consistent with the metabolic impairments observed in *Nkx2.1-Ntrk2−/−* mice on HFD, anorexigenic *Pomc* and orexigenic *Npy* and *Agrp* neuropeptide mRNA levels were measured in the hypothalamus of *Nkx2.1-Ntrk2−/−* mice and wild type controls. Consistent with their obese phenotype, *Pomc* gene expression is significantly lower in *Nkx2.1-Ntrk2−/−* mice compared to controls. *Npy* and *Agrp* expression levels are not different between the genotypes (Figure 3A). No changes in *LepR* or *Mc4R* expression are detected between *Nkx2.1-Ntrk2−/−* mice and wild type controls (Figure 3A). A significant reduction in the expression of genes implicated in thermogenesis and brown adipose determination, including *Ucp1*, *Cidea* and *Pgc1α*, in WAT of *Nkx2.1-Ntrk2−/−* mice is consistent with impaired WAT browning (Figure 3B). Consistent with the elevated body weight and reduced core temperature, *Ucp1* mRNA (data not shown) and *Ucp1* protein (Figure 3C) are significantly lower in BAT of *Nkx2.1-Ntrk2−/−* mice. *Nkx2.1-Ntrk2−/−* mice also have significantly increased liver triglycerides (Figure 3D, E for males and females, respectively) while liver cholesterol levels are similar (1.6 ± 0.2 for wild type males, 1.7 ± 0.1 for *Nkx2.1-Ntrk2−/−* males; 2.0 ± 0.1 for wild type females, 2.5 ± 0.3 for *Nkx2.1-Ntrk2−/−* females).

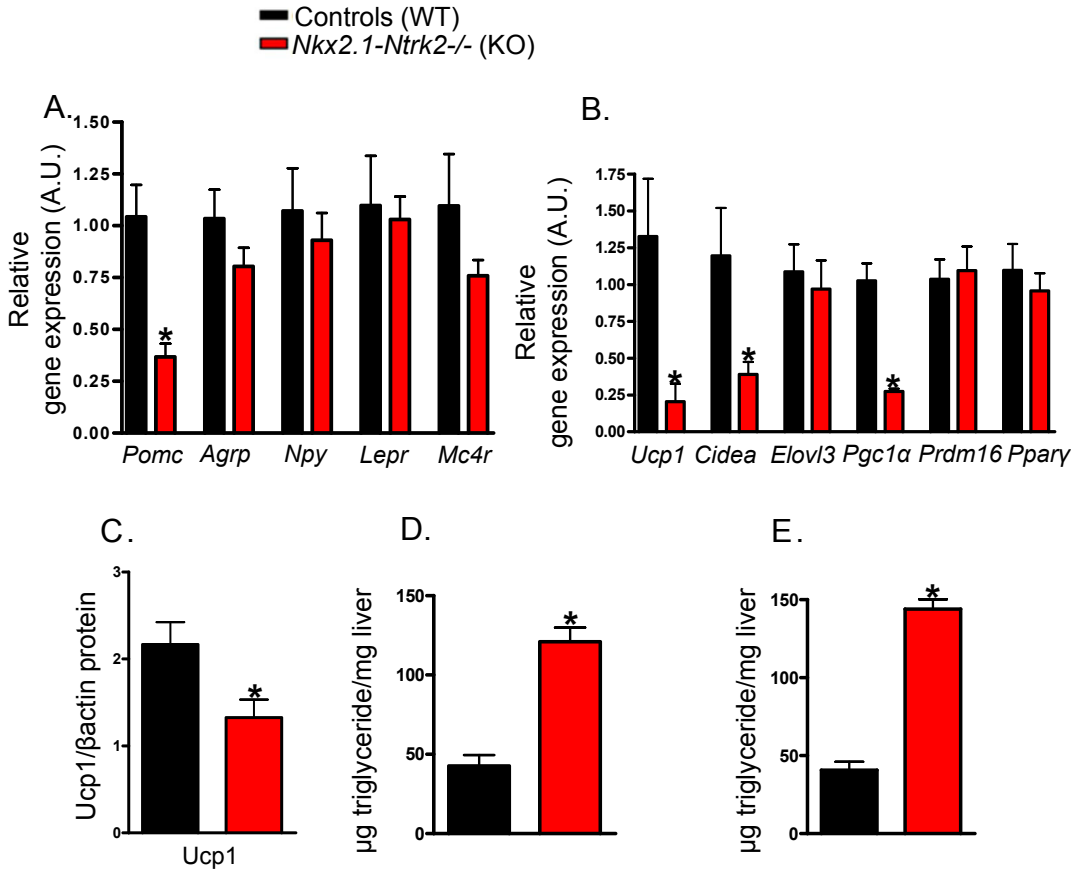

Figure 3: *Nkx2.1-Ntrk2−/−* mice show changes in gene expression consistent with their metabolic impairments on HFD. (**A**) Expression of *Pomc*, *Agrp, Npy*, *LepR*, *Mc4R* in the hypothalamus of female *Nkx2.1-Ntrk2−/−* (n = 6) mice and wild type controls (n = 5). *Hprt1* is used as a housekeeping gene. (**B**) Expression of *Ucp1*, *Cidea*, *Elovl3*, *Pgc1a*, *Prdm16*, *Pparg* in the WAT of female *Nkx2.1-Ntrk2−/−* (n = 6) mice and wild type controls (n = 6). *Hprt1* is used as a housekeeping gene. (**C**) *Ucp1* protein levels in the BAT of female *Nkx2.1-Ntrk2−/−* (n = 6) mice and wild type controls (n = 6). Beta-actin protein levels are used as loading control. (**D**) Liver triglyceride levels of male *Nkx2.1-Ntrk2−/−* (n = 6) mice and wild type controls (n = 6). (**E**) Liver triglyceride levels of female *Nkx2.1-Ntrk2−/−* (n = 6) mice and wild type controls All values are mean ± SEM. All measures are analyzed by unpaired two-tailed Student's *t*-test *p < 0.05.

3.3. *Nkx2.1-Ntrk2−/−* mice display increased body weight, adiposity and body length on chow diet

Weekly body weights of male and female *Nkx2.1-Ntrk2−/−* mice were examined and compared with the *Nkx2.1-Ntrk2+/−* heterozygous mice and wild type control littermates upon weaning. When maintained on a chow diet, male *Nkx2.1-Ntrk2−/−* mice are similar in body weight to their wild type littermates (Figure 4A) whereas female *Nkx2.1-Ntrk2−/−* mice show significantly increased body weight (Figure 4B). Despite the absence of a body weight phenotype, male *Nkx2.1-Ntrk2−/−* mice display a significant increase in fat mass (Figure 4C, D). Lean mass is similar between male *Nkx2.1-Ntrk2−/−* and control mice (Figure 4E). Female *Nkx2.1-Ntrk2−/−* mice also have significantly increased adiposity, as measured by gonadal fat weight (Figure 4F). Both male (Figure 4G) and female (Figure 4H) *Nkx2.1-Ntrk2−/−* mice display a slight increase in body length.

3.4. *Nkx2.1-Ntrk2−/−* mice show increased food intake and leptin resistance

In order to determine the cause of the increased body weight and adiposity of the *Nkx2.1-Ntrk2−/−* mice on chow, daily food intake was measured for a period of five days prior to the onset of any body weight difference. In contrast to mice maintained on HFD, chow-fed *Nkx2.1-Ntrk2−/−* female mice display hyperphagia prior to a significant body weight difference (Figure 5A). *Nkx2.1-Ntrk2−/−* female

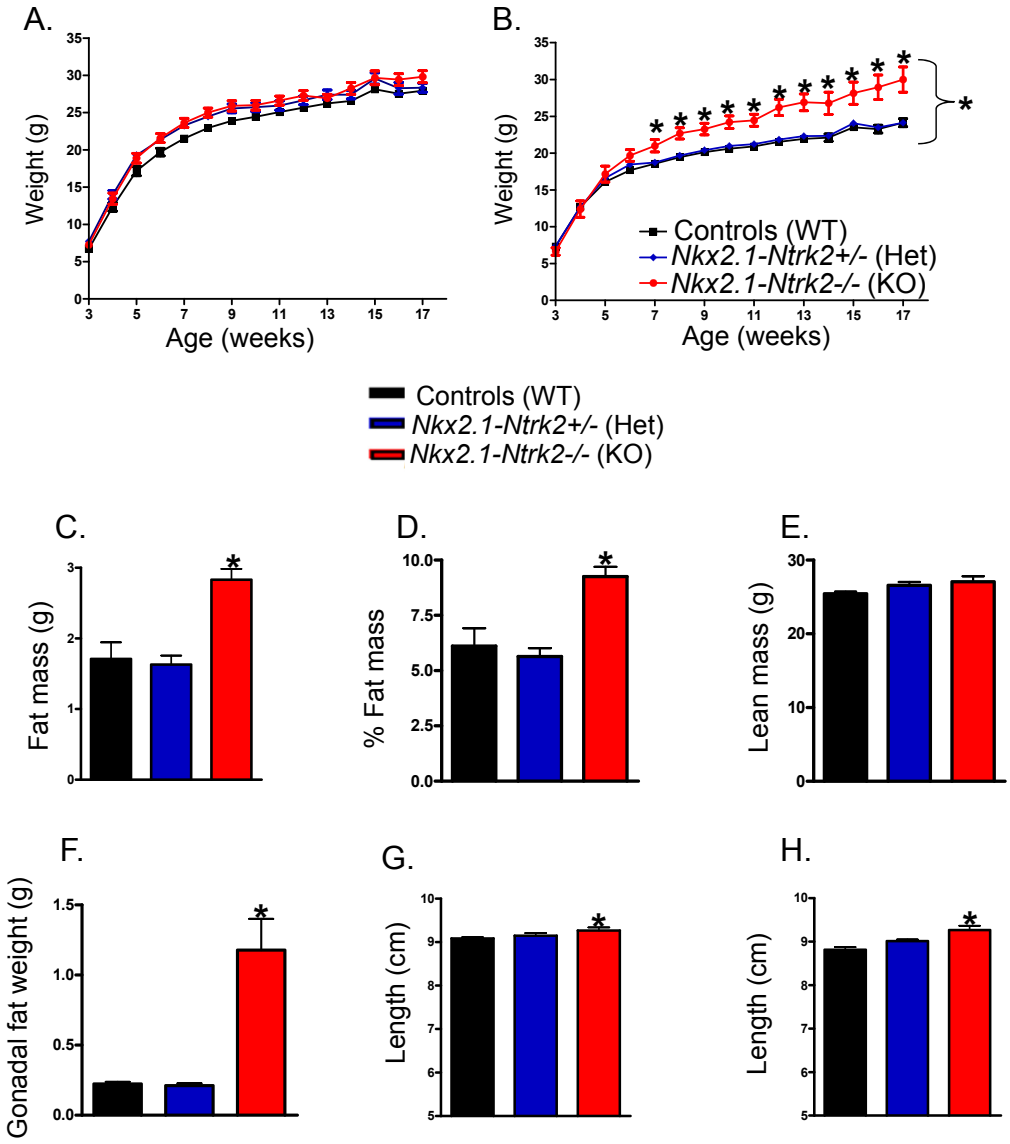

Figure 4: *Nkx2.1-Ntrk2−/−* mice have increased body weight, adiposity and body length on chow diet. (**A**) Body weights of male *Nkx2.1-Ntrk2−/−* (n = 10), *Nkx2.1-Ntrk2+/−* (n = 7) and wild type controls (n = 20) on chow. (**B**) Body weights of female *Nkx2.1-Ntrk2−/−* (n = 6), *Nkx2.1-Ntrk2+/−* (n = 12) and wild type controls (n = 23) on chow. (**C**) Fat mass and (**D**) % fat mass normalized to body weight as determined by NMR of male *Nkx2.1-Ntrk2−/−* (n = 6), *Nkx2.1-Ntrk2+/−* (n = 6), and wild type controls (n = 6). (**E**) Lean mass as determined by NMR of male *Nkx2.1-Ntrk2−/−* (n = 6), *Nkx2.1-Ntrk2+/−* (n = 6), and wild type controls (n = 6). (**F**) Gonadal fat weight of female *Nkx2.1-Ntrk2−/−* (n = 6), *Nkx2.1-Ntrk2+/−* (n = 8), and wild type controls (n = 10). (**G**) Body length for male *Nkx2.1-Ntrk2−/−* (n = 6), *Nkx2.1-Ntrk2+/−* (n = 6), and wild type controls (n = 6). (**H**) Body length for female *Nkx2.1-Ntrk2−/−* (n = 6), *Nkx2.1-Ntrk2+/−* (n = 8), and wild type controls (n = 10). Weight curves are analyzed by two-way ANOVA followed by Bonferroni post-hoc pairwise comparison between *Nkx2.1-Ntrk2−/−* mice and wild type controls. All values are mean ± SEM. Body composition and body length data are analyzed by one-way ANOVA followed by Bonferroni post-hoc pairwise comparison with the wild type controls. *p < 0.05 compared to wild type.

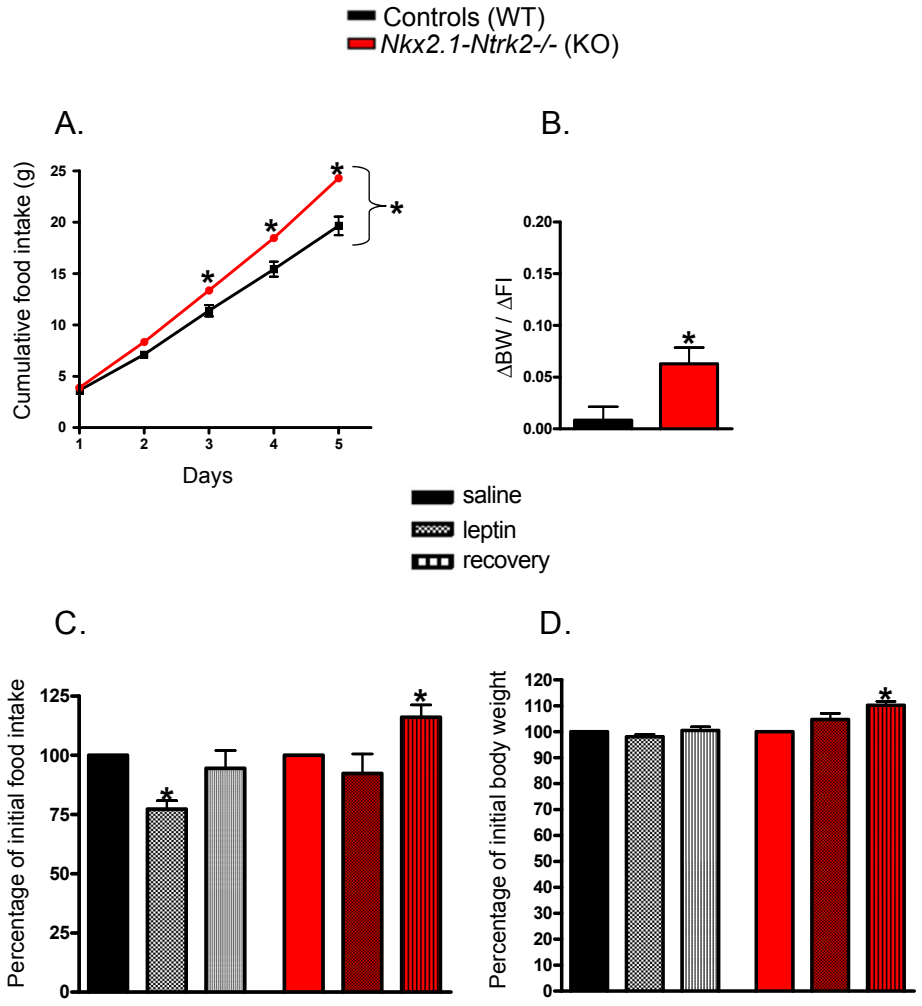

Figure 5: *Nkx2.1-Ntrk2*−/− mice show increased food intake and resistance to leptin. (**A**) Cumulative food intake of 5−6 week old, female *Nkx2.1-Ntrk2*−/− (n = 6) mice and wild type controls (n = 6) on chow. (**B**) 5 day feed efficiency of 5−6 week old, female *Nkx2.1-Ntrk2*−/− (n = 6) mice and wild type controls (n = 6). (**C**) Leptin-induced suppression of food intake in 8 week old male *Nkx2.1-Ntrk2*−/− (n = 5) mice and wild type controls (n = 5). Baseline body weight measurements for the days of saline injections were averaged and used to calculate percent change. Leptin bar refers to the measurement taken 72 h after the first leptin injection. Recovery bar refers to the measurement taken 48 h after the last leptin injection. (**D**) Leptin-induced reduction in body weight in 8 week old male *Nkx2.1-Ntrk2*−/− (n = 5) mice and wild type controls (n = 5). Baseline body weight measurements for the days of saline injections were averaged and used to calculate percent change. Leptin bar refers to the measurements 72 h after the first leptin injection. Recovery bar refers to the measurement taken 48 h after the last leptin injection. All values are mean ± SEM. Cumulative food intake is analyzed by two-way ANOVA followed by Bonferroni post-hoc pairwise comparison between *Nkx2.1-Ntrk2*−/− mice and wild type controls. Feed efficiency, % change in food intake and body weight are analyzed by unpaired two-tailed Student's *t*-test *$p < 0.05$ compared to wild type (and saline control for C and D).

mice also exhibit increased feed efficiency (Figure 5B), suggesting that both food intake and energy expenditure impairments likely play a role in the overall body weight phenotype. In response to exogenous leptin administration, *Nkx2.1-Ntrk2*−/− mice show resistance to leptin-induced suppression of food intake (Figure 5C) suggesting that intact hypothalamic TrkB signaling is required to convey leptin's effects on feeding. With this dose and injection paradigm, there is no significant reduction in body weight in either group (Figure 5D).

3.5. *Nkx2.1-Ntrk2*−/− mice show impaired glucose homeostasis

Given that obesity is often accompanied by glucose intolerance and insulin resistance, blood glucose and serum insulin levels were measured under random fed or overnight fasted conditions in male and female *Nkx2.1-Ntrk2*−/− mice and controls fed a chow diet or HFD. Overall, female *Nkx2.1-Ntrk2*−/− mice show a more severe hyperglycemic and hyperinsulinemic phenotype compared to male *Nkx2.1-Ntrk2*−/− mice and the phenotype is more robust on HFD compared to chow (Tables 1 and 2). Prior to the onset of major body weight differences, *Nkx2.1-Ntrk2*−/− mice have similar blood glucose and serum insulin levels (data not shown) to the controls. Male *Nkx2.1-Ntrk2*−/− mice which do not have a body weight phenotype on chow also do not display any impairment in glucose tolerance test (Table 2, Supplemental 1B). Taken together, these results suggest that the impairments in glucose metabolism that are detected in *Nkx2.1-Ntrk2*−/− mice on both diets are likely secondary to the body weight gain.

3.6. *Phox2b-Ntrk2*−/− mice are perinatal lethal

In order to assess the importance of endogenous hindbrain TrkB receptors to overall energy balance, mice with hindbrain TrkB-deficiency

Table 2 — Metabolic and neuroendocrine parameters of *Nkx2.1-Ntrk2−/−* mice on chow diet. Random fed or overnight fasted *Nkx2.1-Ntrk2−/−*, *Nkx2.1-Ntrk2+/−* and wild type male and female mice are used in this study. Blood glucose, serum insulin and serum leptin measurements are taken on weeks 13 for fed and 15 for fasted conditions. All measurements are analyzed by one-way ANOVA followed by Bonferroni post-hoc pairwise comparison between *Nkx2.1-Ntrk2−/−* mice and wild type controls. *p < 0.05 compared to wild type.

Chow				
	Genotype	Control (WT)	*Nkx2.1-Ntrk2+/−* (Het)	*Nkx2.1-Ntrk2−/−* (KO)
Fed (wk 13)	Blood glucose (mg/dl)	104 ± 5	101 ± 5	103 ± 4
Males	Serum insulin (ng/ml)	1.7 ± 0.1	1.7 ± 0.2	1.9 ± 0.4
	Serum leptin (ng/ml)	5.5 ± 0.5	4.7 ± 0.4	6.0 ± 0.9
Fasted (wk 15)	Blood glucose (mg/dl)	76 ± 9	71 ± 7	58 ± 4
Males	Serum insulin (ng/ml)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
	Serum leptin (ng/ml)	2.2 ± 0.2	2.0 ± 0.2	2.2 ± 0.2
Fed (wk 13)	Blood glucose (mg/dl)	110 ± 6	114 ± 4	129 ± 3*
Females	Serum insulin (ng/ml)	1.4 ± 0.1	1.6 ± 0.1	2.6 ± 0.4*
	Serum leptin (ng/ml)	4.9 ± 0.6	4.8 ± 0.4	20.5 ± 4.6*
Fasted (wk 15)	Blood glucose (mg/dl)	73 ± 9	65 ± 4	56 ± 4
Females	Serum insulin (ng/ml)	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
	Serum leptin (ng/ml)	3.2 ± 0.1	3.6 ± 0.4	6.7 ± 1.3*

were generated by crossing *Ntrk2fl/fl* mice to a line of transgenic *Phox2b-Cre* mice which express *Cre* in the NTS and DVC of the hindbrain but not in the hypothalamus [31]. Notably, despite being born in expected Mendelian ratios (data not shown), *Phox2b-Ntrk2−/−* mice die within 2–3 weeks of postnatal life. *Phox2b-Ntrk2−/−* mice are noticeably smaller (Figure 6A) and weigh significantly less (8.85 g ± 0.14 for wild type (n = 24); 5.02 g ± 0.14 for *Phox2b-Ntrk2−/−* (n = 13), measured at P18) than their wild type control littermates. Immunohistochemistry studies show significant overlap of Phox2b+ and TrkB + cells in the NTS of wild type mice (62.9% ± 9.7 of Phox2b + cells are also TrkB+), and confirm that the *Phox2b-Ntrk2−/−* mice lack TrkB expression in Phox2b + neurons (Figure 6B). *Phox2b* mRNA levels in the NTS are similar between *Phox2b-Ntrk2−/−* and wild type controls (data not shown). *Phox2b-Ntrk2+/−* heterozygous mice have an approximately 40% reduction in TrkB protein within the NTS compared to wild type controls as assessed by immunoblotting of NTS-enriched tissue punches (Figure 6C, D).

3.7. *Phox2b-Ntrk2+/−* mice are hyperphagic in the absence of a body weight phenotype

Phox2b-Ntrk2+/− mice and wild type controls were weaned onto either HFD or chow diet. When maintained on HFD, *Phox2b-Ntrk2+/−* female and male mice are similar in body weight compared to their wild type littermates (Figure 6E for females, Supplemental 2A for males). Adiposity is also similar, as measured by gonadal fat weight (Figure 6F for females, Supplemental 2B for males), as is body length (Figure 6G for females, Supplemental 2C for males). Interestingly, despite the absence of a body weight difference, cumulative and average daily food intake measurements suggest that *Phox2b-Ntrk2+/−* mice are hyperphagic (Figure 7A, B). *Phox2b-Ntrk2+/−* mice are similar to their wild type littermates in body temperature (Figure 7C) and BAT *Ucp1* gene expression (Figure 7D). On chow diet, there are no differences in body weight (Supplemental 3A, B for males and females, respectively), adiposity (Supplemental 3C, D for males and females, respectively) or body length (Supplemental 3E, F for males and females, respectively). *Phox2b-Ntrk2+/−* mice still display increased food intake, albeit to a lesser extent than on HFD (Supplemental 3G, H for cumulative and average daily food intake, respectively). Blood glucose and serum insulin levels are normal in *Phox2b-Ntrk2+/−* mice on either diet, with the exception of increased fasted blood glucose on chow-fed *Ntrk2+/−* male mice (Table 3).

4. DISCUSSION

BDNF and TrkB are known to play a major role in the central regulation of energy homeostasis and are also implicated in human obesity. Despite compelling evidence from rodent models to date emphasizing the role of BDNF/TrkB signaling in central metabolic control, the requirement of *endogenous* regional TrkB signaling is still not established. In this study, we have generated two mouse models of TrkB-deficiency to assess the role of intact endogenous hypothalamic (*Nkx2.1-Ntrk2−/−*) or hindbrain (*Phox2b-Ntrk2−/−*) TrkB signaling in energy balance regulation. Our data clearly demonstrate that *Nkx2.1-Ntrk2−/−* mice display significantly increased body weight and adiposity while *Phox2b-Ntrk2+/−* mice are hyperphagic without alterations in body weight or adiposity.

Both male and female *Nkx2.1-Ntrk2−/−* mice show significantly increased body weight when maintained on HFD although the females have a more severe metabolic phenotype compared to males. Furthermore, only females display significantly increased body weight when maintained on regular rodent chow diet. These findings are consistent with previous studies showing that female mice with BDNF- or TrkB-deficiency exhibit a more robust metabolic phenotype than males [9,11]. These sex-specific differences suggest sexual dimorphism of the hypothalamic TrkB signaling pathway, which has been previously shown in other brain regions [32,33]. For example, there is an estrogen response element within the BDNF gene [34] and estrogen signaling has been reported to induce BDNF expression in the hippocampus [35]. In a reciprocal manner, TrkB signaling has been reported to potentiate estrogen-initiated signaling in the human neuronal SH-SY5Y cells [36]. Both BDNF and estrogen influence synaptic plasticity by promoting dendritic spine growth [37] and there is overlap between estrogen receptor-expressing cells and cells that express BDNF and TrkB in the brain [35]. The more severe metabolic phenotype in female *Nkx2.1-Ntrk2−/−* mice could be due to TrkB-deficiency within the VMH where estrogen receptor alpha, BDNF and TrkB are all highly expressed; if TrkB signaling is absent, estrogen signaling may be blunted leading to increased weight gain. Furthermore, using a BDNF-mimetic to target muscular TrkB receptors results in improvements in energy expenditure and overall reduction in body weight only in female mice, suggesting there may be interactions between sex hormones and BDNF/TrkB pathways in peripheral tissues in addition to brain [38]. Although we did not find differences in estrogen receptor alpha gene expression in the whole

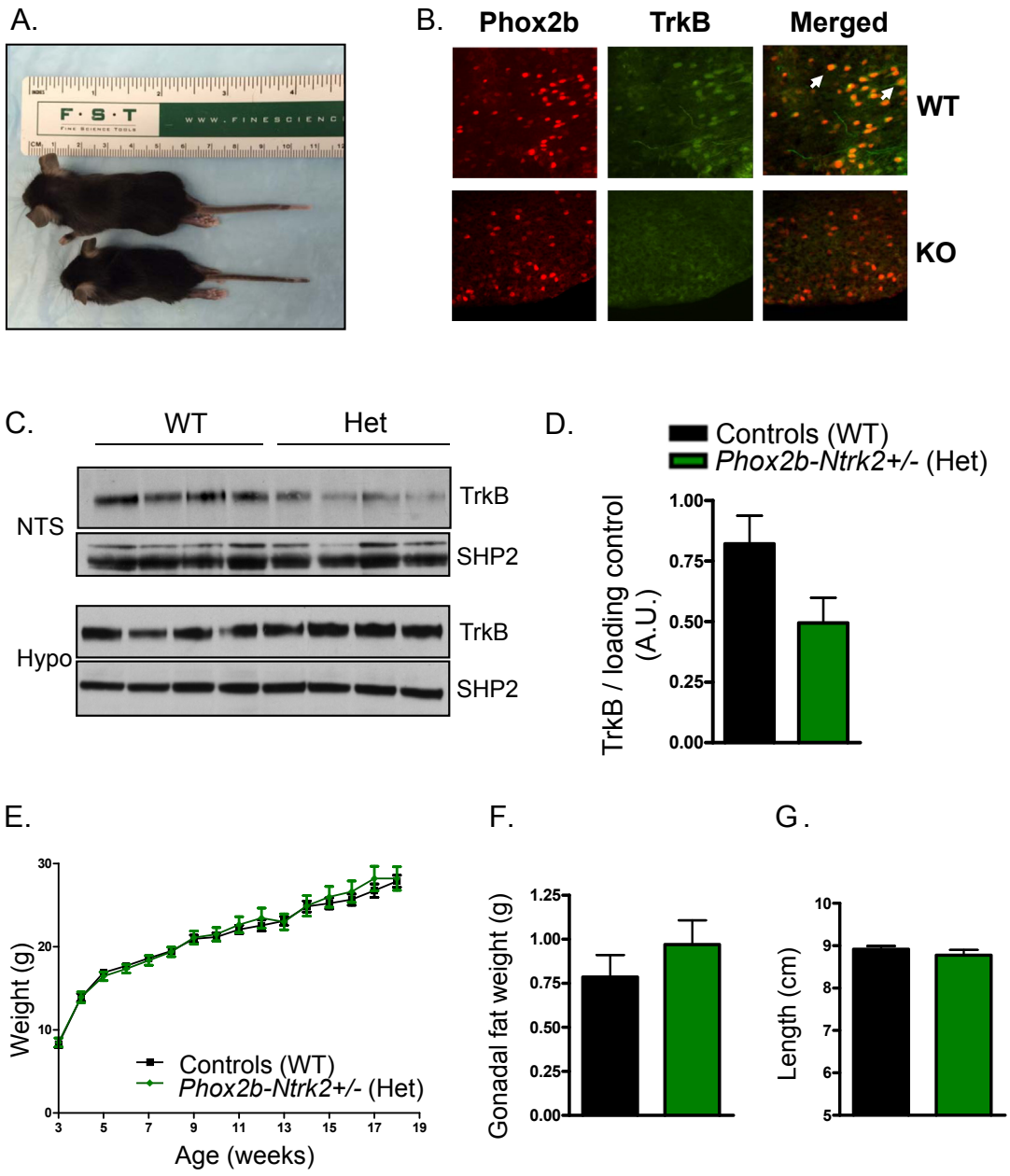

Figure 6: *Phox2b-Ntrk2*−/− mice are perinatal lethal and *Phox2b-Ntrk2*+/− are similar in body weight, adiposity and length compared to wild type controls on HFD. (**A**) Representative image of *Phox2b-Ntrk2*−/− mice and wild type control littermates at P18. (**B**) Representative immunofluorescence of Phox2b+ and TrkB+ cells in the NTS of control (top) and *Phox2b-Ntrk2*−/− (bottom) mice. White arrows indicate representative cells that are Phox2b+ and TrkB+. (**C**) TrkB protein levels in the NTS-enriched lysates (top 2 blots) and the hypothalamus (bottom 2 blots) of *Phox2b-Ntrk2*+/− (Het) and *Cre*-controls (WT). SHP2 protein levels are shown as a loading control. (**D**) Blots are quantified using ImageJ software, n = 7 for each genotype. (**E**) Body weights of female *Phox2b-Ntrk2*+/− (n = 9) mice and wild type controls (n = 17) on HFD. (**F**) Gonadal fat weight of female *Phox2b-Ntrk2*+/− (n = 9) mice and wild type controls (n = 11). (**G**) Body length for female *Phox2b-Ntrk2*+/− (n = 9) mice and wild type controls (n = 11). All values are mean ± SEM. Weight curves are analyzed by two-way ANOVA followed by Bonferroni post-hoc pairwise comparison between *Phox2b-Ntrk2*+/− mice and wild type controls. Body composition and body length data are analyzed by unpaired two-tailed Student's *t*-test. *p < 0.05 compared to wild type.

hypothalamus of *Nkx2.1-Ntrk2*−/− mice and wild type controls (data not shown), further investigation is required to study the interaction between estrogen and BDNF/TrkB signaling within specific nuclei of the hypothalamus.

In addition to the sex-specific differences, there are differences in various parameters of the metabolic phenotype of *Nkx2.1-Ntrk2*−/− mice depending on the diet. Somewhat surprisingly, under HFD-fed conditions *Nkx2.1-Ntrk2*−/− mice do not show any differences in food intake while chow-fed *Nkx2.1-Ntrk2*−/− mice exhibit hyperphagia. It is possible that the HFD-fed mice reach a "ceiling effect" in HFD consumption due to the high palatability of this diet. Alternatively, very high levels of circulating leptin in HFD-fed *Nkx2.1-Ntrk2*−/− mice might counteract the hyperphagia by suppressing food intake. On HFD, the overall body weight phenotype appears to be primarily driven by

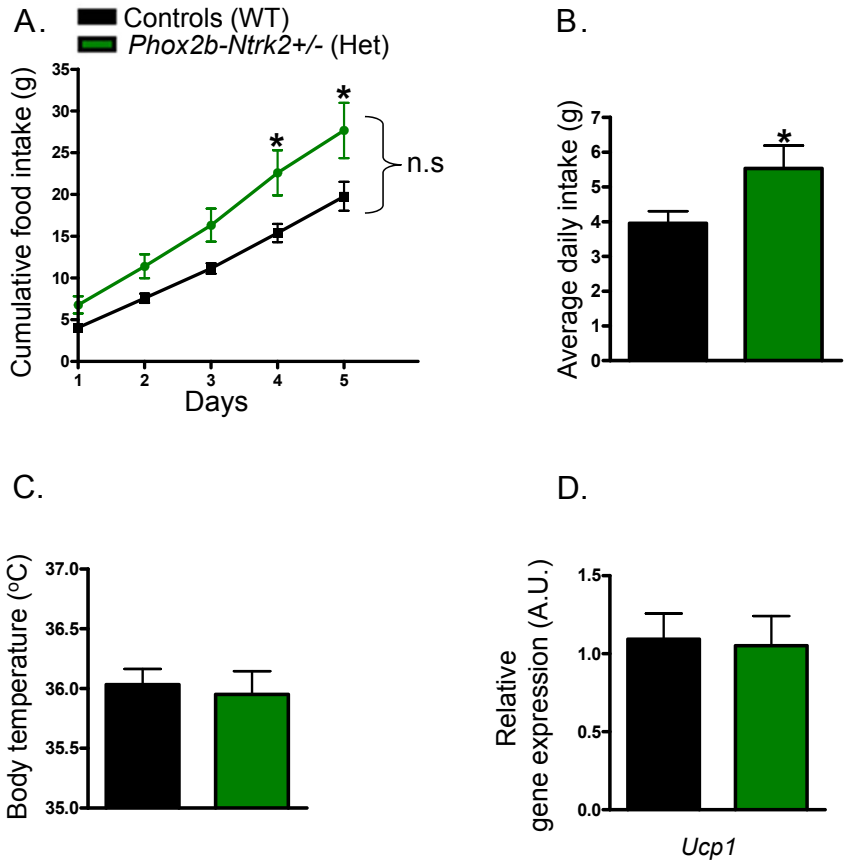

Figure 7: *Phox2b-Ntrk2*+/– mice are hyperphagic on HFD. Cumulative (**A**) and average daily (**B**) food intake of 12–13 week old, female *Phox2b-Ntrk2*+/– (n = 9) mice and wild type controls (n = 10) on HFD. (**C**) Core temperature of 13–14 week old, female *Phox2b-Ntrk2*+/– (n = 5) mice and wild type controls (n = 7). (**D**) *Ucp1* gene expression in the BAT of female *Phox2b-Ntrk2*+/– (n = 9) mice and wild type controls (n = 9). *Hprt1* gene is used as a housekeeping gene. All values are mean ± SEM. Cumulative food intake is analyzed by two-way ANOVA followed by Bonferroni post-hoc pairwise comparison between *Phox2b-Ntrk2*+/– mice and wild type controls. Average daily food intake and energy expenditure measures are analyzed by unpaired two tailed Student's *t*-test *p < 0.05 compared to wild type.

Table 3 – Metabolic and neuroendocrine parameters of *Phox2b-Ntrk2*+/– mice on HFD and chow diet. Random fed or overnight fasted *Phox2b-Ntrk2*+/– and wild type male and female mice are used in this study. Blood glucose and serum insulin measurements are taken on weeks 13 for fed and 15 for fasted conditions. All measurements are analyzed by unpaired two-tailed Student's *t*-test. *p < 0.05 compared to wild type.

HFD			
		Control (WT)	*Phox2b-Ntrk2*+/– (Het)
Fed (wk 13)	Blood glucose (mg/dl)	124 ± 4	119 ± 12
Males	Serum insulin (ng/ml)	3.4 ± 0.4	3.4 ± 0.5
Fasted (wk 15)	Blood glucose (mg/dl)	73 ± 2	74 ± 11
Males	Serum insulin (ng/ml)	2.2 ± 0.1	2.1 ± 0.1
Fed (wk 13)	Blood glucose (mg/dl)	132 ± 7	130 ± 19
Females	Serum insulin (ng/ml)	2.4 ± 0.1	2.5 ± 0.3
Fasted (wk 15)	Blood glucose (mg/dl)	71 ± 6	76 ± 16
Females	Serum insulin (ng/ml)	1.8 ± 0.1	1.9 ± 0.1
Chow			
Fed (wk 13)	Blood glucose (mg/dl)	114 ± 8	108 ± 4
Males	Serum insulin (ng/ml)	1.5 ± 0.1	1.9 ± 0.2
Fasted (wk 15)	Blood glucose (mg/dl)	55 ± 2	75 ± 4*
Males	Serum insulin (ng/ml)	1.0 ± 0.1	1.1 ± 0.1
Fed (wk 13)	Blood glucose (mg/dl)	104 ± 5	119 ± 5
Females	Serum insulin (ng/ml)	1.5 ± 0.1	1.6 ± 0.2
Fasted (wk 15)	Blood glucose (mg/dl)	71 ± 3	68 ± 4
Females	Serum insulin (ng/mlss)	0.9 ± 0.1	0.8 ± 0.1

decreased energy expenditure, specifically reduced locomotor activity and decreased core temperature. Direct administration of BDNF into the VMH or PVH results in increased spontaneous physical activity [17,39] and BDNF delivered to PVH results in increased BAT *Ucp1* gene expression through sympathetic innervation [15]. Thus, the observed reduction in activity and core temperature is consistent with deletion of TrkB in these two brain regions. There are no differences in measures of indirect calorimetry at week 5–6 but this does not rule out the possibility of *Nkx2.1-Ntrk2*–/– mice developing reduced basal metabolic rate at a later time point, which will require further study. A previous paper which examined mice with central TrkB-deficiency using the *Rgs9-Cre* which deletes in the arcuate nucleus, dorsomedial nucleus of the hypothalamus, and lateral hypothalamus [11] reported similar increases in body weight and adiposity. However, *Rgs9-Ntrk2*–/– mice do not show reduced locomotor activity while *Nkx2.1-Ntrk2*–/– mice do. Additionally *Rgs9-Ntrk2*–/– mice, unlike *Nkx2.1-Ntrk2*–/–, show increased V_{02} production and V_{CO2} consumption. One explanation for this discrepancy between the two lines could be that with the *Rgs9-Cre* line, the PVH and VMH are not targeted efficiently and there is additional deletion in the striatum, cortex and hippocampus [11]. Chow-fed male *Rgs9-Ntrk2*–/– mice have significantly increased body weight compared to the controls and *Nkx2.1-Ntrk2*–/– do not. This difference is likely due to the variation in caloric makeup

of the chow diet as the diet used in that study has an intermediate fat percentage (21.6% fat by calories) between the chow diet (12.7% fat by calories) and HFD (54.8% fat by calories) used in this study.

In the current study, we find that *Nkx2.1-Ntrk2−/−* mice exhibit increased linear growth, similar to previously described mouse models of BDNF-deficiency and TrkB hypomorphic mice [7–9], implicating the BDNF/TrkB signaling pathway in growth regulation. The melanocortin pathway also plays an important role in somatic growth [30,40,41], although we did not detect any differences in *Mc4r* gene expression in the hypothalamus of *Nkx2.1-Ntrk2−/−* mice compared to controls. Nevertheless, it is possible that TrkB-deficiency results in impaired melanocortin signaling and increased linear growth indirectly via this pathway.

The increased body weight phenotype in *Nkx2.1-Ntrk2−/−* mice is accompanied by glucose homeostasis impairments as these mice are hyperglycemic and hyperinsulinemic. Notably, at an earlier time point (8–10 weeks of age) there are no significant differences in fed or fasted blood glucose and serum insulin levels (data not shown), suggesting that this phenotype may most likely be secondary to increased body weight. Consistently, *Nkx2.1-Ntrk2−/−* male mice on chow diet which do not have a body weight phenotype also do not display impairments in glucose homeostasis, as measured by fed and fasted blood glucose, serum insulin levels and glucose tolerance test. The increased fed (but not fasted) blood glucose levels in chow-fed *Nkx2.1-Ntrk2−/−* female mice could be due to hyperphagia.

Brain-derived neurotrophic factor (BDNF) is a key neurotrophic factor that is implicated in neural circuit development and plasticity through enhancing axonal arborization and neurite outgrowth [42,43]. BDNF has also been shown to influence neurohormone synthesis, differentiation and release in the hypothalamus [44]. Nissl staining of the *Nkx2.1-Ntrk2−/−* hypothalamus does not reveal a major difference in the overall hypothalamic architecture (data not shown) suggesting that the metabolic phenotype of the *Nkx2.1-Ntrk2−/−* mice is not a result of gross neuroanatomical abnormalities due to embryonic deletion of TrkB (starting at E10) in the hypothalamus. However, given that BDNF has potent neurotrophic effects, we cannot rule out the possibility that TrkB-deficiency within the hypothalamus results in altered neuronal connectivity. In fact, a recent study reported that BDNF-deficient mice exhibit impairments in NPY projections from the arcuate nucleus of the hypothalamus to the PVH as well as impaired POMC projections to the dorsomedial nucleus of the hypothalamus [45]. In the future, it will be very informative to perform neuronal tracing experiments in mouse models of TrkB-deficiency restricted to distinct subpopulations of neurochemically identified neurons to test whether absence of TrkB in these neuronal populations result in impairments in intrahypothalamic projections similar to BDNF-deficient mice.

Studies show that *Nkx2.1* is broadly expressed in the hypothalamus. Within the arcuate nucleus, cells that express *Nkx2.1* give rise to a subset of GABAergic, NPY, POMC and dopaminergic neurons or glia (tanycytes) [46]. Interestingly, a recent study has shown that in the arcuate nucleus, most of the TrkB+ neurons do not express *Pomc* or *Npy*, suggesting that they represent a distinct set of previously uncharacterized neurons [45]. In the future, it will be very informative to identify the neurochemical characteristics of the TrkB-expressing neurons in the hypothalamus to further elucidate their role in the hypothalamic circuitry controlling energy balance.

Previous studies have shown that hypothalamic BDNF expression is regulated by MC4R signaling, that BDNF acts downstream of MC4R signaling to regulate food intake and body weight, and that BDNF is required for leptin to activate hypothalamic neurons and to inhibit food intake [9,10]. Our data show that although there are no changes in the expression of *LepR* and *Mc4R* genes in the whole hypothalamus, *Nkx2.1-Ntrk2−/−* mice are resistant to acute leptin-induced suppression of food intake. Thus, these findings support the notion that BDNF/TrkB signaling is an important effector in conveying leptin's effects on feeding.

Phox2b-Ntrk2−/− mice are perinatal lethal within second to third postnatal week of life despite being born in appropriate Mendelian ratios. We speculate that this is due to the vital role of TrkB signaling in the development of visceral motor neurons in the brainstem that are implicated in cardiovascular, respiratory and digestive functions [47,48]. In the future, it will be crucial to use an inducible *Cre* line to overcome the lethal phenotype and to study the role of endogenous hindbrain TrkB in central metabolic control.

Consistent with the notion that hindbrain TrkB signaling is important to food intake regulation [22], *Phox2b-Ntrk2+/−* mice exhibit hyperphagia in the absence of a body weight phenotype. Whether these animals have increased energy expenditure to counteract the increased food intake similar to mice with hindbrain *LepR* deletion [31] needs further investigation. Hyperphagia observed in both HFD and to a lesser extent chow diet-fed *Phox2b-Ntrk2+/−* mice could be due to local action of BDNF/TrkB signaling in the NTS and DVC or through alterations in the forebrain-hindbrain wiring, likely through the PVH [49] or through projections to the mesolimbic reward centers involved in hedonic feeding [50]. *Phox2b* is expressed in enteric neuronal precursors that migrate from the vagal neural axis in a rostrocaudal manner to innervate the stomach and the intestine, and differentiate into enteric neurons [51,52]. In *Phox2b−/−* mice, enteric neuronal precursors fail to migrate from foregut to midgut and hindgut due to increased apoptosis [47]. Notably, both BDNF and TrkB are expressed in the enteric nervous system innervating the gut. BDNF enhances enteric nervous system signaling by promoting neural activity and synaptic transmission and subsequently increasing gastrointestinal motility [53]. Furthermore, *Ntrk2−/−* mice show deterioration of the normal architecture of the enteric nervous system [54]. Both BDNF and TrkB expression are altered in intestinal pathologies such as Hirschprung disease and infantile hypertrophic pyloric stenosis [55,56]. Thus, it is feasible that impairments resulting from a lack of TrkB in the enteric nervous system of *Phox2b-Ntrk2+/−* mice could lead to a deficiency in intestinal nutrient absorption and subsequent hyperphagia in the absence of body weight differences.

Taken together, our study establishes the importance of the hypothalamus as a key brain region in endogenous BDNF/TrkB signaling and central metabolic control and emphasizes that endogenous hindbrain BDNF/TrkB signaling has a modulatory role in food intake.

DISCLOSURE STATEMENT

The authors have nothing to disclose.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

None declared.

APPENDIX A

Gene	Forward primer	Reverse primer
Pomc	5′ GACACGTGGAGATGCCGAG 3′	5′ CAGCGAGAGGTCGAGTTTGC 3′
Agrp	5′ GCGGAGGTGCTAGATCCACA 3′	5′ AGGACTCGTGCAGCCTTACAC 3′
Npy	5′ CTCCGCTCTGCGACACTACA 3′	5′ AATCAGTGTCTCAGGGCTGGA 3′
Leprb	5′ CAAACCCCAAGAATTGTT CCTGG 3′	5′ TCAGGCTCCAGAAGAAGA GGACC 3′
Hprt1	5′ GCGTCGTGATTAGCGATG ATGAAC 3′	5′ CCTCCCATCTCTCCTTCATG CATCT 3′
Ucp1	5′ GGCATTCAGAGGCAAATCAGCT 3′	5′ CAATGAACACTGCCACACCTC 3′
Cidea	5′ ATCACAACTGGCCTGGTTACG 3′	5′ TACTACCCGGTGTCCATTTCT 3′
Elovl3	5′ GGACCTGATGCAACCCTATGA 3′	5′ TCCGCGTTCTCATGTAGGTCT 3′
Ppargc1a	5′ CCCTGCCATTGTTAAGACC 3′	5′ TGCTGCTGTTCCTGTTTTC 3′
Prdm16	5′ CAGCACGGTGAAGCCATTC 3′	5′ GCGTGCATCCGCTTGTG 3′

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.08.002.

REFERENCES

[1] Flegal, K.M., Carroll, M.D., Kit, B.K., Ogden, C.L., 2012. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999–2010. JAMA: The Journal of the American Medical Association 307:491–497.

[2] Locke, A.E., Kahali, B., Berndt, S.I., Justice, A.E., Pers, T.H., Day, F.R., et al., 2015. Genetic studies of body mass index yield new insights for obesity biology. Nature 518:197–206.

[3] Rios, M., 2013. BDNF and the central control of feeding: accidental bystander or essential player? Trends in Neurosciences 36:83–90.

[4] Vanevski, F., Xu, B., 2013. Molecular and neural bases underlying roles of BDNF in the control of body weight. Frontiers in Neuroscience 7:37.

[5] Gray, J., Yeo, G.S., Cox, J.J., Morton, J., Adlam, A.L., Keogh, J.M., et al., 2006. Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene. Diabetes 55:3366–3371.

[6] Yeo, G.S., Connie Hung, C.C., Rochford, J., Keogh, J., Gray, J., Sivaramakrishnan, S., et al., 2004. A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. Nature Neuroscience 7:1187–1189.

[7] Kernie, S.G., Liebl, D.J., Parada, L.F., 2000. BDNF regulates eating behavior and locomotor activity in mice. The EMBO Journal 19:1290–1300.

[8] Rios, M., Fan, G., Fekete, C., Kelly, J., Bates, B., Kuehn, R., et al., 2001. Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. Molecular Endocrinology 15:1748–1757.

[9] Xu, B., Goulding, E.H., Zang, K., Cepoi, D., Cone, R.D., Jones, K.R., et al., 2003. Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nature Neuroscience 6:736–742.

[10] Liao, G.Y., An, J.J., Gharami, K., Waterhouse, E.G., Vanevski, F., Jones, K.R., et al., 2012. Dendritically targeted Bdnf mRNA is essential for energy balance and response to leptin. Nature Medicine 18:564–571.

[11] Liao, G.Y., Li, Y., Xu, B., 2013. Ablation of TrkB expression in RGS9-2 cells leads to hyperphagic obesity. Molecular Metabolism 2:491–497.

[12] Yan, Q., Radeke, M.J., Matheson, C.R., Talvenheimo, J., Welcher, A.A., Feinstein, S.C., 1997. Immunocytochemical localization of TrkB in the central nervous system of the adult rat. The Journal of Comparative Neurology 378:135–157.

[13] Yan, Q., Rosenfeld, R.D., Matheson, C.R., Hawkins, N., Lopez, O.T., Bennett, L., et al., 1997. Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience 78:431–448.

[14] Wang, C., Bomberg, E., Billington, C., Levine, A., Kotz, C.M., 2007. Brain-derived neurotrophic factor in the hypothalamic paraventricular nucleus reduces energy intake. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 293:R1003–R1012.

[15] Wang, C., Bomberg, E., Billington, C., Levine, A., Kotz, C.M., 2007. Brain-derived neurotrophic factor in the hypothalamic paraventricular nucleus increases energy expenditure by elevating metabolic rate. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 293:R992–R1002.

[16] Wang, C., Bomberg, E., Levine, A., Billington, C., Kotz, C.M., 2007. Brain-derived neurotrophic factor in the ventromedial nucleus of the hypothalamus reduces energy intake. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 293:R1037–R1045.

[17] Wang, C., Bomberg, E., Billington, C.J., Levine, A.S., Kotz, C.M., 2010. Brain-derived neurotrophic factor (BDNF) in the hypothalamic ventromedial nucleus increases energy expenditure. Brain Research 1336:66–77.

[18] Wang, C., Godar, R.J., Billington, C.J., Kotz, C.M., 2010. Chronic administration of brain-derived neurotrophic factor in the hypothalamic paraventricular nucleus reverses obesity induced by high-fat diet. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 298:R1320–R1332.

[19] Godar, R., Dai, Y., Bainter, H., Billington, C., Kotz, C.M., Wang, C.F., 2011. Reduction of high-fat diet-induced obesity after chronic administration of brain-derived neurotrophic factor in the hypothalamic ventromedial nucleus. Neuroscience 194:36–52.

[20] Bariohay, B., Lebrun, B., Moyse, E., Jean, A., 2005. Brain-derived neurotrophic factor plays a role as an anorexigenic factor in the dorsal vagal complex. Endocrinology 146:5612–5620.

[21] Bariohay, B., Roux, J., Tardivel, C., Trouslard, J., Jean, A., Lebrun, B., 2009. Brain-derived neurotrophic factor/tropomyosin-related kinase receptor type B signaling is a downstream effector of the brainstem melanocortin system in food intake control. Endocrinology 150:2646–2653.

[22] discSpaeth, A.M., Kanoski, S.E., Hayes, M.R., Grill, H.J., 2012. TrkB receptor signaling in the nucleus tractus solitarius mediates the food intake-suppressive effects of hindbrain BDNF and leptin. American Journal of Physiology. Endocrinology and Metabolism 302:E1252–E1260.

[23] Minichiello, L., Korte, M., Wolfer, D., Kuhn, R., Unsicker, K., Cestari, V., et al., 1999. Essential role for TrkB receptors in hippocampus-mediated learning. Neuron 24:401–414.

[24] Zhai, J., Zhou, W., Li, J., Hayworth, C.R., Zhang, L., Misawa, H., et al., 2011. The in vivo contribution of motor neuron TrkB receptors to mutant SOD1 motor neuron disease. Human Molecular Genetics 20:4116–4131.

[25] Tsou, R.C., Zimmer, D.J., De Jonghe, B.C., Bence, K.K., 2012. Deficiency of PTP1B in leptin receptor-expressing neurons leads to decreased body weight and adiposity in mice. Endocrinology 153:4227–4237.

[26] Banno, R., Zimmer, D., De Jonghe, B.C., Atienza, M., Rak, K., Yang, W., et al., 2010. PTP1B and SHP2 in POMC neurons reciprocally regulate energy balance in mice. The Journal of Clinical Investigation 120:720–734.

[27] Tsou, R.C., Rak, K.S., Zimmer, D.J., Bence, K.K., 2014. Improved metabolic phenotype of hypothalamic PTP1B-deficiency is dependent upon the leptin receptor. Molecular Metabolism 3:301–312.

[28] Klaman, L.D., Boss, O., Peroni, O.D., Kim, J.K., Martino, J.L., Zabolotny, J.M., et al., 2000. Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. Molecular and Cellular Biology 20:5479–5489.

[29] Delibegovic, M., Zimmer, D., Kauffman, C., Rak, K., Hong, E.G., Cho, Y.R., et al., 2009. Liver-specific deletion of protein-tyrosine phosphatase 1B (PTP1B) improves metabolic syndrome and attenuates diet-induced endoplasmic reticulum stress. Diabetes 58:590–599.
[30] Ring, L.E., Zeltser, L.M., 2010. Disruption of hypothalamic leptin signaling in mice leads to early-onset obesity, but physiological adaptations in mature animals stabilize adiposity levels. The Journal of Clinical Investigation 120: 2931–2941.
[31] Scott, M.M., Williams, K.W., Rossi, J., Lee, C.E., Elmquist, J.K., 2011. Leptin receptor expression in hindbrain Glp-1 neurons regulates food intake and energy balance in mice. The Journal of Clinical Investigation 121:2413–2421.
[32] Liu, Y., Rutlin, M., Huang, S., Barrick, C.A., Wang, F., Jones, K.R., et al., 2012. Sexually dimorphic BDNF signaling directs sensory innervation of the mammary gland. Science 338:1357–1360.
[33] Lucas, E.K., Jegarl, A., Clem, R.L., 2014. Mice lacking TrkB in parvalbumin-positive cells exhibit sexually dimorphic behavioral phenotypes. Behavioural Brain Research 274:219–225.
[34] Sohrabji, F., Miranda, R.C., Toran-Allerand, C.D., 1995. Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. Proceedings of the National Academy of Sciences of the United States of America 92:11110–11114.
[35] Solum, D.T., Handa, R.J., 2002. Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience 22:2650–2659.
[36] Wong, J., Woon, H.G., Weickert, C.S., 2011. Full length TrkB potentiates estrogen receptor alpha mediated transcription suggesting convergence of susceptibility pathways in schizophrenia. Molecular and Cellular Neurosciences 46:67–78.
[37] Scharfman, H.E., MacLusky, N.J., 2006. Estrogen and brain-derived neurotrophic factor (BDNF) in hippocampus: complexity of steroid hormone-growth factor interactions in the adult CNS. Frontiers in Neuroendocrinology 27:415–435.
[38] Chan, C.B., Tse, M.C., Liu, X., Zhang, S., Schmidt, R., Otten, R., et al., 2015. Activation of muscular TrkB by its small molecular agonist 7,8-dihydroxyflavone sex-dependently regulates energy metabolism in diet-induced obese mice. Chemistry & Biology 22:355–368.
[39] An, J.J., Liao, G.Y., Kinney, C.E., Sahibzada, N., Xu, B., 2015. Discrete BDNF neurons in the paraventricular hypothalamus control feeding and energy expenditure. Cell Metabolism 22:175–188.
[40] Yen, T.T., Gill, A.M., Frigeri, L.G., Barsh, G.S., Wolff, G.L., 1994. Obesity, diabetes, and neoplasia in yellow A(vy)/- mice: ectopic expression of the agouti gene. FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology 8:479–488.
[41] Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., et al., 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88:131–141.
[42] Park, H., Poo, M.M., 2013. Neurotrophin regulation of neural circuit development and function. Nature Reviews. Neuroscience 14:7–23.
[43] Jeanneteau, F., Deinhardt, K., Miyoshi, G., Bennett, A.M., Chao, M.V., 2010. The MAP kinase phosphatase MKP-1 regulates BDNF-induced axon branching. Nature Neuroscience 13:1373–1379.
[44] Tapia-Arancibia, L., Rage, F., Givalois, L., Arancibia, S., 2004. Physiology of BDNF: focus on hypothalamic function. Frontiers in Neuroendocrinology 25: 77–107.
[45] Liao, G.Y., Bouyer, K., Kamitakahara, A., Sahibzada, N., Wang, C.H., Rutlin, M., et al., 2015. Brain-derived neurotrophic factor is required for axonal growth of selective groups of neurons in the arcuate nucleus. Molecular Metabolism 4: 471–482.
[46] Yee, C.L., Wang, Y., Anderson, S., Ekker, M., Rubenstein, J.L., 2009. Arcuate nucleus expression of NKX2.1 and DLX and lineages expressing these transcription factors in neuropeptide Y(+), proopiomelanocortin(+), and tyrosine hydroxylase(+) neurons in neonatal and adult mice. The Journal of Comparative Neurology 517:37–50.
[47] Pattyn, A., Morin, X., Cremer, H., Goridis, C., Brunet, J.F., 1999. The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399:366–370.
[48] Coppola, E., d'Autreaux, F., Rijli, F.M., Brunet, J.F., 2010. Ongoing roles of Phox2 homeodomain transcription factors during neuronal differentiation. Development 137:4211–4220.
[49] Grill, H.J., Hayes, M.R., 2012. Hindbrain neurons as an essential hub in the neuroanatomically distributed control of energy balance. Cell Metabolism 16: 296–309.
[50] Cordeira, J.W., Frank, L., Sena-Esteves, M., Pothos, E.N., Rios, M., 2010. Brain-derived neurotrophic factor regulates hedonic feeding by acting on the mesolimbic dopamine system. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience 30:2533–2541.
[51] Pattyn, A., Morin, X., Cremer, H., Goridis, C., Brunet, J.F., 1997. Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development 124:4065–4075.
[52] Young, H.M., Hearn, C.J., Ciampoli, D., Southwell, B.R., Brunet, J.F., Newgreen, D.F., 1998. A single rostrocaudal colonization of the rodent intestine by enteric neuron precursors is revealed by the expression of Phox2b, Ret, and p75 and by explants grown under the kidney capsule or in organ culture. Developmental Biology 202:67–84.
[53] Boesmans, W., Gomes, P., Janssens, J., Tack, J., Vanden Berghe, P., 2008. Brain-derived neurotrophic factor amplifies neurotransmitter responses and promotes synaptic communication in the enteric nervous system. Gut 57: 314–322.
[54] Levanti, M.B., Esteban, I., Ciriaco, E., Perez-Pinera, P., Cabo, R., Garcia-Suarez, O., et al., 2009. Enteric glial cells express full-length TrkB and depend on TrkB expression for normal development. Neuroscience Letters 454:16–21.
[55] Hoehner, J.C., Wester, T., Pahlman, S., Olsen, L., 1996. Alterations in neurotrophin and neurotrophin-receptor localization in Hirschsprung's disease. Journal of Pediatric Surgery 31:1524–1529.
[56] Guarino, N., Yoneda, A., Shima, H., Puri, P., 2001. Selective neurotrophin deficiency in infantile hypertrophic pyloric stenosis. Journal of Pediatric Surgery 36:1280–1284.

