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Dendritically targeted *Bdnf* mRNA is essential for energy balance and response to leptin

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Abstract

Mutations in the *Bdnf* gene, which produces transcripts with either short or long 3′ untranslated regions (3′UTRs), cause human obesity; however, the precise role of BDNF in the regulation of energy balance remains unknown. Here we show the relationship between long 3′UTR *Bdnf* mRNA, leptin, neuronal activation and body weight. We found that long 3′UTR *Bdnf* mRNA was enriched in dendrites of hypothalamic neurons and that insulin and leptin could stimulate its translation in dendrites. Furthermore, mice harboring a truncated long *Bdnf* 3′UTR developed severe hyperphagic obesity, which was completely rescued by viral expression of long 3′UTR *Bdnf* mRNA in the hypothalamus. In these animals the ability of leptin to activate hypothalamic neurons and to inhibit food intake was compromised despite normal activation of leptin receptors. These results reveal a novel mechanism linking leptin action to BDNF expression during hypothalamic-mediated regulation of body weight, while also implicating dendritic protein synthesis in this process.

INTRODUCTION

Owing to its high prevalence, costly associated disorders, and lack of effective drugs for treatments, obesity has become a leading health problem[1]. The biological system controlling energy balance is composed of several organs, including adipose tissues, the pancreas, the

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Author Contributions:

GYL performed experiments addressing the obesity syndrome, local protein synthesis, viral rescue of obesity, co-expression of BDNF and TrkB with the leptin receptor, STAT3 activation, and c-Fos induction. JJA analyzed *Bdnf* mRNA subcellular localization and gene expression. KG characterized the obesity phenotype of $Bdnf^{klox/klox}$ mice. EGW created viral constructs and helped GYL in stereotaxic AAV microinjection. FV generated the reporter constructs for local BDNF synthesis. KRJ provided $Bdnf^{klox/klox}$ mouse strain. BX supervised the project. BX and GYL wrote the manuscript.

COMPETING INTERESTS STATEMENT

BX, JJA, and EGW are co-inventors on a patent application that has been filed by Georgetown University related to the technology that is described in this paper.

gastrointestinal tract, and the brain. Peripheral tissues produce signals reflecting the state of nutrition and fat stores, such as leptin[2], insulin[3], ghrelin[4–7], glucagon-like peptide-1 ([8,9]), peptide tyrosine tyrosine ([10]), and certain metabolites (e.g. glucose, fatty acids, and amino acids)[11–14]. These signals are integrated in several brain regions, including the arcuate nucleus (ARC), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and paraventricular hypothalamus[15,16]. These brain regions act to control food intake and energy expenditure in several peripheral tissues[16,17]. Elucidation of the intricate interaction between neural circuits in these brain regions and factors important for the control of energy balance may provide new strategies for developing effective obesity therapies.

Brain-derived neurotrophic factor (BDNF) is a potent regulator of neuronal development and synaptic function[18], and has recently been implicated in the control of energy balance. The first evidence for a role of BDNF in energy balance came from the observation that *Bdnf* heterozygous mice exhibit hyperphagia and moderate obesity[19,20]. This finding was confirmed and extended by the severe obesity phenotypes observed in mice expressing the BDNF receptor, TrkB, at ~25% of the normal amount[21] and in mice where the *Bdnf* gene is deleted in neurons expressing Ca^{2+}/calmodulin-dependent protein kinase II alpha (CaMKIIα)[22]. Since CaMKIIα is a brain-specific protein[23], these observations demonstrate that BDNF acts on neurons of the central nervous system to affect energy balance. More recently, loss of a functional *Bdnf* allele or a dominant-negative TrkB mutation has been found to cause severe hyperphagia and obesity in children[24–26]. Furthermore, *Bdnf* gene variants have been linked to human obesity in large-scale genome-wide association studies[27,28]. However, the means by which BDNF inhibits food intake remain unclear.

The *Bdnf* gene in humans and rodents produces two populations of transcripts with either a short (~0.4 kb) or long (~2.9 kb) 3′ untranslated region (3′UTR) due to two alternative polyadenylation sites (Supplementary Fig. 1a)[29]. Our previous results show that short 3′UTR *Bdnf* mRNA is restricted to neuronal cell bodies whereas long 3′UTR *Bdnf* mRNA is also localized to dendrites in cortical and hippocampal neurons[30]. Numerous mRNA species have been found in neuronal dendrites[31], and these dendritic transcripts serve as templates for local translation in response to synaptic activity[32]. While it has been shown that local protein synthesis in dendrites is required for lasting synaptic plasticity[33–36], it is unknown whether local protein synthesis is important for a physiological process like energy homeostasis. Here we report that BDNF translated from long 3′UTR *Bdnf* mRNA is necessary for leptin-mediated regulation of energy balance.

RESULTS

Truncation of the long *Bdnf* 3′UTR leads to severe hyperphagic obesity

We previously described a mouse mutant, $Bdnf^{klox/klox}$, in which long 3′UTR *Bdnf* mRNA is not generated due to an insertion of three tandem SV40 polyadenylation signals into the genomic sequence encoding the long *Bdnf* 3′UTR (Supplementary Fig. 1b)[30]. In these animals the truncation of the long *Bdnf* 3′UTR led to impairments in dendritic localization of *Bdnf* mRNA in cortical and hippocampal neurons[30]. Surprisingly, $Bdnf^{klox/klox}$ mice also developed severe obesity, starting to show higher body weight at 5–6 weeks of age compared to their WT littermates (Fig. 1a, b). By 16 weeks of age, female and male *Bdnf*

mutants were 171% and 90% heavier, respectively, than sex-matched WT mice. Increased weight gain was also observed in male *Bdnf*$^{klox/+}$ mice (Fig. 1b) and older female *Bdnf*$^{klox/+}$ mice (Supplementary Fig. 2a). Furthermore, these animals exhibited increased linear growth (Fig. 1c). The high body weight of *Bdnf*$^{klox/klox}$ mice was associated with hyperleptinemia (Supplementary Fig. 2b), greatly enlarged adipose tissues (Supplementary Fig. 2c), and impaired glucose homeostasis (Supplementary Fig. 2d–f). These results show that truncation of the long *Bdnf* 3′UTR leads to an obesity syndrome.

The development of obesity in *Bdnf*$^{klox/klox}$ mice could result from increased energy intake and/or decreased energy expenditure. Effects on energy intake were examined by determining the daily food intake of these animals from 6 to 8 weeks of age. Both female and male *Bdnf*$^{klox/klox}$ mice exhibited a marked hyperphagia, consuming 69–80% more food than WT mice (Fig. 1d). To determine if decreased energy expenditure also contributes to obesity in these mice, *Bdnf*$^{klox/klox}$ mice were pair-fed to restrict their daily food intake to WT levels. Each day, female *Bdnf*$^{klox/klox}$ mice were provided the amount of food consumed by female WT littermates on the previous day, starting at 4 weeks of age when *Bdnf*$^{klox/klox}$ mice were not obese. Concurrently one group of *Bdnf*$^{klox/klox}$ mice was not pair-fed and was given *ad libitum* access to food. As expected, non-pair-fed *Bdnf*$^{klox/klox}$ mice became severely obese (Fig. 1e). However, body weights of pair-fed *Bdnf*$^{klox/klox}$ mice were not significantly different from those of WT mice throughout the 12-week pair-feeding period. These results demonstrate that hyperphagia is the sole cause of obesity in *Bdnf*$^{klox/klox}$ mice. Hyperphagia has also been found to cause moderate obesity in *Bdnf* heterozygous mice[37].

Levels of *Bdnf* mRNA are reduced in the VMH of *Bdnf*$^{klox/klox}$ mice

BDNF is expressed in the VMH, and fasting drastically and selectively reduces levels of *Bdnf* mRNA in this region[21,38]. Furthermore, deletion of the *Bdnf* gene in the VMH and DMH using Cre-expressing virus leads to increased weight gain[38], whereas BDNF overexpression in these region reduces body weight[39]. These results highlight the importance of VMH BDNF in the control of energy balance. By employing radioactive *in situ* hybridization, we found that young *Bdnf*$^{klox/klox}$ and WT mice had similar amounts of *Bdnf* mRNA in the cerebral cortex and hippocampal CA1 region (Fig. 2a, b). However, the level of VMH *Bdnf* mRNA in *Bdnf*$^{klox/klox}$ mice was only one third of that in WT littermates (Fig. 2b). This reduction in *Bdnf* mRNA levels was unlikely due to stability issues of the truncated long 3′UTR *Bdnf* mRNA, because the ratio of this mRNA species to short 3′UTR *Bdnf* mRNA in the *Bdnf*$^{klox/klox}$ hypothalamus appeared similar to the ratio of long 3′UTR *Bdnf* mRNA to short 3′UTR *Bdnf* mRNA in the WT hypothalamus (Fig. 2c). These results suggest that the long 3′UTR is required for the maintenance of normal levels of *Bdnf* mRNA in the VMH.

Viral expression of long 3′UTR *Bdnf* mRNA in the hypothalamus blunts the obesity phenotype of *Bdnf*$^{klox/klox}$ mice

Since the level of total VMH *Bdnf* mRNA in *Bdnf*$^{klox/klox}$ mice was reduced, it was not clear if these animals developed obesity due to diminished *Bdnf* expression in the VMH or the lack of long 3′UTR *Bdnf* mRNA. To distinguish between these two possibilities, we investigated whether viral expression of either short or long 3′UTR *Bdnf* mRNA in the

VMH would rescue the obesity phenotype observed in *Bdnf*$^{klox/klox}$ mice. We generated two adeno-associated viral (AAV) constructs by linking a Myc-tagged *Bdnf* coding sequence to either sequence "A" that encodes the short *Bdnf* 3′UTR (AAV-BDNF-A) or sequence "A*B" that encodes the entire long *Bdnf* 3′UTR (AAV-BDNF-A*B, where the first polyadenylation signal of the long *Bdnf* 3′UTR was mutated) (Fig. 2d). These two viruses expressed BDNF well in hypothalamic neurons (Fig. 2e). Because it remains unclear whether BDNF regulates neuronal development or neuronal function to affect body weight, we decided to inject stereotaxically AAV into the VMH of WT and *Bdnf*$^{klox/klox}$ pups at 2 weeks of age so that overexpressed BDNF has an effect on synaptic development and/or synaptic function. In addition to the VMH, viral infection was also detected in the ARC and DMH. Viral expression of either short or long 3′UTR *Bdnf* mRNA did not affect body weights of WT mice, as both female and male WT mice infected with either AAV-BDNF-A or AAV-BDNF-A*B had comparable body weights to sex-matched WT mice infected with a control virus, AAV-GFP ($P > 0.05$ at all ages by two-way ANOVA Bonferroni posttests) (Fig. 2f, g). Viral expression of short 3′UTR *Bdnf* mRNA significantly reduced body weights of female *Bdnf*$^{klox/klox}$ mice (female k/k-BDNF-A mice vs. female k/k-GFP mice: $P < 0.01$ at 7 weeks of age or later by two-way ANOVA Bonferroni posttests) (Fig. 2f). Viral expression of short 3′UTR *Bdnf* mRNA also reduced body weights of male *Bdnf*$^{klox/klox}$ mice, but the effect was not statistically significant ($P > 0.05$ at all ages by two-way ANOVA Bonferroni posttests) (Fig. 2g). Remarkably, viral expression of long 3′UTR *Bdnf* mRNA completely rescued the obesity phenotype in both female and male *Bdnf*$^{klox/klox}$ mice (WT-GFP mice vs. k/k-BDNF-A*B mice: $P > 0.05$ at all ages for both genders by two-way ANOVA Bonferroni posttests) (Fig. 2f, g). It also normalized body length, food intake, and blood glucose levels in *Bdnf*$^{klox/klox}$ mice (Fig. 2h–j). These results, along with the obesity phenotype of *Bdnf*$^{klox/klox}$ mice, indicate that long 3′UTR *Bdnf* mRNA plays more important roles than short 3′UTR *Bdnf* mRNA in the control of food intake.

Long 3′UTR *Bdnf* mRNA is localized to dendrites of hypothalamic neurons

Long 3′UTR *Bdnf* mRNA is targeted to the dendrites of hippocampal neurons where it plays an important role in regulating spine morphology and synaptic plasticity[30]. To investigate if long 3′UTR *Bdnf* mRNA is also targeted to the dendrites of hypothalamic neurons, we performed fluorescent *in situ* hybridization (FISH) on cultured rat hypothalamic neurons using RNA probes derived from the *Bdnf* coding region or a 1.9 kb cDNA fragment corresponding to the 3′ end of the long *Bdnf* 3′UTR (Fig. 3a). The coding region probe recognizes both populations of *Bdnf* mRNA whereas the 3′UTR probe detects long 3′UTR *Bdnf* mRNA. We found that the ratio of the FISH signal in the initial 50 μm segment of dendrites to the somatic FISH signal from the 3′UTR probe was ~4-fold higher than that from the coding region probe (Fig. 3b). Thus, long 3′UTR *Bdnf* mRNA is preferentially targeted to the dendrites of hypothalamic neurons.

Insulin stimulates local BDNF synthesis in hypothalamic neurons via the mTOR pathway

Our previous results indicate that the long *Bdnf* 3′UTR is sufficient to direct dendritic localization and local translation of mRNA in cultured hippocampal neurons[30]. On the basis of this observation, we reasoned that factors important for the control of energy balance, such as insulin and leptin, might regulate translation of long 3′UTR *Bdnf* mRNA in the

dendrites of hypothalamic neurons. If insulin directly stimulates local BDNF synthesis, BDNF neurons should also express the receptor for insulin in the hypothalamus. We performed immunohistochemistry against insulin receptor β (IRβ) and β-galactosidase on brain sections of *Bdnf*$^{LacZ/+}$ knockin mice in which the *LacZ* coding sequence replaces the *Bdnf* coding region, and found that the majority of BDNF neurons also express IRβ in the DMH and VMH (Fig. 3c–h).

To test the possibility that insulin regulates local BDNF synthesis in hypothalamic neurons, we generated two local protein synthesis reporter constructs by attaching the myr-d1GFP-nls coding sequence to sequence "A" (myr-d1GFP-nls-A) or sequence "A*B" (myr-d1GFP-nls-A*B). The membrane-anchoring myristoylation peptide (myr), the nuclear localization sequence (nls), and the short half-life of the destabilized d1GFP protein impede GFP synthesized in cell bodies from diffusing to distal dendrites, so that GFP in distal dendrites better reflects local protein synthesis[40]. Application of insulin to hypothalamic cultures increased the amount of GFP in distal dendrites of neurons expressing myr-d1GFP-nls-A*B, but not in neurons expressing myr-d1GFP-nls-A (Fig. 3i; Supplementary Fig. 3a), indicating that insulin stimulates local protein synthesis through the long *Bdnf* 3′UTR. Importantly, the stimulation was specific to dendritic protein synthesis, as insulin did not increase levels of GFP in cell bodies (Supplementary Fig. 3b). One important regulator of local protein synthesis is the mammalian target of rapamycin (mTOR), which can be activated by phosphoinositol-3 kinase (PI3K)[32]. As insulin strongly activates PI3K[41], we examined whether insulin stimulated local translation of transcripts containing the long *Bdnf* 3′UTR via the mTOR pathway. Pretreatment of neuronal cultures with an mTOR specific inhibitor, rapamycin, abolished the stimulating effect of insulin on dendritic protein synthesis (Fig. 3j). These results indicate that insulin can directly stimulate local BDNF synthesis from long 3′UTR *Bdnf* mRNA in the dendrites of hypothalamic neurons via an mTOR-dependent mechanism.

Leptin activates hypothalamic BDNF-expressing neurons through network activity

Unlike the insulin receptor, we did not detect co-expression of BDNF and the leptin receptor (LepR) in the DMH or VMH. Although alternative mRNA splicing produces multiple isoforms of the LepR, the long leptin receptor form (LepRb) is the one that mediates leptin's physiological action[42,43]. It has been shown that after leptin administration the immunoreactivity for phosphorylated signal transducer and activator of transcription protein 3 (pSTAT3) sensitively and reliably identifies neurons that express LepRb[44]. Double immunohistochemistry against pSTAT3 and β-galactosidase on brain sections of leptin-treated *Bdnf*$^{LacZ/+}$ mice revealed that there was little co-expression of pSTAT3 and BDNF in the DMH (Fig. 4a) or VMH (Fig. 4b, c). To further examine if BDNF and LepRb are co-expressed in the hypothalamus, we introduced the *LepR-Cre* allele to *Bdnf*$^{klox/+}$ mice. In these mice, the *IRES-Cre* sequence was inserted into the 3′UTR for LepRb[45], and BDNF neurons start to express β-galactosidase once the floxed *Bdnf* allele is deleted by Cre-mediated recombination[46]. In *LepR-Cre;Bdnf*$^{klox/+}$ mice, we found many neurons expressing β-galactosidase in the dentate gyrus (Fig. 4d), which is consistent with previous observations that these neurons express BDNF as well as LepRb[46,47]. However, very few β-galactosidase-expressing neurons were detected in the VMH (Fig. 4e) and DMH (Fig. 4f),

confirming lack of co-expression between BDNF and LepRb in these two brain regions. These results suggest that it is unlikely that leptin would directly stimulate local BDNF synthesis in these two hypothalamic regions.

We then asked if leptin could stimulate local BDNF synthesis *in vivo* via network activity. Leptin administration has been shown to induce STAT3 activation and c-Fos expression in distinct populations of neurons in the DMH and VMH[48]. By using c-Fos expression as a marker for neuronal activation, we found that leptin administration activated many BDNF-expressing neurons in the DMH (Fig. 4g) and VMH (Fig. 4h). Quantification revealed that 32% (46/142) in the DMH and 33% (54/163) in the VMH of BDNF neurons expressed c-Fos. Finally, we employed KCl-induced neuronal depolarization to determine if neuronal activity is sufficient to stimulate local BDNF synthesis in cultured hypothalamic neurons using the *in vitro* reporter assay described earlier. We found that KCl treatment increased GFP synthesis in dendrites of cultured hypothalamic neurons expressing GFP transcripts containing the long *Bdnf* 3′UTR, and this stimulation was independent of mTOR (Fig. 4i). Taken together, our results indicate that leptin can activate BDNF-expressing hypothalamic neurons through activated neural circuits, which in turn stimulates dendritic BDNF synthesis.

Mice lacking long 3′UTR *Bdnf* mRNA fail to respond to leptin administration

Because long 3′UTR *Bdnf* mRNA is required for the control of energy balance (Fig. 1, 2) and leptin can stimulate local translation of transcripts containing the long *Bdnf* 3′UTR indirectly through neuronal activation (Fig. 4), we reasoned that $Bdnf^{klox/klox}$ mice might not respond to leptin. To test this hypothesis, we intraperitoneally injected young and lean $Bdnf^{klox/klox}$ and WT mice with leptin three times over a 24-h period. The leptin administration significantly reduced food intake by 26% over the 24-h period in WT mice (Fig. 5a). The same treatment, however, did not affect food intake in $Bdnf^{klox/klox}$ mice (Fig. 5a), although young WT and $Bdnf^{klox/klox}$ mice at 5–6 weeks of age had comparable serum leptin levels (female: 0.34 ± 0.03 ng/ml for WT and 0.33 ± 0.03 ng/ml for $Bdnf^{klox/klox}$, P=0.859, n=8 mice per genotype; male: 0.44 ± 0.05 ng/ml for WT and 0.76 ± 0.16 ng/ml for $Bdnf^{klox/klox}$, P=0.076, n=8 mice per genotype). These observations suggest that local BDNF synthesis is required for the anorexigenic effect of leptin.

Leptin receptor activation is normal in the hypothalamus of $Bdnf^{klox/klox}$ mice

Leptin resistance in young lean $Bdnf^{klox/klox}$ mice might result from impaired leptin signaling in TrkB-expressing hypothalamic neurons. To address this possibility, we first examined co-expression of TrkB and LepRb in the ARC, VMH, and DMH by using immunohistochemistry against β-galactosidase and pSTAT3 on brain sections of leptin-injected $TrkB^{LacZ/+}$ mice. We detected many TrkB-expressing neurons in the ARC (Fig. 5b) and DMH (Supplementary Fig. 4a), a small percentage of which also contained pSTAT3 immunoreactivity (Fig. 5b–d; Supplementary Fig. 4a–c). In the VMH, few TrkB-expressing neurons were detected and there was little co-expression of TrkB and pSTAT3 (Supplementary Fig. 4d–f). The low co-expression of TrkB and LepRb suggests that deficits in BDNF-to-TrkB signaling are unlikely to impair leptin signaling in LepRb-expressing hypothalamic neurons in $Bdnf^{klox/klox}$ mice. To test this prediction, we examined the ability

of leptin to activate STAT3, a major signaling component in the leptin pathway, in hypothalami of young *Bdnf*$^{klox/klox}$ mice (Fig. 5e; Supplementary Fig. 4g–j). Cell counting revealed that STAT3 activation in the ARC, VMH, and DMH of *Bdnf*$^{klox/klox}$ mice was not significantly different from WT littermates (Fig. 5f). Furthermore, *Bdnf*$^{klox/klox}$ mice had normal levels of mRNAs for suppressor of cytokine signaling 3 (SOCS3), pro-opiomelanocortin (POMC), neuropeptide Y (NPY), and agouti-related protein (AgRP) in the ARC (Supplementary Fig. 5), which are all directly regulated by LepRb signaling[49]. Thus, lack of long 3′UTR *Bdnf* mRNA does not impair the leptin receptor activation.

Leptin-induced neuronal activation is impaired in *Bdnf*$^{klox/klox}$ mice

It has been shown that leptin induces STAT3 activation and c-Fos expression in distinct neuronal populations of several hypothalamic nuclei[48]. We observed a similar phenomenon in the DMH and VMH (Fig. 4a–c, g, h). This observation suggests that LepRb-expressing neurons send inputs to non-LepRb-expressing neurons and subsequently induce c-Fos expression in these cells. If the BDNF protein translated from long 3′UTR *Bdnf* mRNA is required for this information flow, the truncation of the long 3′UTR may lead to leptin resistance in *Bdnf*$^{klox/klox}$ mice. To test this possibility, we examined c-Fos induction in young and lean WT and *Bdnf*$^{klox/klox}$ mice after leptin administration (Fig. 6a; Supplementary Fig. 6). Cell counting revealed that c-Fos induction was abolished in the DMH and significantly impaired in the ARC and VMH (Fig. 6b). These results indicate that BDNF derived from long 3′UTR *Bdnf* mRNA controls the information flow from leptin-sensing neurons to non-LepRb-expressing neurons, likely by regulating the formation or function of neuronal connections. In support of this argument, the projection of anorexgenic POMC neurons, the majority of which express LepRb[49], into the DMH was significantly reduced in *Bdnf*$^{klox/klox}$ mice (Supplementary Fig. 7).

To determine if the impairment in leptin-induced c-Fos expression is restricted to TrkB-expressing neurons, we examined co-localization of c-Fos and β-galactosidase in brain sections of leptin-administered *TrkB*$^{LacZ/+}$ mice. We focused our study on the DMH as a result of our finding that leptin-induced c-Fos expression in the DMH was completely abolished in *Bdnf*$^{klox/klox}$ mice (Fig. 6b). We found that only 20% (23/113) of c-Fos expression was induced in TrkB-expressing DMH neurons (Fig. 6c). This result indicates that information flow to both TrkB- and non-TrkB-expressing neurons is impaired in the absence of BDNF synthesis from long 3′UTR *Bdnf* mRNA.

Discussion

Local protein synthesis offers a mechanism to selectively and quickly strengthen or weaken individual synapses in response to neuronal activity. It is required for long-lasting synaptic plasticity in the hippocampus[33–35]. In this study we showed that *Bdnf*$^{klox/klox}$ mice lacking the dendritically targeted long 3′UTR *Bdnf* mRNA developed hyperphagic obesity. We further showed that both leptin and insulin could stimulate local translation of long 3′UTR *Bdnf* mRNA in the dendrites of hypothalamic neurons. These observations implicate for the first time dendritic local protein synthesis in the control of feeding behavior. This finding

suggests that mutations in the *Bdnf* 3′UTR and proteins important for controlling dendritic localization and translation of *Bdnf* mRNA may increase susceptibility to obesity.

Our results indicate that the lack of long 3′UTR *Bdnf* mRNA rather than the reduced level of VMH *Bdnf* mRNA is the main cause of the obesity syndrome observed in $Bdnf^{klox/klox}$ mice. First, the obesity syndrome in $Bdnf^{klox/klox}$ mice is not identical to the one observed in conditional mutant mice where the *Bdnf* gene is deleted in CaMKIIα-expressing neurons[22]. For example, the conditional mutant showed severe hyperglycemia[22], whereas blood glucose levels were only modestly elevated in female $Bdnf^{klox/klox}$ mice and normal in male $Bdnf^{klox/klox}$ mice. Furthermore, $Bdnf^{klox/klox}$ mice developed more severe obesity than the *Bdnf* conditional mutant mice, which is likely due to incomplete *Bdnf* deletion in the hypothalamus of the conditional mutant[22]. Second, obese $Bdnf^{+/-}$ mice are only approximately 20% heavier than WT mice at 4 months of age, although the level of VMH *Bdnf* mRNA is drastically reduced in these heterozygous mice[20]. Lastly and most importantly, we showed that viral expression of long 3′UTR *Bdnf* mRNA in the ventromedial hypothalamic region completely rescued hyperphagic obesity in $Bdnf^{klox/klox}$ mice, whereas viral expression of short 3′UTR *Bdnf* mRNA only partially rescued obesity in female $Bdnf^{klox/klox}$ mice and did not have significant effects on body weight in male $Bdnf^{klox/klox}$ mice. Given that overexpression usually diminishes the specificity of protein action, it is possible that BDNF derived from the endogenous short 3′UTR *Bdnf* mRNA in the ventromedial hypothalamic region would have a minimal effect on body weight, even in females. Therefore, we conclude that BDNF synthesized from long 3′UTR *Bdnf* mRNA has more important roles than BDNF derived from short 3′UTR *Bdnf* mRNA in the control of energy balance.

Since dendritic mRNAs are packaged into transport granules and are translationally repressed in the cytoplasm and during dendritic transport[32], translation of long 3′UTR *Bdnf* mRNA should mainly occur in dendrites in response to stimulation. If this inference is correct, the obesity phenotype in $Bdnf^{klox/klox}$ mice should result from lack of dendritic local BDNF synthesis. It is possible that the long 3′UTR controls BDNF synthesis in the cytoplasm in response to stimulation, and loss of this specific control in $Bdnf^{klox/klox}$ mice also contributes to the development of obesity. Further studies employing new techniques are needed to address the relative contribution of somatic vs. dendritic translation of long 3′UTR *Bdnf* mRNA in the control of energy balance.

Our results show that lack of long 3′UTR *Bdnf* mRNA leads to leptin resistance, a primary risk factor for obesity, without affecting LepRb signaling. This leptin resistance is likely due to defective neural circuits, as leptin-induced c-Fos expression was impaired or abolished in the ARC, DMH and VMH of $Bdnf^{klox/klox}$ mice while leptin activated LepRb normally in these brain regions. Leptin induces LepRb signaling and c-Fos expression in distinct populations of DMH neurons[48]; however, it remains unknown whether LepRb-expressing neurons within or outside the DMH innervate the DMH neurons that express c-Fos in response to leptin administration. If POMC neurons in the ARC induce c-Fos expression in their target neurons within the DMH, our results may provide one mechanism underlying leptin resistance in young $Bdnf^{klox/klox}$ mice. We detected significantly fewer POMC fibers within the DMH in these animals. This impairment in axonal projection and innervation

could diminish the ability of POMC neurons to activate DMH neurons in response to leptin. Similar projection deficits from LepRb-expressing neurons may occur in other brain regions. This projection defect would indicate that BDNF controls energy balance in part by regulating the formation and/or maintenance of hypothalamic connections. In light of low co-expression of TrkB and LepRb in the adult ARC, we were surprised to observe the impairment in the projection of POMC neurons to the DMH in $Bdnf^{klox/klox}$ mice. Further studies are needed to investigate whether TrkB is more widely expressed in the developing ARC.

On the basis of our results, we propose that leptin and BDNF have a linked role in the control of energy balance (Supplementary Fig. 8). Leptin can stimulate translation of long 3′UTR *Bdnf* mRNA in neuronal dendrites through neuronal activity. BDNF derived from this form of transcripts in turn is required for leptin-induced neuronal activity in several hypothalamic areas, likely by regulating the formation, maintenance, and/or function of neuronal connections. When BDNF signaling is compromised, neural circuits in these hypothalamic areas are dysfunctional, leading to leptin resistance and obesity.

METHODS

Animals

$Bdnf^{klox/klox}$, $Bdnf^{LacZ/+}$ and $TrkB^{LacZ/+}$ mouse strains were on the C57BL6/J genetic background. All animal procedures were approved by the Georgetown University Animal Care and Use Committee.

In situ hybridization

Fluorescent *in situ* hybridization of cultured neurons was performed using digoxigenin-labeled riboprobes and the TSA Plus Fluorescein System (PerkinElmer, Waltham, MA) as previously described[30]. Radioactive *in situ* hybridization of brain sections was performed using ^{35}S-labeled riboprobes as previously described[21].

Viral BDNF overexpression

To generate BDNF-expressing AAV constructs, we first sequentially subcloned the CMV promoter, the mouse *Bdnf* coding sequence that is extended at its 3′ end with a sequence encoding the Myc tag (gccGAACAAAAACTCATCTCAGAAGAGGATCTGaatagctag, where the Myc-encoding sequence is shown in capital), and the mouse genomic sequence encoding the short *Bdnf* 3′UTR (A) or the long *Bdnf* 3′UTR (A*B) into pBluescript II KS (−). The whole CMV-Bdnf-Myc-A or CMV-Bdnf-Myc-A*B fragment could be released from the plasmids using NotI restriction enzyme. We digested pAAV-MCS vector (Stratagene, Cedar Creek, TX, USA) with NotI to remove a 1.7-kb fragment and subcloned the two NotI fragments, CMV-Bdnf-Myc-A (1.8 kb) and CMV-Bdnf-Myc-A*B (4.2 kb), into pAAV-MCS to generate pAAV-BDNF-A and pAAV-BDNF-A*B, respectively. In this way, the construct expressing long 3′UTR *Bdnf* mRNA was still within the AAV packaging capacity. Virus titers were determined by quantitative PCR, and 1μl of viral preparation (~1 × 10^8 viral particles) was stereotaxically injected to each VMH of mice at P14 using the

following coordinates: anteroposterior, −1.2 mm; mediolateral, ±0.5 mm; dorsoventral, −5.9 mm.

In vitro local protein synthesis assays

Hypothalamic neurons were isolated and cultured according to a previously described procedure[50] with some modifications. Culture medium was replaced with fresh medium omitting glucose and insulin at 6 day in vitro (DIV). Cells were transfected using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) at 7 DIV, and treated with insulin (50 nM for 2 h; Sigma-Aldrich) or KCl (50 mM for 30 min) at 8 DIV with or without the rapamycin (25 μM for 30 min; Calbiochem, San Diego, CA) pretreatment. Cultures were fixed and stained using antibodies against MAP2. GFP intensity in cell bodies and dendrites was quantified using NIH ImageJ software.

Leptin treatment of mice

Mice were maintained on a low-fat diet (Purina diet 5001) to limit the development of obesity. To activate STAT3, we intraperitoneally injected mice at 6 weeks of age with either saline or murine leptin (R&D Systems, Minneapolis, MN) at 5 μg/g of body weight, and perfused the animals 45 min later. Averaged body weight was 18.2 ± 0.7 and 23.3 ± 0.7 g for +/+ and k/k mice, respectively. To induce c-Fos, we handled mice daily at 5 weeks of age for one week, and then intraperitoneally injected mice with either saline or leptin at 5 μg/g of body weight and perfused the animals 2 hr later. The averaged body weight was 18.6 ± 0.8 and 20.6 ± 1.3 g (P=0.22) for +/+ and k/k mice, respectively. For food intake assays, mice were singly housed one week before leptin administration, switched to the standard chow (Purina diet 5058), and injected intraperitoneally with saline daily for a week to reduce their stress response to the injection procedure. Leptin at 3 μg/g of body weight or saline was injected intraperitoneally at 12:00, 18:00 and 1:30. Pre-weighed mouse chow was given to each mouse immediately after the first leptin injection. Food intake was measured 24 h after the first leptin injection. Average body weight for each group was as follows: 23.2 ± 0.7 g for the +/+ saline group (12 mice), 23.1 ± 0.8 g for the +/+ leptin group (12 mice), 25.6 ± 1.5 g for the k/k saline group (9 mice), and 25.9 ± 1.1 g for the k/k leptin group (8 mice).

Immunohistochemistry

Immunohistochemistry was performed as previously described[30,51]. For pSTAT3 immunohistochemistry, brain sections were sequentially pretreated in 1% NaOH + 1% H_2O_2 for 20 min, 0.3% glycine for 10 min, and 0.03% SDS for 10 min as previously described[44]. The primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA; rabbit anti-pSTAT3, 1:2,000), Calbiochem (San Diego, CA; rabbit anti-cFOS, 1:10,000), Promega Corporation (Madison, WI; mouse anti-β-galactosidase, 1:300), and Santa Cruz Biotechnology (Santa Cruz, CA; rabbit anti-MAP2, 1:200).

Counting of immunoreactive neurons

Every fourth brain section from each animal was processed for pSTAT3 or c-Fos immunohistochemistry. Hypothalamic areas corresponding to the ARC, VMH, or DMH were outlined, and immunoreactive neurons were counted using Stereo Investigator software

(MicroBrightField Inc, Williston, VT). The total number of pSTAT3- or c-Fos-positive cells for each animal was calculated by multiplying the counted number by 4.

Statistical analysis

All data are expressed as mean ± SEM. Data was analyzed using unpaired Student's *t*-tests or two-way ANOVA.

Additional methods

Additional methodology is described in the Supplementary Methods online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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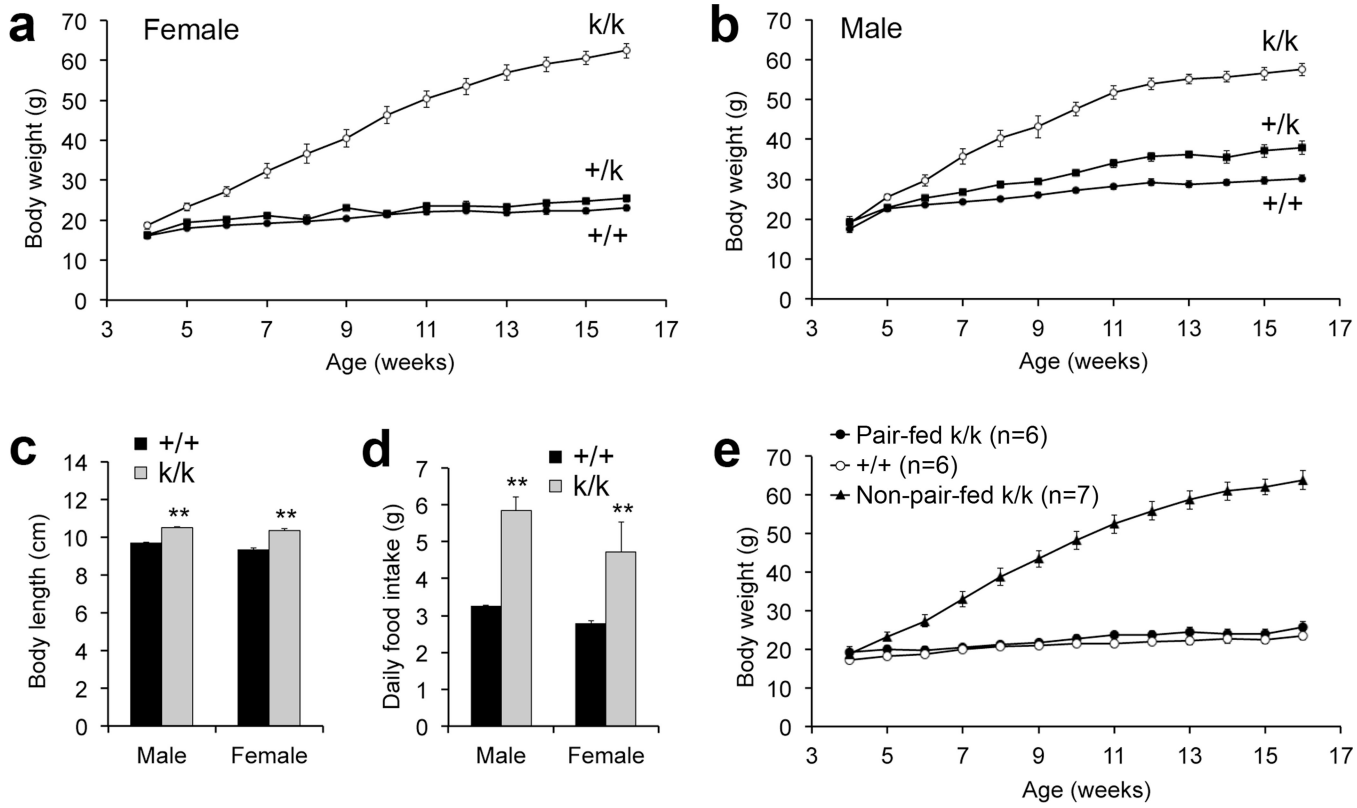

Figure 1.

Mice lacking long 3′UTR *Bdnf* mRNA display severe hyperphagic obesity. (**a**) Body weight curves of female WT (+/+, n=10), heterozygous $Bdnf^{klox/+}$ (+/k, n=8), and homozygous $Bdnf^{klox/klox}$ (k/k, n=10) mice. There was a significant effect of genotypes on body weight (two-way ANOVA: $F_{(2, 325)} = 56.47$, $P < 0.0001$). (**b**) Body weight curves of male +/+ (n=6), +/k (n=6), and k/k (n=8) mice. There was a significant effect of genotypes on body weight (two-way ANOVA: $F_{(2, 221)} = 42.12$, $P < 0.0001$). (**c**) Body length of +/+ and k/k mice at 5 months of age. (**d**) Daily food intake of individually housed mice. (**e**) Normal body weights of pair-fed female k/k mice. Error bars represent the standard error of the mean. ** $P < 0.01$ by Student's *t* test.

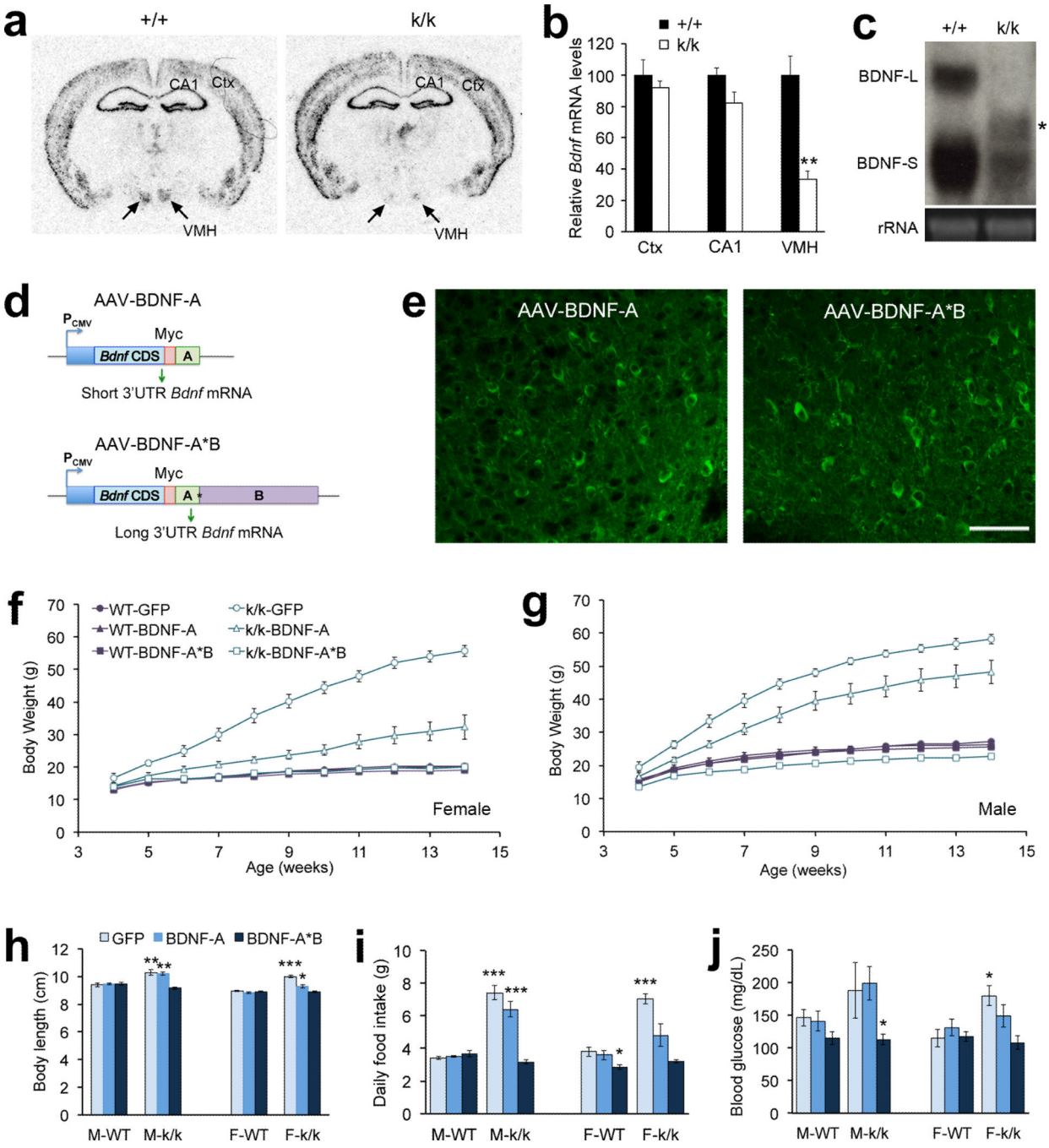

Figure 2.

Viral expression of long 3′UTR *Bdnf* mRNA in the ventromedial hypothalamic region rescues the obesity syndrome in *Bdnf*$^{klox/klox}$ mice. (**a**) Representative *Bdnf in situ* hybridization images of brain sections from +/+ and k/k mice at 5–6 weeks of age. (**b**) Quantification of *in situ* hybridization signals in the cerebral cortex (Ctx), CA1 region, and VMH (n=4 mice per genotype). (**c**) Northern blot analysis of *Bdnf* mRNA isolated from hypothalami of +/+ and k/k mice. BDNF-L and BDNF-S mark long and short 3′UTR *Bdnf* mRNA, and the asterisk denotes a new *Bdnf* mRNA species. Lower panel: 18S rRNA as a

loading control. (**d**) Diagram of AAV constructs expressing either short or long 3′UTR *Bdnf* mRNA. The *Bdnf* coding region (CDS) is linked to a Myc tag sequence at its 3' end. (**e**) Myc immunoreactivity in the ventromedial hypothalamic region of k/k mice injected with either AAV-BDNF-A or AAV-BDNF-A*B viral particles. Scale bar, 50 μm. (**f**) Body weight curves of female +/+ and k/k mice that received injection of AAV viral particles expressing GFP, short 3′UTR *Bdnf* mRNA (BDNF-A), or long 3′UTR *Bdnf* mRNA (BDNF-A*B) (n=6 mice per group). There was a significant effect from genotype and viral injection (two-way ANOVA: $F_{(5, 330)} = 57.46$, $P < 0.0001$). (**g**) Body weight curves of male +/+ and k/k mice that received injection of AAV viral particles expressing GFP, short 3′UTR *Bdnf* mRNA (BDNF-A), or long 3′UTR *Bdnf* mRNA (BDNF-A*B) (n=4 mice for the k/k-GFP group and 6 mice for other groups). There was a significant effect from genotype and viral injection (two-way ANOVA: $F_{(5, 308)} = 25.14$, $P < 0.0001$). (**h–j**) Body length, daily food intake, and fasting blood glucose of male +/+ (M-WT) and k/k (M-k/k) mice and female +/+ (F-WT) and k/k (F-k/k) mice injected with AAV-GFP, AAV-BDNF-A, or AAV-BDNF-A*B. Error bars represent the standard error of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test.

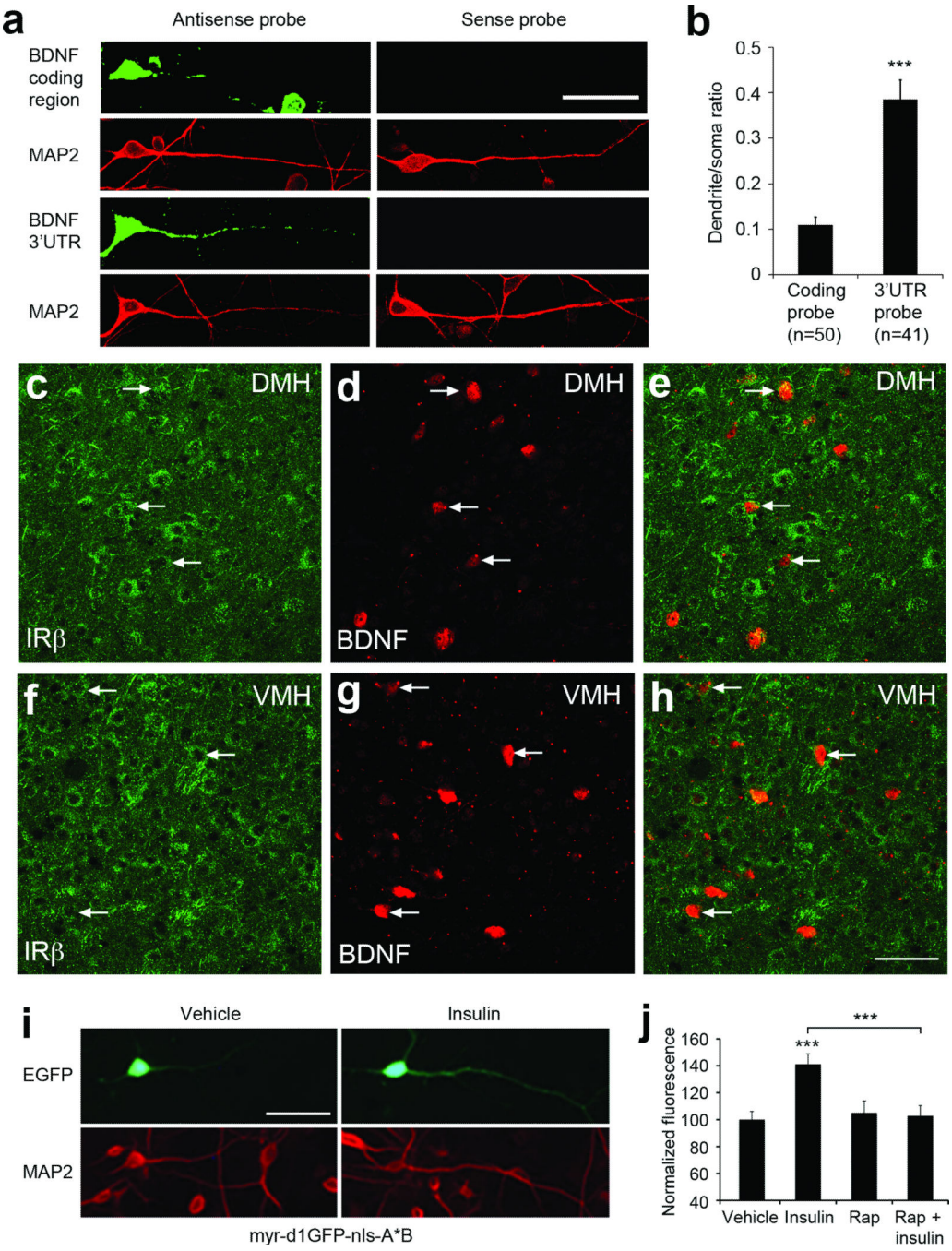

Figure 3.

Insulin stimulates local translation of transcripts containing the long *Bdnf* 3′UTR in the dendrites of hypothalamic neurons. (**a**) Fluorescent *in situ* hybridization showing the distribution of *Bdnf* mRNA in cell bodies and dendrites of cultured rat hypothalamic neurons. MAP2 immunostaining marked cell bodies and dendrites. Scale bar, 50 μm. (**b**) Ratio of the FISH signal in dendrites to that in cell bodies. (**c–h**) Co-expression of BDNF and the insulin receptor in the DMH and VMH of $Bdnf^{LacZ/+}$ mice. Arrows denote neurons expressing both BDNF and the insulin receptor. Scale bar, 50 μm. (**i**) Representative images

of hypothalamic neurons expressing myr-d1GFP-nls-A*B treated with either vehicle or insulin. MAP2 immunohistochemistry was used to reveal cell bodies and dendrites of cultured neurons. Scale bar, 100 μm. (**j**) Stimulatory effects of insulin on dendritic translation of myr-d1GFP-nls-A*B mRNA. Dendritic GFP fluorescence was measured at 150–200 μm away from the soma [n=38, 57, 21, and 42 neurons for the vehicle, insulin, rapamycin (Rap), and Rap + insulin treatments, respectively]. Error bars indicate standard errors of the mean. *** $P < 0.001$ by Student's *t* test.

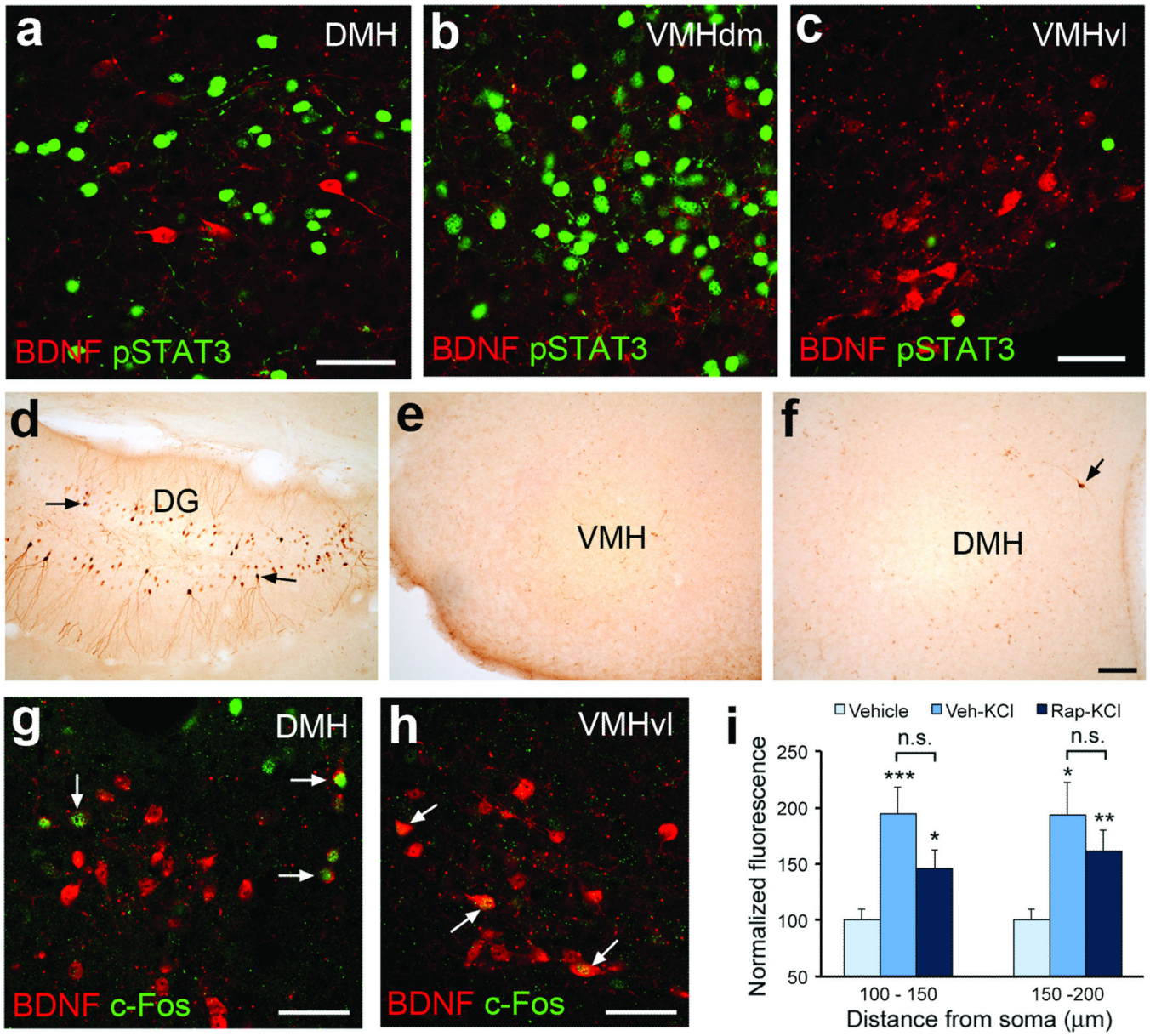

Figure 4.

Leptin activates hypothalamic BDNF-expressing neurons through network activity. (**a–c**) Confocal images showing lack of co-localization of BDNF and pSTAT3 in the DMH, dorsomedial VMH (VMHdm), and ventrolateral VMH (VMHvl) of $Bdnf^{LacZ/+}$ mice injected with leptin. Scale bar, 50 μm. (**d–f**) Microscopic images showing β-galactosidase immunoreactivity in the dentate gyrus (DG), VMH, and DMH in *LepR-Cre/+;*$Bdnf^{klox/+}$ mice. Arrows denote β-galactosidase-expressing neurons. Scale bar, 100 μm. (**g, h**) Confocal images showing some colocalization of BDNF and c-Fos in the VMH and DMH of leptin-injected $Bdnf^{LacZ/+}$ mice. Arrows denote representative BDNF-expressing neurons that are positive for c-Fos immunoreactivity. Scale bar, 50 μm. (**l**) KCl stimulation of dendritic local translation of myr-d1GFP-nls-A*B mRNA in hypothalamic neurons (n=16, 25, and 20 neurons for vehicle, vehicle + KCl, and Rap + KCl, respectively). Error bars represent

standard errors of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test; n.s., not significantly different ($P > 0.05$).

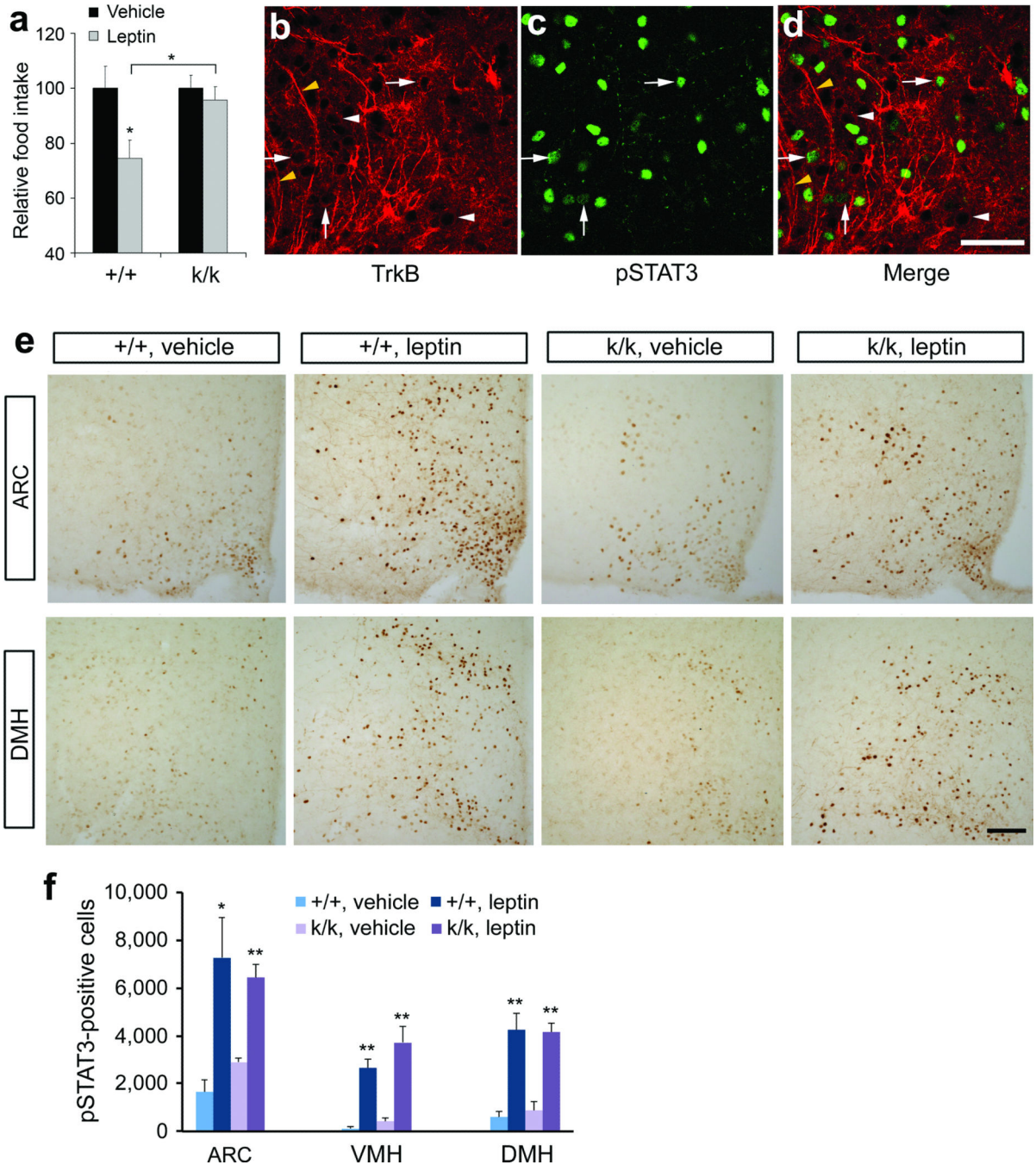

Figure 5.

Leptin normally activates the LepRb in *Bdnf*$^{klox/klox}$ mice. (**a**) Food intake response of young and lean *Bdnf*$^{klox/klox}$ (k/k) mice to leptin administration. (**b–d**) Co-expression of TrkB and pSTAT3 in the ARC of leptin-injected *TrkB*$^{LacZ/+}$ mice. White arrows denote representative neurons that contain both TrkB and pSTAT3. White arrowheads indicate representative neurons that express TrkB but are negative for pSTAT3. Yellow arrowheads indicate the TrkB^{+} processes of tanycytes that align the ventral 3rd ventricle. Scale bar, 50 μm. (**e**) Representative images of basal or leptin-induced pSTAT3 immunoreactivity in the ARC and

DMH of +/+ or k/k mice. Scale bar, 50 μm. (**f**) Quantification of pSTAT3-positive cells in the ARC, VMH, and DMH of +/+ and k/k mice (n=3 mice per group). Error bars represent standard errors of the mean. Comparison between the vehicle and leptin groups for each genotype was analyzed using Student's *t* test: * $P<0.05$ and ** $P<0.01$.

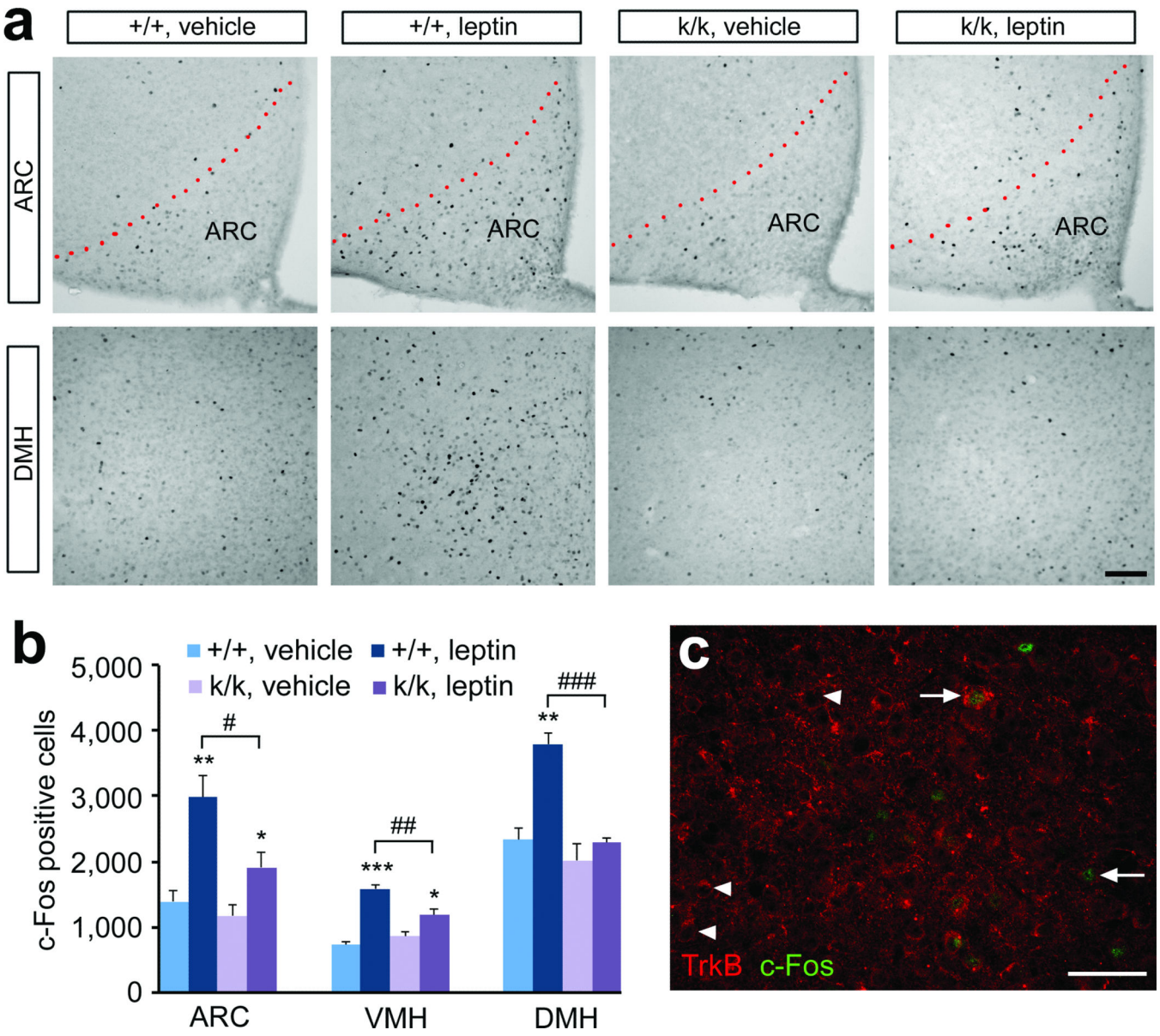

Figure 6.

Leptin-induced neuronal activation is impaired in *Bdnf*$^{klox/klox}$ mice. (**a**) Representative images of c-Fos immunoreactivity in the ARC and DMH of +/+ and k/k mice under basal or leptin-stimulated condition. Scale bar, 100 μm. (**b**) Counts of cells expressing c-Fos in the ARC, VMH, and DMH (n= 3 or 4 mice per group). Student's *t* test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with the vehicle group of the same genotype; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, and $^{\#\#\#}$ $P < 0.001$ when the two leptin groups of different genotypes were compared. Error bars represent standard errors of the mean. (**c**) Localization of c-Fos and TrkB in the DMH of a leptin-injected *TrkB*$^{LacZ/+}$ mouse. Arrows denote representative cells expressing both c-Fos and TrkB, whereas arrowheads denote representative cells expressing TrkB but not c-Fos. Scale bar, 50 μm.