Role of the central and peripheral BDNF/TrkB axes in metabolic regulation

(中枢・末梢組織の BDNF/TrkB axis による代謝制御の解明)

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Background/Objectives: Brain-derived neurotrophic factor (BDNF) and its receptor (tropomyosin-related kinase B: TrkB, also known as Ntrk2) play a key role in central regulation of the energy balance. BDNF and TrkB are also expressed in peripheral tissues, including adipose tissue, but their peripheral role has been unclear. Here I report on the functional significance of the adipose tissue BDNF/TrkB axis in metabolic homeostasis.

Methods: To examine the role of the BDNF/TrkB axes in central and peripheral tissues, I generated adipocyte-specific or neuron-specific BDNF/TrkB conditional knockout (CKO) mice. Then I compared the feeding behavior and metabolic profile between CKO mice and their littermates.

Results: Expression of *Bdnf/Ntrk2* in adipose tissue showed dramatic changes in obese mice. The first line of adipocyte-specific BDNF/TrkB CKO mice created using *Fabp4*-Cre mice displayed hyperphagia, obesity, and aggressiveness, probably due to ectopic *Fabp4*-Cre mediated gene recombination in brain. In the second line of adipocyte-specific BDNF/TrkB CKO mice, adipose tissue expression of *Ntrk2*, but not *Bdnf*, was reduced by *Adipoq*-Cre mediated gene recombination, indicating that adipocytes only expressed TrkB. When adipocyte-specific TrkB CKO mice were fed a normal diet, no phenotypic changes were detected, while female CKO mice on a high-calorie diet showed a decrease of food intake and resistance to obesity.

Conclusions: The adipose tissue BDNF/TrkB axis has a substantial influence on feeding behavior and obesity in female mice.

Keywords: Neuropeptides, feeding behavior, obesity, adipose tissue

Introduction

Obesity has become a serious health problem because of its growing prevalence, numerous associated diseases, and lack of effective treatment[1]. The main cause of obesity is an imbalance between calorie intake and energy expenditure, resulting in accumulation of body fat. Although regulation of food intake is potentially an effective therapeutic strategy, difficulty in achieving control usually leads to failure. Physiological regulation of the energy balance involves input from both the brain and peripheral tissues. For example, adipose tissue releases molecules that reflect the nutritional state and fat accumulation, such as leptin[2], adiponectin[3], and certain metabolites like fatty acids[4]. These molecules transmit signals to the energy homeostasis center in the hypothalamus, which in turn regulates food intake and energy expenditure in several peripheral tissues. Identifying the complicated interaction between the brain and peripheral tissues involved in regulating food intake may provide new effective therapeutic options for obesity.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic factor family that shows high-affinity binding to its receptor (tropomyosin-related kinase B: TrkB, also known as Ntrk2) and plays a key role in regulating neuronal survival, neuronal differentiation, and synaptic plasticity[5-8]. BDNF and the TrkB receptor are expressed in various brain regions, including energy homeostasis centers within the hypothalamus and the hindbrain in adult animals[9-13]. Consistent with this pattern of expression, BDNF has an essential role in regulating body weight and energy homeostasis. Chronic infusion of BDNF into the cerebral ventricles has been shown to reduce food intake and body weight[14, 15], while heterozygous *Bdnf*-deficient mice display increased locomotor activity, aggressiveness, hyperphagia, and obesity[16, 17]. In addition, mice expressing TrkB at approximately one quarter of the normal level exhibit hyperphagia and obesity[9]. Similarly, mice lacking neuronal *Bdnf* develop obesity and anxiety[18]. In humans, genome-wide

association studies[19-22] and rare cases of genetic variants of *BDNF*[23] and *NTRK2* (ref. 24) have shown an association between the BDNF/TrkB axis and obesity in both adults and children.

A number of studies have demonstrated that BDNF and TrkB are also expressed in non-neural tissues and have an influence on eating behavior and metabolic homeostasis. It was reported that skeletal muscle BDNF is induced by exercise and enhances fat oxidation by muscle[25]. In addition, mice with hepatocyte-specific *Bdnf* deficiency show normal food intake and body weight, but are protected against diet-induced metabolic abnormalities[26]. Furthermore, it has been demonstrated that BDNF-expressing hematopoietic cells regulate feeding behavior and energy balance by migrating from bone marrow to the paraventricular nucleus of the hypothalamus [27]. Moreover, it was reported that BDNF is expressed in adipose tissue [28, 29]. It is well known that humoral factors released by adipose tissue, collectively termed adipokines, participate in the regulation of energy expenditure and glucose/lipid metabolism through various systemic actions[2, 30, 31]. Taken together, these reports suggest the possibility that the BDNF/TrkB axis in adipose tissue may play a role in the regulation of systemic metabolism, but its actual role has been unclear. To test this hypothesis, I investigated adipose tissue expression of BDNF and TrkB in a dietary obesity model and generated various conditional knockout (CKO) mouse models by using the Cre-loxP system. Here I report that the adipose tissue BDNF/TrkB axis has a critical role in regulating metabolic homeostasis.

Methods

Animal Models

All animal care and experimental procedures were approved by the Chiba University review board. Mice were housed in individual cages in a temperature-controlled facility with a 12-h day/night cycle and free access to water. The mice were fed a normal chow diet (CLEA Rodent diet CE-2; CLEA Japan, Inc., Tokyo, Japan) or a high-fat/high-sucrose diet (F2HFHSD, Oriental Yeast, Suita, Japan) from 6 weeks of age. C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Floxed *Bdnf* mice, *Syn1*-Cre mice, and *Fabp4*-Cre mice (with a C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). Generation and genotyping of floxed *Ntrk2* mice (a gift from Luis F. Parada, University of Texas Southwestern Medical Center, Dallas, TX) and *Adipoq*-Cre mice (a gift from Evan D. Rosen, Beth Israel Deaconess Medical Center, Boston, MA) have been described previously[32, 33].

The genotypes of the mice and Cre-mediated recombination were assessed by PCR using genomic DNA harvested from tail tips and various other tissues of mutant mice. To examine tissue-specific deletion of BDNF or TrkB in CKO mice, I analyzed male mice homozygous for the floxed allele with one copy of the Cre recombinase transgene (CKO), littermate mice homozygous for the floxed allele without the Cre recombinase transgene (littermate controls), and wild-type C57BL/6 mice using the following primers: Cre recombinase, 5’-GTTCGCAAGAACCTGATGGACA-3’ and 5’-CTAGAGCCTGTTTTGCACGTTC-3’; wild-type or floxed *Bdnf* allele, 5'-TGTGATTGTGTTTCTGGTGAC-3’, 5’-GATACATCATGGGCAGTGGA-3’; wild-type or floxed *Ntrk2* allele, 5’-ATGTACTCGTTCTACAAATCCTGC-3’, 5’-TCCAGACACATACACGTGCGTGC-3’, and 5’-CAAGAAGTCAGAGACCAGAGAGA-3’.

RNA analysis

Total RNA was extracted from adipose tissue using an RNeasy Plus Universal kit (Qiagen) and was extracted from other tissues with RNA-Bee (Molecular Research Center). Then reverse transcription was performed using a QuantiTect reverse transcription kit (Qiagen), and real-time PCR was done with a LightCycler (Roche), the Taqman Universal Probe Library, and Light Cycler Master (Roche) according to the manufacturer's instructions. For normalization of gene expression, *36B4* mRNA was measured as the internal control. Data were analyzed by the 2-1ΔΔCT method. The following primers were employed: *36B4*, forward GATGCCCAGGGAAGACAG, reverse ACAATGAAGCATTTTGGATAATCA; *Bdnf*, forward GCCTTTGGAGCCTCCTCTAC, reverse GCGGCATCCAGGTAATTTT; *Ntrk2*, forward CGAACCTGCAGATACCCAAT, reverse TGCAGGAAAGGGTCACAGA; *Ntrk2-tk*, forward TTCTGCCTGCTGGTGATGT, reverse TCCAGTGGGATCTTATGAAACA.

Measurement of body weight and food intake

Body weight was measured every two weeks. Food intake was assessed by housing mice individually. The animals were fed a normal diet or a high-calorie diet ad libitum, and the amount of residual food was weighed for a total of 7 days. In pair-feeding experiments, the same amount of a high-calorie diet was given to CKO mice and their littermates each day.

Assessment of locomotor activity

Locomotor activity was assessed by using an implantable intra-abdominal radiofrequency probe and receiver (TA10TA-F20 and RPC-1, Data Sciences International) (Barber et al., 2004). Mice (14-16 weeks old) were placed individually into cages and baseline locomotor

activity was monitored. Data were sampled in the continuous mode and analyzed by Dataquest ART2.1.

CT scanning

The adipose tissue of mice was examined by CT (LaTheta, ALOCA) according to the manufacturer's protocol. I obtained CT scans at 2 mm intervals from diaphragm to the floor of the pelvic cavity.

Laboratory Tests

In the glucose tolerance test, mice were fasted for 16 hours and D-glucose (2 g kg^{-1} body weight) was administered by intraperitoneal injection at 3:00 p.m. To perform the insulin tolerance test, mice were given human insulin intraperitoneally (1 U kg^{-1} body weight) at 3:00 p.m. without fasting. Blood was collected from the tail vein at 0, 15, 30, 60, and 120 minutes after administration of glucose or insulin. Then blood glucose concentrations were measured with a Glutest Mint (Sanwa Kagaku Kenkyusho, Nagoya, Japan), while plasma insulin levels were measured by immunoassay (Morinaga).

Statistical Analysis

Results are shown as the mean ± SEM. Differences between groups were examined by Student's t-test for comparison of mean values. In all analyses, $p < 0.05$ was considered statistically significant.

Results

Expression of *Bdnf* and *Ntrk2* in peripheral tissues

I first examined the expression of *Bdnf* and *Ntrk2* in various tissues of mice fed a normal diet. *Bdnf* was highly expressed in the brain, whereas intermediate expression was found in the heart, lung, and kidney (Fig.1A). *Bdnf* was also expressed in adipose tissue, although its expression was much lower than in neural tissues. *Ntrk2* (the gene coding for TrkB) was also expressed by adipose tissue at the highest level among the peripheral tissues that I examined (Fig. 1B). To investigate the role of the adipose tissue BDNF/TrkB axis in obesity, I examined *Bdnf* and *Ntrk2* expression in mice with dietary obesity fed a high-fat/high-sucrose (HFHS) diet. I found that *Bdnf* expression was markedly up-regulated in the adipose tissue of these mice (Fig. 1C), while no significant change of *Bdnf* expression was observed in other peripheral tissues (data not shown). Unlike *Bdnf*, expression of *Ntrk2* was significantly down-regulated in the adipose tissue of obese mice (Fig. 1D). Based on these findings and previous observations[28, 29], I speculated that the adipose tissue BDNF/TrkB axis might play a role in regulating metabolic homeostasis.

Mice with adipocyte-specific *Bdnf* or *Ntrk2* deficiency exhibit obesity and hyperphagia

To study the role of the BDNF/TrkB axis in adipose tissue, I generated mice lacking *Bdnf* or *Ntrk2* in adipocytes by breeding $Bdnf^{flox/flox}$ or $Ntrk2^{flox/flox}$ mice with *Fabp4*-Cre mice (Fabp4-BDNF CKO mice and Fabp4-TrkB CKO mice, respectively). Both lines of mice exhibited age-dependent obesity (Fig. 2A, 2B), hyperphagia (Fig. 2C, 2D), and aggressiveness (data not shown). These phenotypic changes suggested that the CKO mice had abnormalities of the central nervous system. Although Fabp4 was originally identified as an adipocyte-specific protein, recent studies have shown that it is also expressed by other types of cells, including neurons[34]. Since BDNF acts on the hypothalamus to regulate

energy homeostasis[9, 13, 15], I investigated the specificity of *Fabp4*-Cre-mediated gene recombination. Genomic PCR detected the excised allele of *Bdnf*$^{flox/flox}$ mice in the hypothalamus as well as in adipose tissue of Fabp4-BDNF CKO mice (Fig. 2E). Likewise, the excised allele of *Ntrk2*$^{flox/flox}$ mice was observed in the hypothalamus of Fabp4-TrkB CKO mice (Fig. 2F). Adipose tissue expression of both *Bdnf* and *Ntrk2* was markedly reduced in these CKO mice (Fig. 2G, 2H). Consistent with the genomic PCR data, hypothalamic expression of *Ntrk2* was down-regulated in Fabp4-TrkB CKO mice (Fig. 2I), indicating that *Fabp4*-Cre-mediated gene recombination in the hypothalamus could affect the metabolic phenotype of Fabp4-TrkB CKO mice.

Neuronal *Bdnf* deficiency causes a similar phenotype to those of Fabp4-BDNF/TrkB CKO mice

To examine whether *Fabp4*-Cre mediated recombination in the brain accounted for the obese phenotype of Fabp4-BDNF/TrkB CKO mice, I next generated mice lacking neuronal *Bdnf* by breeding *Bdnf*$^{flox/flox}$ mice with *Syn1*-Cre mice (Syn1-BDNF CKO mice). I confirmed that *Syn1*-Cre mediated recombination was only detected in the nervous system by genomic PCR (Fig. 3A), in accordance with a previous report[35]. *Bdnf* expression was exclusively decreased in the brains of Syn1-BDNF CKO mice (Fig. 3B). Syn1-BDNF CKO mice exhibited a higher body weight compared with their littermate controls (Fig. 3C), which was associated with hyperphagia (Fig. 3D) and increased locomotor activity (Fig. 3E). These results suggest that *Fabp4*-Cre mediated gene recombination in the brain affects the metabolic phenotype of Fabp4-BDNF/TrkB CKO mice.

Adipocyte-specific *Bdnf/Ntrk2* deletion does not cause obesity in *Adipoq*-Cre mice

To eliminate the possibility of genetic recombination occurring in the brain, I generated a

second line of adipocyte-specific Bdnf/Ntrk2 CKO mice by breeding *Bdnf*$^{flox/flox}$ or *Ntrk2*$^{flox/flox}$ mice with *Adipoq*-Cre mice (Adipoq-BDNF CKO mice and Adipoq-TrkB CKO mice, respectively). It has been reported that Cre expression driven by the *Adipoq* promoter leads to recombination exclusively affecting adipocytes[33, 36], and I confirmed that Adipoq-TrkB CKO mice exhibited adipose tissue-specific recombination (Fig. 4A) and that *Ntrk2* expression was specifically deleted from adipose tissue (Fig. 4B). Although Adipoq-BDNF CKO mice revealed adipose tissue-specific recombination (Fig. 4C), *Bdnf* expression was unexpectedly not suppressed in adipose tissue (Fig. 4D), suggesting that adipocytes do not contribute to its expression by adipose tissue. In contrast, the pattern of expression in Adipoq-TrkB CKO mice supported the concept that *Ntrk2* is predominantly expressed by adipocytes in fatty tissues.

I then focused on TrkB in adipose tissue to investigate the role of the BDNF/TrkB axis in metabolism. Since decreased adipose tissue expression of TrkB was associated with obesity (Fig. 1E–F), I hypothesized that BDNF might positively regulate energy metabolism by acting on the adipocyte TrkB. I monitored physiological parameters in male and female mice on a normal diet, revealing that Adipoq-TrkB CKO mice and their littermate controls showed no differences of body weight (Fig. 5A, 5B), fat mass (Fig. 5C, 5D), food intake (Fig. 5E, 5F), or locomotor activity (Fig. 5G, 5H). Consistent with the results for *Bdnf* expression, Adipoq-Bdnf CKO mice did not display any phenotypic changes (data not shown). To assess the role of the adipose tissue BDNF/TrkB axis in glucose homeostasis, I performed a glucose tolerance test and an insulin tolerance test, and I measured plasma levels of insulin during the glucose tolerance test. Normal glucose metabolism was observed in both sexes (Fig. 5I–L). Taken together, these results suggest that adipose tissue *Ntrk2* only has a minor role in regulating eating behavior and metabolism in mice receiving a normal diet.

Adipoq-TrkB CKO female mice on a high-calorie diet show decreased food intake and resistance to obesity

I next examined the effect of a high-calorie diet on the metabolic phenotype of Adipoq-TrkB CKO mice. I found that female, but not male, Adipoq-TrkB CKO mice fed a high-calorie diet had a lower body weight (Fig. 6A, 6B) and less accumulation of adipose tissue (Fig. 6C, 6D) than their littermate controls. Adipoq-TrkB CKO female mice (but not male mice) showed a decrease of food intake (Fig. 6E, 6F), suggesting that TrkB deficiency in adipose tissue leads to weight loss due to lower calorie intake. To test this hypothesis, I next performed a pair-feeding experiment. This showed that providing equal food intake diminished the difference of body weight between female Adipoq-TrkB CKO mice and their littermate controls (Fig. 6G), suggesting that TrkB may have a role in regulating food intake. In Adipoq-TrkB CKO mice, I assessed glucose metabolism at the age of 12–14 weeks and at 24–26 weeks. There were no differences of fat mass, glucose tolerance, insulin tolerance, and the plasma insulin level between Adipoq-TrkB CKO mice and their littermate controls at 12–14 weeks of age (Fig. 6H–6L). In contrast, Adipoq-TrkB CKO female mice aged 24–26 weeks exhibited improvement of glucose tolerance and had lower insulin levels (Fig. 6M, 6N), presumably secondary to a decrease of fat mass. Collectively, these results indicate that TrkB deficiency in adipose tissue leads to decreased food intake and weight gain in female mice on a high-calorie diet, which in turn improves glucose metabolism.

Discussion

In the present study, I investigated the pathophysiological role of BDNF/TrkB signaling in peripheral tissues. I found significant changes of *Bdnf* and *Ntrk2* expression in the adipose tissue of mice with dietary obesity, with *Bdnf* being up-regulated and *Ntrk2* being down-regulated. The results of adipocyte-specific CKO models have indicated that *Ntrk2* is predominantly expressed by adipocytes, while *Bdnf* is mainly expressed by other cells. Adipocyte-specific deletion of *Ntrk2* in female mice, but not male mice, receiving a high-calorie diet led to reduced food intake and less weight gain than in littermate controls due to a decrease of fat mass, indicating a potential role of adipose tissue BDNF/TrkB signaling in regulating both appetite and fat storage.

Deletion of TrkB mediated by *Adipoq*-Cre led to changes of eating behavior without any alteration of neuronal *Ntrk2* expression. Since it is known that food intake is regulated by the hypothalamic feeding center[37], my results suggested that the adipose tissue BDNF/TrkB axis remotely regulates feeding behavior in obese mice. It is noteworthy that the hypothalamic BDNF/TrkB axis negatively regulates the appetite in contrast to positive regulation by the adipose tissue BDNF/TrkB axis. The impact of the adipose tissue BDNF/TrkB axis on feeding behavior appears to be relatively small, since deletion of the hypothalamic and adipose BDNF/TrkB axes by *Fabp4*-Cre mediated recombination led to an increase of body weight with hyperphagia. It has been reported that adipose tissue regulates feeding behavior by secretion of adipokines[2, 3] or by releasing metabolites such as fatty acids[4]. Thus, regulation of metabolism by the adipose tissue BDNF/TrkB axis could involve such adipokines and metabolites, but the molecular mechanisms remain to be determined.

It is unclear why deletion of adipose tissue TrkB had different effects on the feeding behavior of male and female mice. Estrogen regulates the expression of BDNF and

TrkB and has functions that overlap those of BDNF in the central nervous system[38]. In addition, the plasma BDNF level differs between men and women[39-41] and is positively correlated with the estrogen level in female subjects[42]. These data suggest that there are gender differences in the biological activity of the BDNF/TrkB axis. It is widely accepted that estrogen regulates food intake and adiposity via the estrogen receptor[43]. Because adipocytes express the estrogen receptor[44], it may mediate the effects of estrogen on food intake and adiposity[45, 46]. My results also raise the possibility that interaction of the BDNF/TrkB axis and estrogen on adipocytes, as well as in the brain, may contribute to the regulation of appetite and fat accumulation.

It has been reported that BDNF is expressed by adipocytes[29] and might influence energy metabolism as one of the adipokines[47]. Although I confirmed that the expression of *Bdnf* was increased in the adipose tissue of obese mice, I did not detect a significant decrease of *Bdnf* expression in the adipose tissue of Adipoq-BDNF CKO mice. There is evidence that Cre expression driven by the *Fabp4* promoter leads to recombination in macrophages as well as in adipocytes[48], and it has been reported that macrophages express *Bdnf*[49], suggesting that it may be mainly expressed by macrophages in adipose tissue. Accordingly, it is possible that an increase of infiltrating macrophages accounts for up-regulation of *Bdnf* expression in the adipose tissue of obese mice.

In summary, I found that the expression of BDNF/TrkB in adipose tissue was altered by a high-calorie diet. In contrast to the known role of the BDNF/TrkB axis in the central nervous system, deletion of TrkB from adipocytes led to decreased food intake and fat accumulation in mice fed a high-calorie diet, thereby improving various metabolic abnormalities associated with obesity. Thus, inhibition of the peripheral BDNF/TrkB axis may be a potential strategy for the treatment of dietary metabolic abnormalities.

References

1. Kopelman PG. Obesity as a medical problem. *Nature*. 2000; 404: 635-643.
2. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature*. 1998; 395: 763-770.
3. Kubota N, Yano W, Kubota T, Yamauchi T, Itoh S, Kumagai H, et al. Adiponectin stimulates amp-activated protein kinase in the hypothalamus and increases food intake. *Cell Metab*. 2007; 6: 55-68.
4. Pocai A, Lam TK, Obici S, Gutierrez-Juarez R, Muse ED, Arduini A, et al. Restoration of hypothalamic lipid sensing normalizes energy and glucose homeostasis in overfed rats. *J Clin Invest*. 2006; 116: 1081-1091.
5. Knusel B, Winslow JW, Rosenthal A, Burton LE, Seid DP, Nikolics K, et al. Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. *Proc Natl Acad Sci U S A*. 1991; 88: 961-965.
6. Wright EM, Vogel KS, Davies AM. Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. *Neuron*. 1992; 9: 139-150.
7. Cowansage KK, LeDoux JE, Monfils MH. Brain-derived neurotrophic factor: A dynamic gatekeeper of neural plasticity. *Curr Mol Pharmacol*. 2010; 3: 12-29.
8. Yoshii A, Constantine-Paton M. Postsynaptic bdnf-trkb signaling in synapse maturation, plasticity, and disease. *Dev Neurobiol*. 2010; 70: 304-322.
9. Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, et al. Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. *Nat Neurosci*. 2003; 6: 736-742.
10. Bariohay B, Lebrun B, Moyse E, Jean A. Brain-derived neurotrophic factor plays a role as an anorexigenic factor in the dorsal vagal complex. *Endocrinology*. 2005; 146: 5612-5620.
11. Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S. Distribution of brain-derived neurotrophic factor (bdnf) protein and mrna in the normal adult rat cns: Evidence for anterograde axonal transport. *J Neurosci*. 1997; 17: 2295-2313.
12. Yan Q, Radeke MJ, Matheson CR, Talvenheimo J, Welcher AA, Feinstein SC. Immunocytochemical localization of trkb in the central nervous system of the adult rat. *J Comp Neurol*. 1997; 378: 135-157.
13. Unger TJ, Calderon GA, Bradley LC, Sena-Esteves M, Rios M. Selective deletion of bdnf

in the ventromedial and dorsomedial hypothalamus of adult mice results in hyperphagic behavior and obesity. *J Neurosci*. 2007; 27: 14265-14274.

14. Lapchak PA, Hefti F. Bdnf and ngf treatment in lesioned rats: Effects on cholinergic function and weight gain. *Neuroreport*. 1992; 3: 405-408.
15. Toriya M, Maekawa F, Maejima Y, Onaka T, Fujiwara K, Nakagawa T, et al. Long-term infusion of brain-derived neurotrophic factor reduces food intake and body weight via a corticotrophin-releasing hormone pathway in the paraventricular nucleus of the hypothalamus. *J Neuroendocrinol*. 2010; 22: 987-995.
16. Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, et al. Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proc Natl Acad Sci U S A*. 1999; 96: 15239-15244.
17. Kernie SG, Liebl DJ, Parada LF. Bdnf regulates eating behavior and locomotor activity in mice. *Embo j*. 2000; 19: 1290-1300.
18. Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, et al. Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol Endocrinol*. 2001; 15: 1748-1757.
19. Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadottir A, et al. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. *Nat Genet*. 2009; 41: 18-24.
20. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nat Genet*. 2010; 42: 937-948.
21. Okada Y, Kubo M, Ohmiya H, Takahashi A, Kumasaka N, Hosono N, et al. Common variants at cdkal1 and klf9 are associated with body mass index in east asian populations. *Nat Genet*. 2012; 44: 302-306.
22. Wen W, Cho YS, Zheng W, Dorajoo R, Kato N, Qi L, et al. Meta-analysis identifies common variants associated with body mass index in east asians. *Nat Genet*. 2012; 44: 307-311.
23. Han JC, Liu QR, Jones M, Levinn RL, Menzie CM, Jefferson-George KS, et al. Brain-derived neurotrophic factor and obesity in the wagr syndrome. *N Engl J Med*. 2008; 359: 918-927.
24. Yeo GS, Connie Hung CC, Rochford J, Keogh J, Gray J, Sivaramakrishnan S, et al. A de

novo mutation affecting human trkb associated with severe obesity and developmental delay. *Nat Neurosci*. 2004; 7: 1187-1189.

25. Matthews VB, Astrom MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek O, et al. Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of amp-activated protein kinase. *Diabetologia*. 2009; 52: 1409-1418.
26. Teillon S, Calderon GA, Rios M. Diminished diet-induced hyperglycemia and dyslipidemia and enhanced expression of pparalpha and fgf21 in mice with hepatic ablation of brain-derived neurotropic factor. *J Endocrinol*. 2010; 205: 37-47.
27. Urabe H, Kojima H, Chan L, Terashima T, Ogawa N, Katagi M, et al. Haematopoietic cells produce bdnf and regulate appetite upon migration to the hypothalamus. *Nat Commun*. 2013; 4: 1526.
28. Hausman GJ, Poulos SP, Richardson RL, Barb CR, Andacht T, Kirk HC, et al. Secreted proteins and genes in fetal and neonatal pig adipose tissue and stromal-vascular cells. *J Anim Sci*. 2006; 84: 1666-1681.
29. Bernhard F, Landgraf K, Kloting N, Berthold A, Buttner P, Friebe D, et al. Functional relevance of genes implicated by obesity genome-wide association study signals for human adipocyte biology. *Diabetologia*. 2013; 56: 311-322.
30. Havel PJ. Update on adipocyte hormones: Regulation of energy balance and carbohydrate/lipid metabolism. *Diabetes*. 2004; 53 Suppl 1: S143-151.
31. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating amp-activated protein kinase. *Nat Med*. 2002; 8: 1288-1295.
32. Luikart BW, Nef S, Shipman T, Parada LF. In vivo role of truncated trkb receptors during sensory ganglion neurogenesis. *Neuroscience*. 2003; 117: 847-858.
33. Eguchi J, Wang X, Yu S, Kershaw EE, Chiu PC, Dushay J, et al. Transcriptional control of adipose lipid handling by irf4. *Cell Metab*. 2011; 13: 249-259.
34. Martens K, Bottelbergs A, Baes M. Ectopic recombination in the central and peripheral nervous system by ap2/fabp4-cre mice: Implications for metabolism research. *FEBS Lett*. 2010; 584: 1054-1058.
35. Zhu Y, Romero MI, Ghosh P, Ye Z, Charnay P, Rushing EJ, et al. Ablation of nf1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. *Genes Dev*. 2001; 15: 859-876.

36. Lee KY, Russell SJ, Ussar S, Boucher J, Vernochet C, Mori MA, et al. Lessons on conditional gene targeting in mouse adipose tissue. *Diabetes*. 2013; 62: 864-874.
37. Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature*. 2000; 404: 661-671.
38. Sohrabji F, Lewis DK. Estrogen-bdnf interactions: Implications for neurodegenerative diseases. *Front Neuroendocrinol*. 2006; 27: 404-414.
39. Lommatzsch M, Zingler D, Schuhbaeck K, Schloetcke K, Zingler C, Schuff-Werner P, et al. The impact of age, weight and gender on bdnf levels in human platelets and plasma. *Neurobiol Aging*. 2005; 26: 115-123.
40. Komulainen P, Pedersen M, Hanninen T, Bruunsgaard H, Lakka TA, Kivipelto M, et al. Bdnf is a novel marker of cognitive function in ageing women: The dr's extra study. *Neurobiol Learn Mem*. 2008; 90: 596-603.
41. Golden E, Emiliano A, Maudsley S, Windham BG, Carlson OD, Egan JM, et al. Circulating brain-derived neurotrophic factor and indices of metabolic and cardiovascular health: Data from the baltimore longitudinal study of aging. *PLoS One*. 2010; 5: e10099.
42. Begliuomini S, Casarosa E, Pluchino N, Lenzi E, Centofanti M, Freschi L, et al. Influence of endogenous and exogenous sex hormones on plasma brain-derived neurotrophic factor. *Hum Reprod*. 2007; 22: 995-1002.
43. Brown LM, Clegg DJ. Central effects of estradiol in the regulation of food intake, body weight, and adiposity. *J Steroid Biochem Mol Biol*. 2010; 122: 65-73.
44. Mayes JS, Watson GH. Direct effects of sex steroid hormones on adipose tissues and obesity. *Obes Rev*. 2004; 5: 197-216.
45. Tanaka M, Nakaya S, Kumai T, Watanabe M, Tateishi T, Shimizu H, et al. Effects of estrogen on serum leptin levels and leptin mrna expression in adipose tissue in rats. *Horm Res*. 2001; 56: 98-104.
46. Davis KE, M DN, Sun K, W MS, J DB, J AZ, et al. The sexually dimorphic role of adipose and adipocyte estrogen receptors in modulating adipose tissue expansion, inflammation, and fibrosis. *Mol Metab*. 2013; 2: 227-242.
47. Sornelli F, Fiore M, Chaldakov GN, Aloe L. Adipose tissue-derived nerve growth factor and brain-derived neurotrophic factor: Results from experimental stress and diabetes. *Gen Physiol Biophys*. 2009; 28 Spec No: 179-183.
48. Fu Y, Luo N, Lopes-Virella MF. Oxidized ldl induces the expression of albp/ap2 mrna

and protein in human thp-1 macrophages. *J Lipid Res*. 2000; 41: 2017-2023.

49. Barouch R, Appel E, Kazimirsky G, Brodie C. Macrophages express neurotrophins and neurotrophin receptors. Regulation of nitric oxide production by nt-3. *J Neuroimmunol*. 2001; 112: 72-77.

Figure legends

Figure 1. Expression of *Bdnf* and *Ntrk2* in peripheral tissues.

(**A, B**) Relative expression of *Bdnf* (A) and *Ntrk2* (B) in mouse tissues was determined by real-time PCR. Expression in various tissues is shown relative to that in the cerebral cortex. n = 5. EWAT, epididymal white adipose tissue; BAT, brown adipose tissue.

(**C, D**) Expression of *Bdnf* (C) and *Ntrk2* (D) in epididymal white adipose tissue (EWAT), perinephric white adipose tissue (RWAT), and inguinal white adipose tissue (IWAT) assessed by real-time PCR in 16-week-old mice fed normal chow (NC) or a high-fat/high-sucrose (HFHS) diet. n = 9–10 for EWAT, n = 4 for RWAT, and n = 4 for IWAT. *p < 0.05, ***p < 0.001. Data are shown as the mean ± SEM.

Figure 2. Obesity and hyperphagia in mice with adipocyte-specific deficiency of *Bdnf* or *Ntrk2* developed using *Fabp4*-Cre mice.

(**A, B**) Body weight of Fabp4-BDNF CKO mice (A) and Fabp4-TrkB CKO mice (B) compared with their littermate controls (Control). n = 3 for A, n = 7–11 for B. *p < 0.05, **p < 0.01, ***p < 0.001.

(**C, D**) Food intake of Fabp4-BDNF CKO mice (C) and Fabp4-TrkB CKO mice (D) compared with their littermate controls (Control) at 8–12 weeks of age. n = 4–6 for C, n = 6–8 for D. *p < 0.05, ***p < 0.001.

(**E, F**) PCR analysis of genomic DNA isolated from the hypothalamus, epididymal white adipose tissue (EWAT), and brown adipose tissue (BAT) of Fabp4-BDNF CKO mice (E) and Fabp4-TrkB CKO mice (F), and from EWAT of their littermate controls and wild-type mice. Littermate controls were homozygous for the floxed *Bdnf* (E) or *Ntrk2* (F) allele, but did not carry Cre recombinase.

(**G**) Real-time PCR analysis of *Bdnf* expression by epididymal white adipose tissue

(EWAT) in Fabp4-BDNF CKO mice and their littermate controls (Control). n = 3. *p < 0.05.

(**H, I**) Real-time PCR analysis of *Ntrk2* expression in epididymal white adipose tissue (EWAT) (H) and the hypothalamus (I) of Fabp4-TrkB CKO mice and their littermate controls (Control). n = 3–4. *p < 0.05, ***p < 0.001. Data are shown as the mean ± SEM.

Figure 3. Neuronal Bdnf deficiency leads to similar phenotypes to those of Fabp4-BDNF/TrkB CKO mice.

(**A**) PCR analysis of genomic DNA isolated from various tissues of Syn1-BDNF CKO mice and from the cerebral cortex of their littermate controls and wild-type mice. Littermates were homozygous for the floxed *Bdnf* allele, but did not carry Cre recombinase.

(**B**) Real-time PCR analysis of *Bdnf* expression in various tissues of Syn1-BDNF CKO mice and their littermate controls (Control). n = 5. ***p < 0.001.

(**C**) Body weight of Syn1-BDNF CKO mice and their littermate controls (Control). n = 22–25. *p < 0.05.

(**D**) Food intake of Syn1-BDNF CKO mice and their littermate controls (Control) at 8–12 weeks of age. n = 12–14. *p < 0.05.

(**E**) Locomotor activity of Syn1-BDNF CKO mice and littermate controls (Control) at 14–16 weeks of age. n = 8. *p < 0.05. Data are shown as the mean ± SEM.

Figure 4. Genomic recombination and gene expression in adipocyte-specific Bdnf/Ntrk2 deletion models created using *Adipoq*-Cre mice.

(**A**) PCR analysis of genomic DNA isolated from various tissues of Adipoq-TrkB CKO mice and from epididymal white adipose tissue (EWAT) of their littermate controls and wild-type mice. Littermate controls were homozygous for the floxed *Ntrk2* allele, but did

not carry Cre recombinase.

(**B**) Real-time PCR analysis of *Ntrk2* expression in various tissues of Adipoq-TrkB CKO mice and their littermate controls (Control). n = 5. **p < 0.01, ***p < 0.001.

(**C**) PCR analysis of genomic DNA isolated from various tissues of Adipoq-BDNF CKO mice and from epididymal white adipose tissue (EWAT) of their littermate controls and wild-type mice. Littermate controls were homozygous for the floxed *Bdnf* allele, but did not carry Cre recombinase.

(**D**) Real-time PCR analysis of *Bdnf* expression in various tissues of Adipoq-BDNF CKO mice and their littermate controls (Control). n = 5. Data are shown as the mean ± SEM.

Figure 5. Adipocyte-specific deletion of Ntrk2 does not cause obesity.

(A, B) Body weight of Adipoq-TrkB CKO male mice (A) and female mice (B) receiving a normal diet compared with their littermate controls (Control). n = 11–12 for A, n = 10–11 for B.

(C, D) CT analysis of Adipoq-TrkB CKO male mice (C) and female mice (D) receiving a normal diet compared with their littermate controls (Control) at 12–14 weeks of age. The percent fat tissue/body weight ratio is shown for visceral fat and subcutaneous fat. n = 6 for C, n = 9–10 for D.

(E, F) Food intake of Adipoq-TrkB CKO male mice (E) and female mice (F) receiving a normal diet compared with their littermate controls (Control) at 8–12 weeks of age. n = 9–10 for E, n = 9–10 for F.

(G, H) Locomotor activity of Adipoq-TrkB CKO male mice (G) and female mice (H) receiving a normal diet compared with their littermate controls (Control) at 14–16 weeks of age. n = 6 for G, n = 6 for H.

(I) Glucose tolerance test (GTT) and insulin tolerance test (ITT) in Adipoq-TrkB CKO male

mice receiving a normal diet compared with their littermate controls (Control) at 12–14 weeks of age. n = 9–13 for GTT, n = 10–13 for ITT.

(J) Plasma insulin level during the GTT shown in Figure 5I. n = 9–10.

(K) Glucose tolerance test (GTT) and insulin tolerance test (ITT) in Adipoq-TrkB CKO female mice receiving a normal diet compared with their littermate controls (Control) at 12–14 weeks of age. n = 15–16 for GTT, n = 7–10 for ITT.

(L) Plasma insulin level during the GTT shown in Figure 5K. n = 7–10. Data are shown as the mean ± SEM.

Figure 6. Adipoq-TrkB CKO female mice on a high-calorie diet show decreased food intake and resistance to obesity.

(A, B) Body weight of Adipoq-TrkB CKO male mice (A) and female mice (B) receiving a high-fat/high-sucrose (HFHS) diet compared with their littermate controls (Control). n = 14−16 for A, n = 26—33 for B. *$p < 0.05$, **$p < 0.01$.

(C, D) CT analysis of Adipoq-TrkB CKO male mice (C) and female mice (D) receiving the HFHS diet compared with their littermate controls (Control) at 24 weeks of age. The percent fat tissue/body weight ratio is shown for visceral fat and subcutaneous fat. n = 5 for C, n = 7 for D. *$p < 0.05$.

(E, F) Food intake of Adipoq-TrkB CKO male mice (F) and female mice (G) receiving the HFHS diet compared with their littermate controls (Control) at 14 weeks of age. n = 10–13 for F, n = 14–16 for G. *$p < 0.05$.

(G) Pair-feeding experiments in Adipoq-TrkB CKO female mice receiving the HFHS diet compared with their littermate controls (Control). n = 6.

(H) CT analysis of Adipoq-TrkB CKO female mice receiving the HFHS diet compared with their littermate controls (Control) at 12–14 weeks of age. The percent fat tissue/body

weight ratio is shown for visceral fat and subcutaneous fat. n = 7.

(I) Glucose tolerance test (GTT) and insulin tolerance test (ITT) in Adipoq-TrkB CKO male mice receiving the HFHS diet compared with their littermate controls (Control) at 12–14 weeks of age. n = 17–18 for GTT, n = 15–18 for ITT.

(J) Plasma insulin level during the GTT shown in Figure 6I. n = 12–15.

(K) Glucose tolerance test (GTT) and insulin tolerance test (ITT) in Adipoq-TrkB CKO male mice receiving the HFHS diet compared with their littermate controls (Control) at 12–14 weeks of age. n = 10–13 for GTT, n = 10 for ITT.

(L) Plasma insulin level during the GTT shown in Figure 6K. n = 14–15.

(M) Glucose tolerance test (GTT) and insulin tolerance test (ITT) in Adipoq-TrkB CKO male mice receiving the HFHS diet compared with their littermate controls (Control) at 24–26 weeks of age. (GTT: n = 14–15 for GTT, n = 12–13 for ITT. *$p < 0.05$.

(N) Plasma insulin level during the GTT shown in Figure 6M. n = 10. *$p < 0.05$. Data are shown as the mean ± SEM.

Figure 1

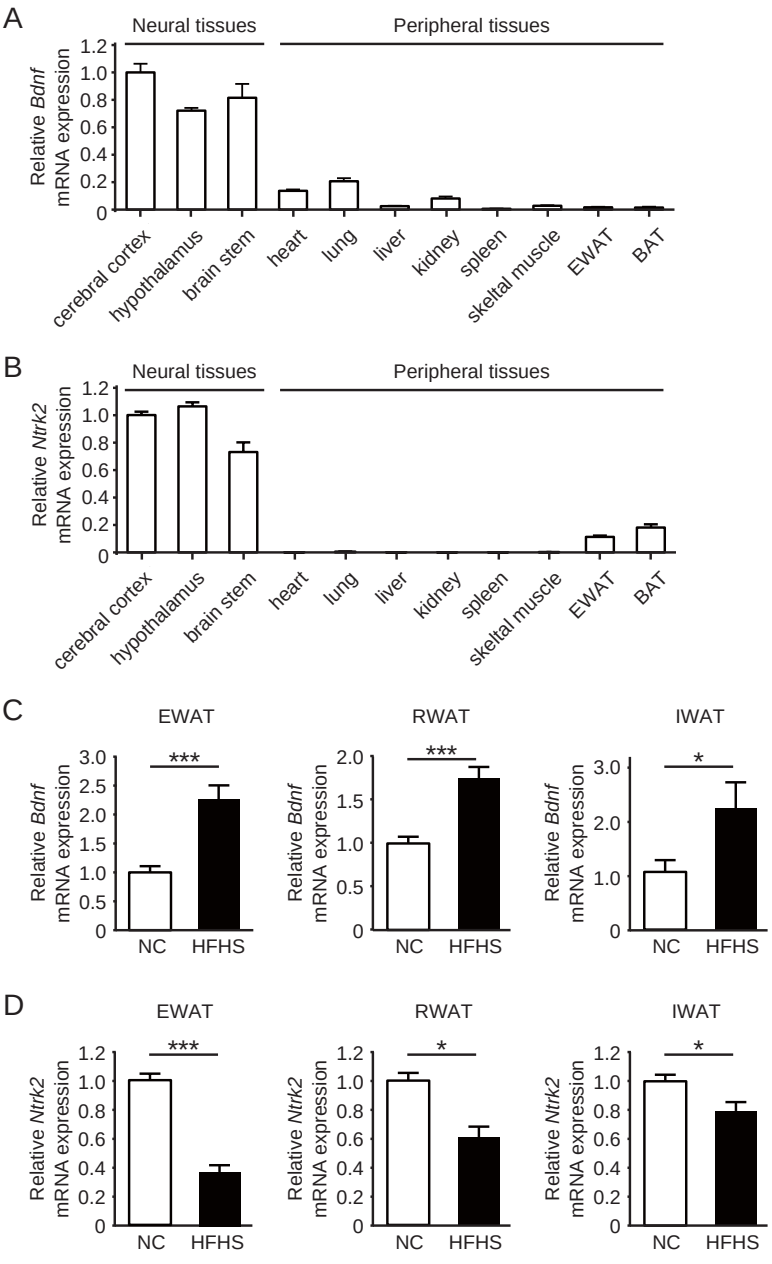
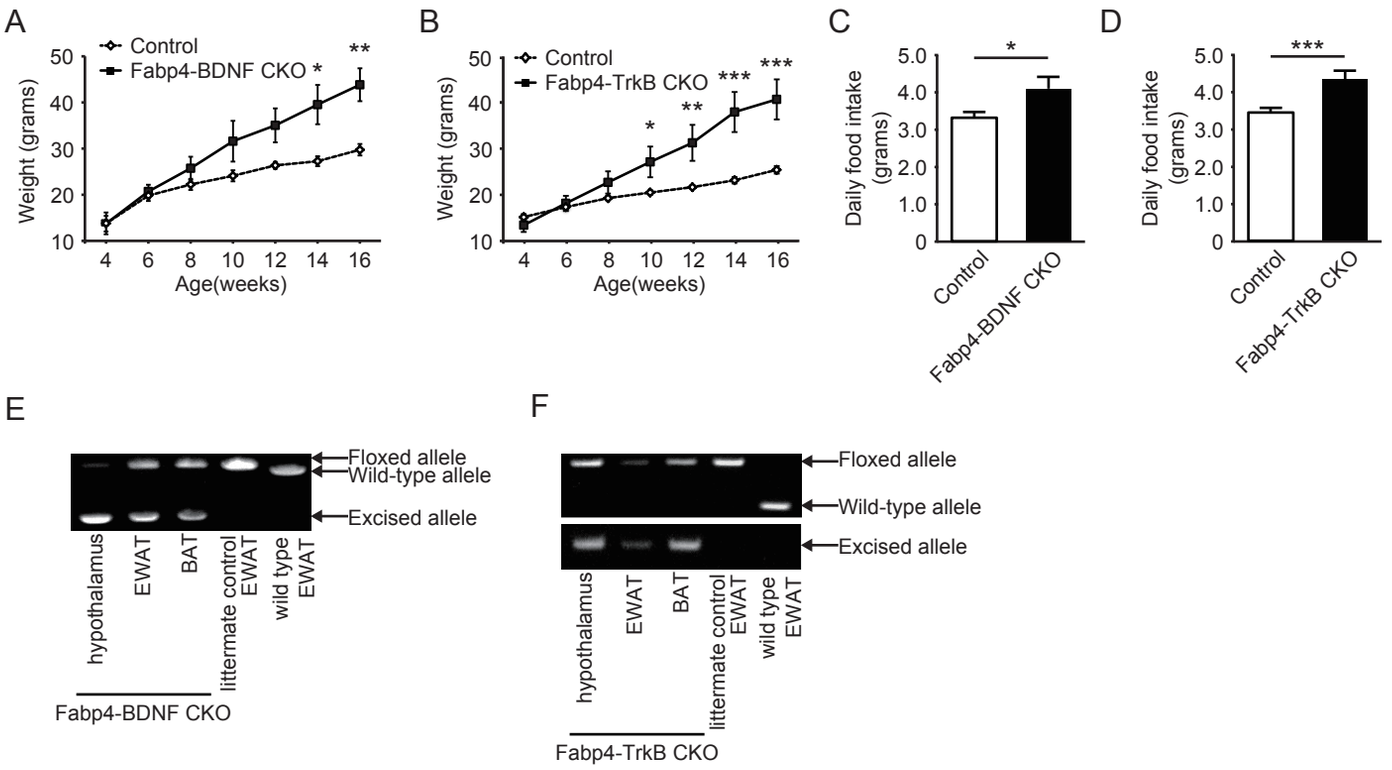

A
Neural tissues
Peripheral tissues
Relative Bdnf mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
cerebral cortex
hypothalamus
brain stem
heart
lung
liver
kidney
spleen
skeltal muscle
EWAT
BAT
B
Neural tissues
Peripheral tissues
Relative Ntrk2 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
cerebral cortex
hypothalamus
brain stem
heart
lung
liver
kidney
spleen
skeltal muscle
EWAT
BAT
C
EWAT
RWAT
IWAT
Relative Bdnf mRNA expression
***
***
*
3.0
2.5
2.0
1.5
1.0
0.5
0
2.0
1.5
1.0
0.5
0
3.0
2.0
1.0
0
NC
HFHS
NC
HFHS
NC
HFHS
D
EWAT
RWAT
IWAT
Relative Ntrk2 mRNA expression
***
*
*
1.2
1.0
0.8
0.6
0.4
0.2
0
1.2
1.0
0.8
0.6
0.4
0.2
0
1.2
1.0
0.8
0.6
0.4
0.2
0
NC
HFHS
NC
HFHS
NC
HFHS

Figure2


A
Control
Fabp4-BDNF CKO
Weight (grams)
Age(weeks)
B
Control
Fabp4-TrkB CKO
Weight (grams)
Age(weeks)
C
Daily food intake (grams)
Control
Fabp4-BDNF CKO
D
Daily food intake (grams)
Control
Fabp4-TrkB CKO

E
Floxed allele
Wild-type allele
Excised allele
hypothalamus
EWAT
BAT
littermate control EWAT
wild type EWAT
Fabp4-BDNF CKO
F
Floxed allele
Wild-type allele
Excised allele
hypothalamus
EWAT
BAT
littermate control EWAT
wild type EWAT
Fabp4-TrkB CKO

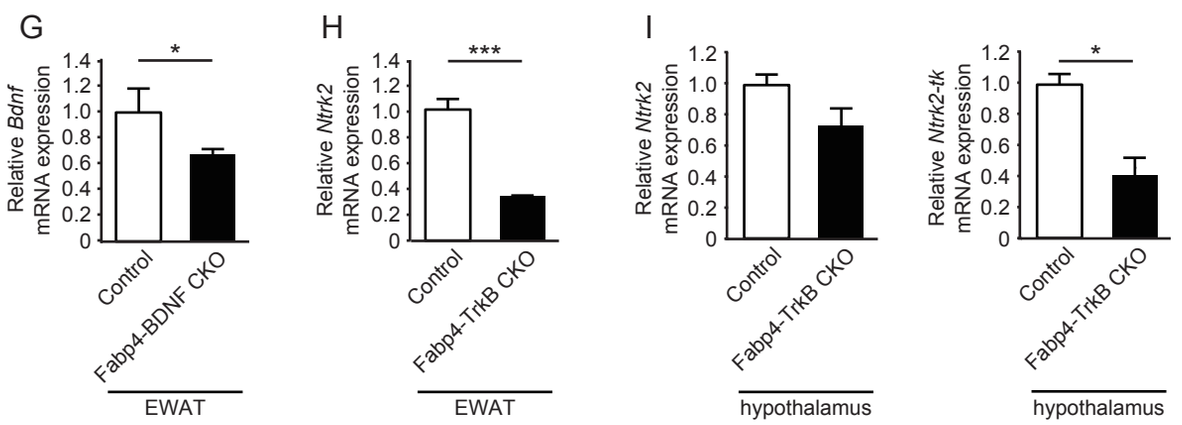
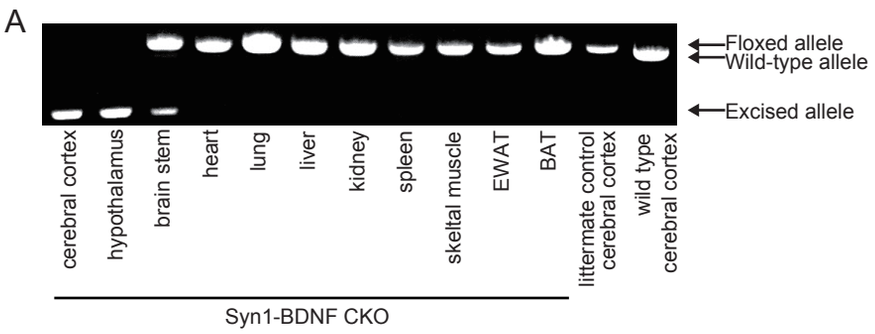
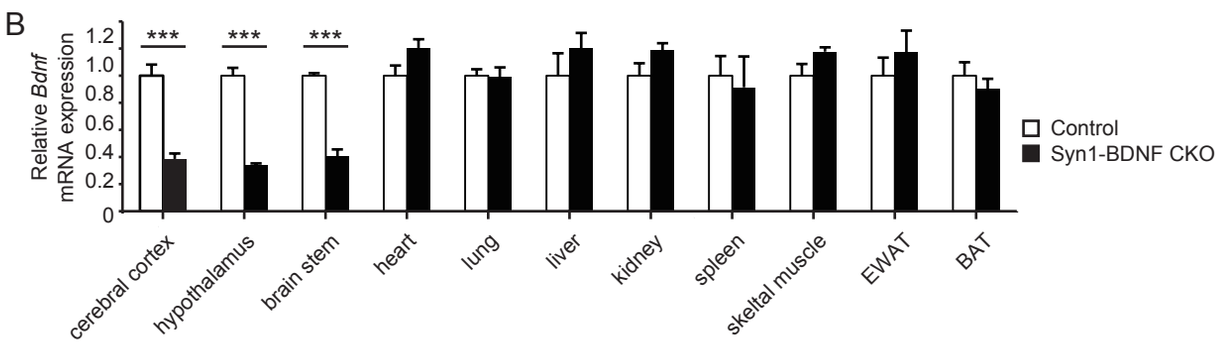
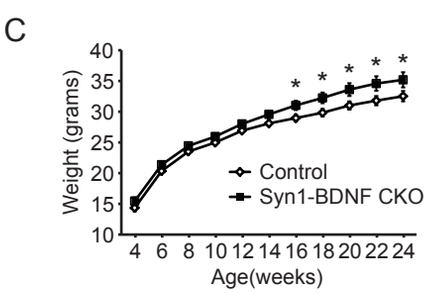
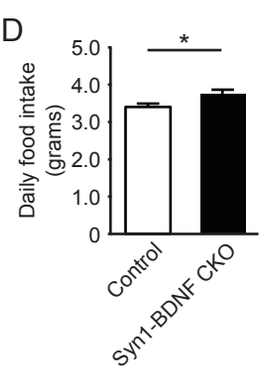

G
Relative Bdnf mRNA expression
Control
Fabp4-BDNF CKO
EWAT
H
Relative Ntrk2 mRNA expression
Control
Fabp4-TrkB CKO
EWAT
I
Relative Ntrk2 mRNA expression
Control
Fabp4-TrkB CKO
hypothalamus
Relative Ntrk2-tk mRNA expression
Control
Fabp4-TrkB CKO
hypothalamus

Figure3


A
Floxed allele
Wild-type allele
Excised allele
cerebral cortex
hypothalamus
brain stem
heart
lung
liver
kidney
spleen
skeltal muscle
EWAT
BAT
littermate control cerebral cortex
wild type cerebral cortex
Syn1-BDNF CKO


B
Relative Bdnf mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
***
***
***
Control
Syn1-BDNF CKO
cerebral cortex
hypothalamus
brain stem
heart
lung
liver
kidney
spleen
skeltal muscle
EWAT
BAT


C
Weight (grams)
40
35
30
25
20
15
10
4 6 8 10 12 14 16 18 20 22 24
Age(weeks)
Control
Syn1-BDNF CKO


D
Daily food intake (grams)
5.0
4.0
3.0
2.0
1.0
0
*
Control
Syn1-BDNF CKO

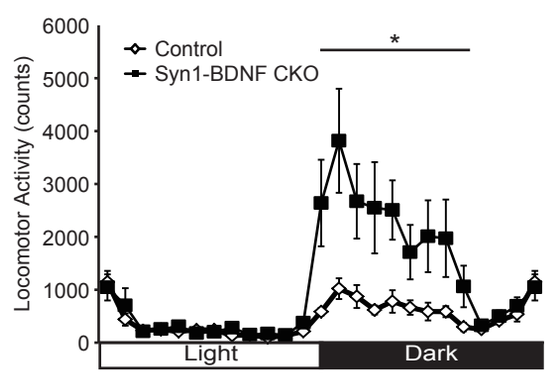
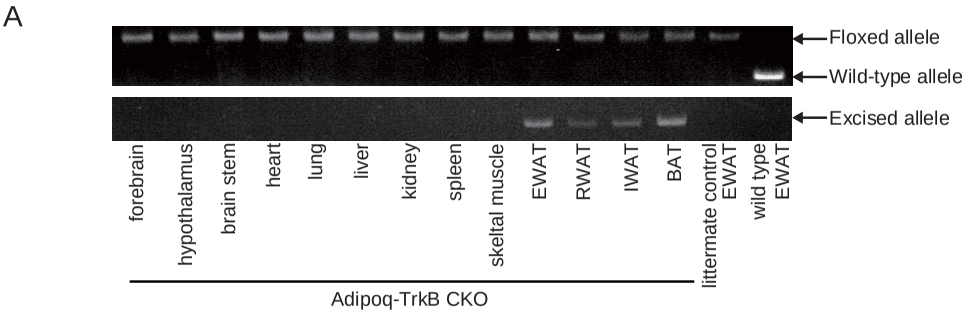
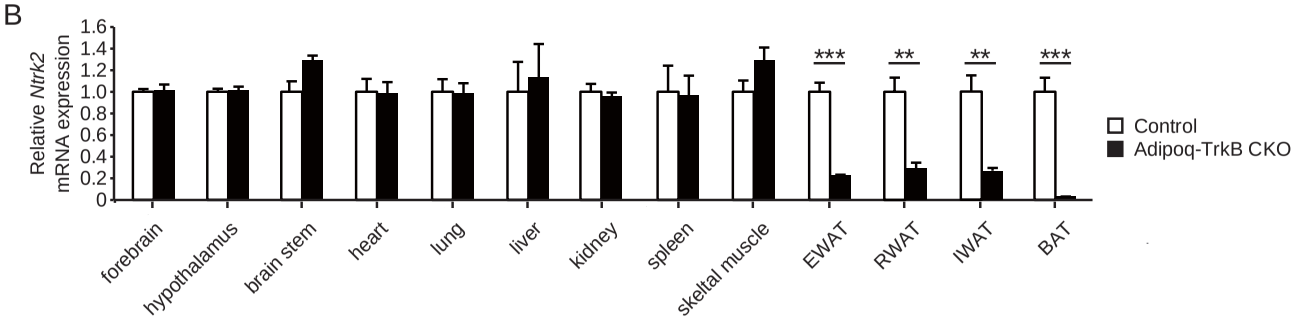
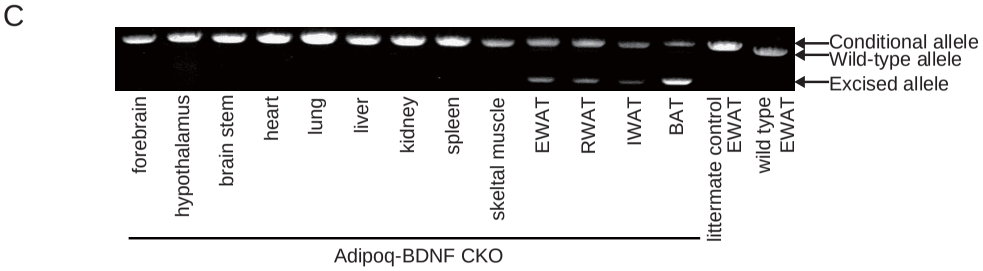

E
Locomotor Activity (counts)
6000
5000
4000
3000
2000
1000
0
Control
Syn1-BDNF CKO
*
Light
Dark

Figure 4


A
Floxed allele
Wild-type allele
Excised allele
forebrain
hypothalamus
brain stem
heart
lung
liver
kidney
spleen
skeltal muscle
EWAT
RWAT
IWAT
BAT
littermate control EWAT
wild type EWAT
Adipoq-TrkB CKO


B
Relative Ntrk2 mRNA expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
***
**
**
***
forebrain
hypothalamus
brain stem
heart
lung
liver
kidney
spleen
skeltal muscle
EWAT
RWAT
IWAT
BAT
Control
Adipoq-TrkB CKO


C
Conditional allele
Wild-type allele
Excised allele
forebrain
hypothalamus
brain stem
heart
lung
liver
kidney
spleen
skeltal muscle
EWAT
RWAT
IWAT
BAT
littermate control EWAT
wild type EWAT
Adipoq-BDNF CKO

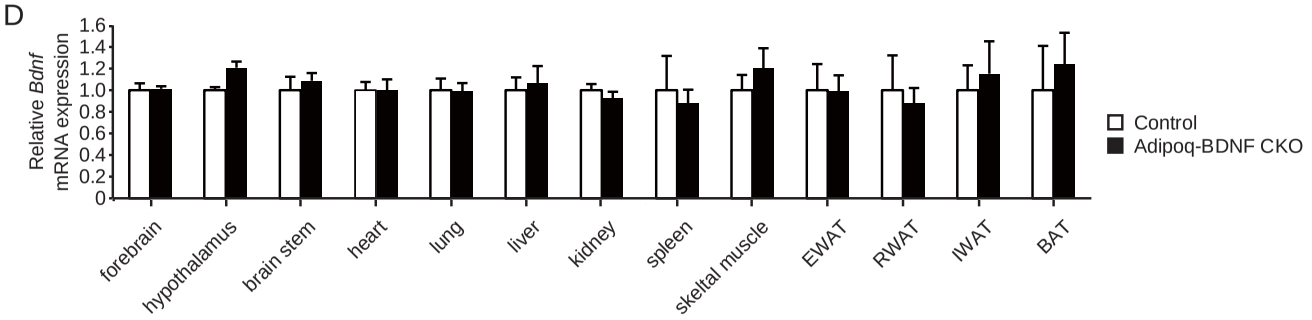

D
Relative Bdnf mRNA expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
forebrain
hypothalamus
brain stem
heart
lung
liver
kidney
spleen
skeltal muscle
EWAT
RWAT
IWAT
BAT
Control
Adipoq-BDNF CKO

Figure5

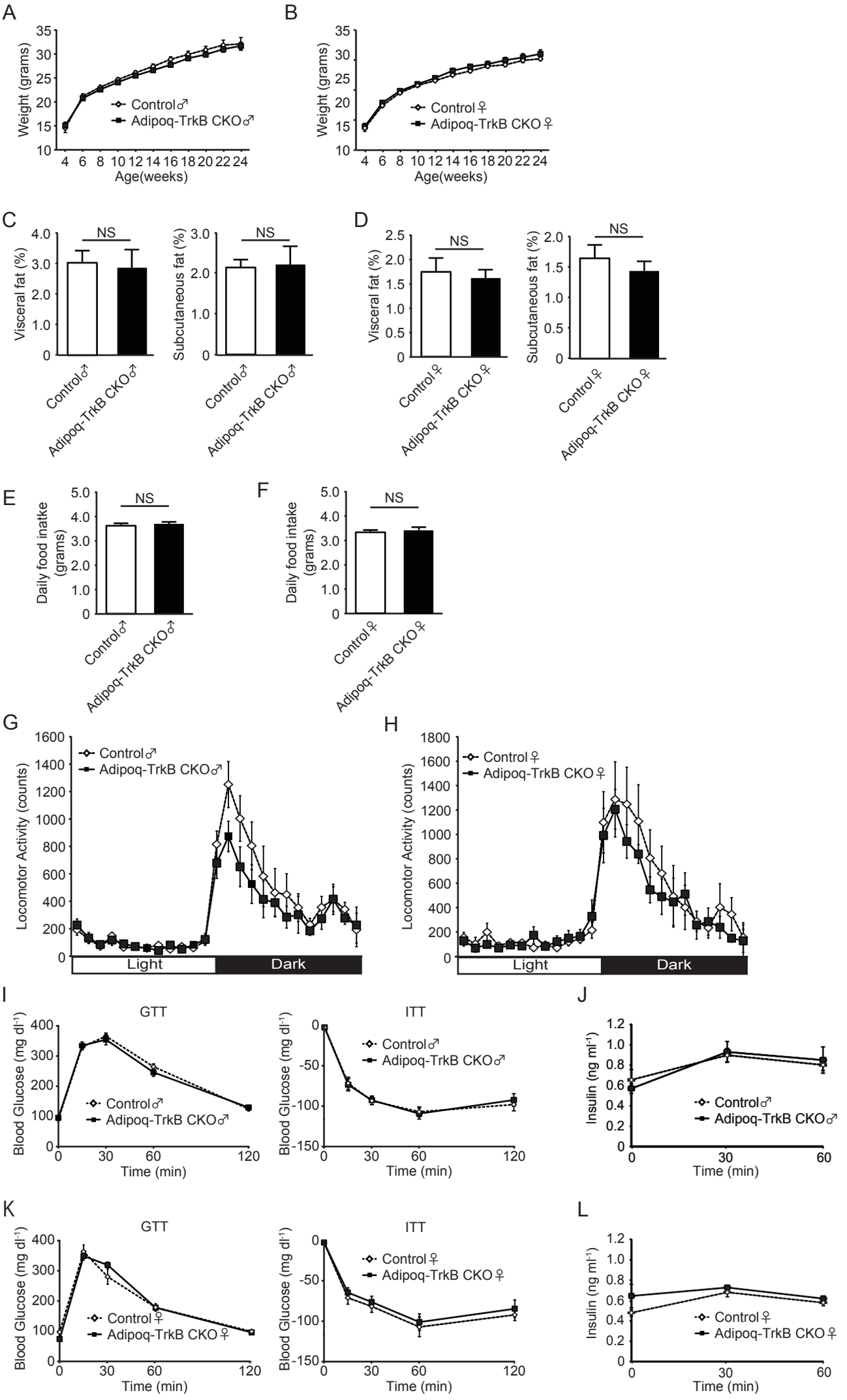
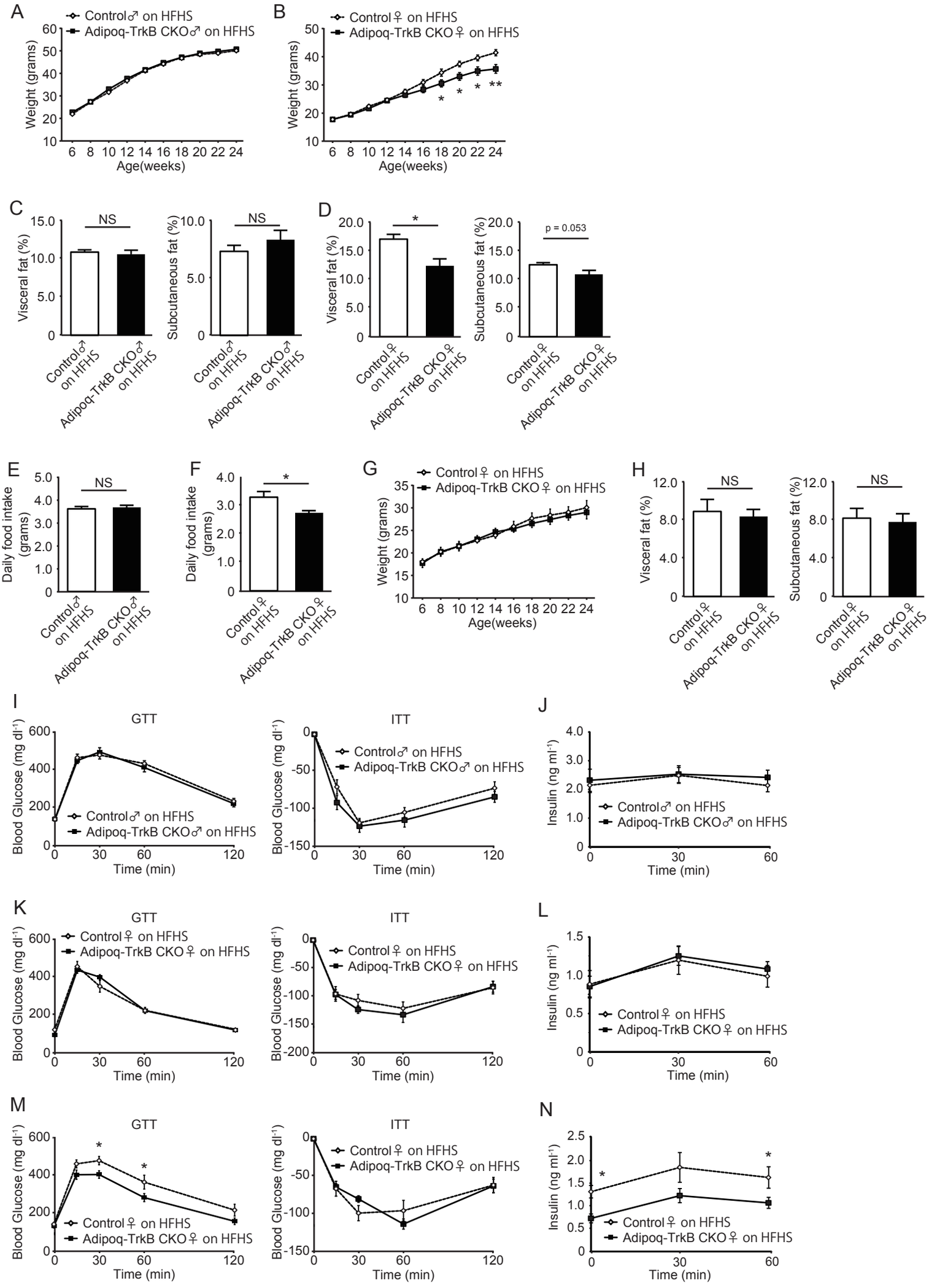

A
Weight (grams)
Control♂
Adipoq-TrkB CKO♂
Age(weeks)
B
Weight (grams)
Control♀
Adipoq-TrkB CKO♀
Age(weeks)
C
Visceral fat (%)
NS
Control♂
Adipoq-TrkB CKO♂
Subcutaneous fat (%)
NS
Control♂
Adipoq-TrkB CKO♂
D
Visceral fat (%)
NS
Control♀
Adipoq-TrkB CKO♀
Subcutaneous fat (%)
NS
Control♀
Adipoq-TrkB CKO♀
E
Daily food inatke (grams)
NS
Control♂
Adipoq-TrkB CKO♂
F
Daily food intake (grams)
NS
Control♀
Adipoq-TrkB CKO♀
G
Locomotor Activity (counts)
Control♂
Adipoq-TrkB CKO♂
Light
Dark
H
Locomotor Activity (counts)
Control♀
Adipoq-TrkB CKO♀
Light
Dark
I
GTT
Blood Glucose (mg dl-1)
Control♂
Adipoq-TrkB CKO♂
Time (min)
ITT
Blood Glucose (mg dl-1)
Control♂
Adipoq-TrkB CKO♂
Time (min)
J
Insulin (ng ml-1)
Control♂
Adipoq-TrkB CKO♂
Time (min)
K
GTT
Blood Glucose (mg dl-1)
Control♀
Adipoq-TrkB CKO♀
Time (min)
ITT
Blood Glucose (mg dl-1)
Control♀
Adipoq-TrkB CKO♀
Time (min)
L
Insulin (ng ml-1)
Control♀
Adipoq-TrkB CKO♀
Time (min)

Figure6


A
Control♂ on HFHS
Adipoq-TrkB CKO♂ on HFHS
Weight (grams)
Age(weeks)
B
Control♀ on HFHS
Adipoq-TrkB CKO♀ on HFHS
Weight (grams)
Age(weeks)
C
Visceral fat (%)
Subcutaneous fat (%)
NS
Control♂ on HFHS
Adipoq-TrkB CKO♂ on HFHS
D
Visceral fat (%)
Subcutaneous fat (%)
*
p = 0.053
Control♀ on HFHS
Adipoq-TrkB CKO♀ on HFHS
E
Daily food intake (grams)
NS
F
Daily food intake (grams)
*
G
Control♀ on HFHS
Adipoq-TrkB CKO♀ on HFHS
Weight (grams)
Age(weeks)
H
Visceral fat (%)
Subcutaneous fat (%)
NS
I
GTT
ITT
Blood Glucose (mg dl-1)
Time (min)
Control♂ on HFHS
Adipoq-TrkB CKO♂ on HFHS
J
Insulin (ng ml-1)
Time (min)
K
GTT
ITT
Blood Glucose (mg dl-1)
Time (min)
Control♀ on HFHS
Adipoq-TrkB CKO♀ on HFHS
L
Insulin (ng ml-1)
Time (min)
M
GTT
ITT
Blood Glucose (mg dl-1)
Time (min)
N
Insulin (ng ml-1)
Time (min)

Aging and Mechanisms of Disease
平成 27 年 2 月　投稿中