Molecular Therapy of Melanocortin-4-Receptor Obesity by an Autoregulatory BDNF Vector

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Mutations in the melanocortin-4-receptor (MC4R) comprise the most common monogenic form of severe early-onset obesity, and conventional treatments are either ineffective long-term or contraindicated. Immediately downstream of MC4R—in the pathway for regulating energy balance—is brain-derived neurotrophic factor (BDNF). Our previous studies show that adeno-associated virus (AAV)-mediated hypothalamic BDNF gene transfer alleviates obesity and diabetes in both diet-induced and genetic models. To facilitate clinical translation, we developed a built-in autoregulatory system to control therapeutic gene expression mimicking the body’s natural feedback systems. This autoregulatory approach leads to a sustainable plateau of body weight after substantial weight loss is achieved. Here, we examined the efficacy and safety of autoregulatory BDNF gene therapy in Mc4r heterozygous mice, which best resemble MC4R obese patients. Mc4r heterozygous mice were treated with either autoregulatory BDNF vector or YFP control and monitored for 30 weeks. BDNF gene therapy prevented the development of obesity and metabolic syndromes characterized by decreasing body weight and adiposity, suppressing food intake, alleviating hyperleptinemia and hyperinsulinemia, improving glucose and insulin tolerance, and increasing energy expenditure, without adverse cardiovascular function or behavioral disturbances. These safety and efficacy data provide preclinical evidence that BDNF gene therapy is a compelling treatment option for MC4R-deficient obese patients.

INTRODUCTION

Obesity afflicts 17.3% of children and 36.5% of adults in the US,1 with an estimated annual cost of $147 billion.2 It is a major risk factor for other chronic illnesses, such as diabetes, cardiovascular disease, depression, sleep apnea, and cancer.3–5 Current treatments include a combination of diet, exercise, behavioral therapy, and pharmaceuticals, which are limited in efficacy partly due to low patient compliance.6–8 In extreme circumstances, bariatric surgery is recommended; however, the procedure is permanent and irreversible, with peri- and post-operative complications not limited to nutrient deficiency.9 Six forms of human monogenic obesity are primarily due to inactivating mutations in leptin-melanocortin signaling. Of these, inactivation of melanocortin-4-receptor (MC4R) comprise the most common monogenic form of severe early-onset obesity, with more than 150 distinct mutations reported to date.10,11 Moreover, conventional treatments for these patients are either ineffective long-term or contraindicated. Therefore, MC4R obesity represents an unmet need and a new approach is clearly needed.

Genetic studies demonstrate that brain-derived neurotrophic factor (BDNF) and its receptor TrkB are one of three known receptor-ligand pairs that have profound effects on body weight and food intake in both mice and humans; the other two pairs are leptin/leptin receptor and α-melanocyte-stimulating hormone (MSH), β-MSH, and Agrp with MC4R. BDNF is believed to function downstream of the leptin-proopiomelanocortin signaling pathway, playing a key role in the regulation of energy balance.12–15 In animal studies, MC4R appears to serve an intermediary role within the leptin pathway, acting downstream of the leptin receptor and upstream of BDNF signaling. Leptin-receptor-deficient db/db mice have decreased hypothalamic Bdnf expression, and their obesity and impaired glucose metabolism are ameliorated by intracerebroventricular administration of BDNF.16,17 Mc4r knockout mice are hyperphagic, obese, and have decreased hypothalamic Bdnf expression.12 The anorexigenic effects of MC4R activation can be blocked by administration of an anti-BDNF antibody into the third ventricle,13 and the orexigenic effects of MC4R antagonism are attenuated by BDNF co-administration into the fourth ventricle.14 Bdnf heterozygous knockout mice and humans also display hyperphagia and obesity.14–16 Conditional knockout of Bdnf in the postnatal brain15 as well as selective depletion of BDNF in the ventromedial and dorsomedial hypothalamus of adult mice also results in hyperphagia and obesity.22 Moreover, animals that are hypomorphic for TrkB, the high affinity receptor for BDNF, are obese,12 similar to humans.23 These data suggest an important role for BDNF serving as a final downstream mediator of the leptin-proopiomelanocortin pathway. In addition to genetic evidence, pharmacological studies also support the effects of BDNF on body weight.
BDNF on appetite and energy balance; moreover, it is likely crucial to deliver BDNF directly into the CNS and ideally into the hypothalamus.\textsuperscript{17,24–29} Recently, we have demonstrated that \textit{Bdnf} gene transfer to the arcuate/ventromedial nuclei (ARC/VMH) of the hypothalamus by adeno-associated virus (AAV) robustly reduces adiposity, improves glycemic control, alleviates metabolic syndromes, and decreases central inflammation while not affecting circadian activity and heart or bone function in diet-induced obesity (DIO) mice.\textsuperscript{30} To titrate \textit{Bdnf} gene dosage appropriately with decreasing body weight, we introduce a second gene cassette—within the same AAV vector—that could be physiologically autoregulated. This second cassette, microRNA against \textit{Bdnf} (miR-\textit{Bdnf}) driven by the orexigenic AGRP promoter, is activated in the hypothalamus following weight loss. Consequently, excessive BDNF-induced weight loss is therefore prevented by autoregulation of constitutive \textit{BDNF} transgene expression. This autoregulatory vector has been characterized in \textit{db/db} mice, in which mutated genes lie upstream of MC4R signaling. It is currently unknown whether modulating hypothalamic \textit{BDNF} signaling downstream of MC4R can attenuate obesity in \textit{MC4R-deficient} patients who are refractory to current treatments. Here, we determine the effects of \textit{BDNF} gene transfer to the ARC/VMH on attenuating \textit{Mc4r-deficient} obesity and evaluate the safety of a physiological autoregulatory vector to treat \textit{Mc4r} obesity.

RESULTS

Efficacy and Safety Assessments

To test our hypothesis, we used \textit{Mc4r\textsuperscript{loxTR/\textsuperscript{o}}/\textsuperscript{o}} mice, which are phenotypically heterozygous for the \textit{Mc4r} gene\textsuperscript{31} and representative of the human patient population afflicted with this monogenetic obesity.\textsuperscript{11} Constructs expressing the autoregulatory \textit{BDNF} cassette (auto\textit{BDNF}) or destabilized yellow fluorescent protein (YFP) control were packaged into serotype 1 AAV capsids\textsuperscript{30} (Figure 1A). Then, age-matched 3-month-old female and male heterozygous mice were randomized into the two treatment groups to receive bilateral hypothalamic injections of either AAV-auto\textit{BDNF} or AAV-YFP into the ARC/VMH nuclei of the hypothalamus (Figure 8A). At 18–20 weeks post-injection (wpi), metabolic parameters were measured (Figure 1B, above). On the other hand, the same group of mice were assessed for side effects due to CNS gene therapy and chronic hypothalamic-sympathoneural-adipocyte axis activation (Figure 1B, below).\textsuperscript{32} The safety studies examined behavioral affect as well as blood pressure before mice were sacrificed for tissue and serum analysis.

\textbf{BDNF Gene Therapy Prevents Obesity of \textit{Mc4r} Heterozygous Mice}

YFP-treated \textit{Mc4r} heterozygous mice rapidly gained body weight and developed obesity. In contrast, \textit{BDNF} treatment completely prevented obesity (Figures 2A and 2B). The \textit{BDNF}-treated cohort appeared lean compared to YFP-treated controls at 15 wpi (Figure 2A), and the normal body weight was maintained throughout the 30 weeks duration of the study (Figures 2B and S1A). At 30 wpi, the \textit{BDNF}-treated female mice gained 1.82 \(\pm\) 1.53 g in body mass, whereas a 19.43 \(\pm\) 2.37 g increase in body weight was observed in the YFP group over the same amount of time (Figure 2D). Similarly, \textit{BDNF}-injected males gained 12.56 \(\pm\) 4.53\% of their original body weight relative to a 61.99 \(\pm\) 11.26\% increase in starting body weight for YFP controls (Figure S1C). By the end of the experiment, the
average body weight of BDNF-treated females was 53.07% ± 2.20% of controls (Figure 2E); BDNF-treated males were 67.71% ± 5.25% of controls (Figure S1D).

**BDNF Gene Therapy Reverses Hyperphagia and Low Energy Expenditure of Mc4r Heterozygous Mice**

Next, to determine what attributed to the discrepancy in body mass between the two groups, home cage food intake was monitored over the course of the trial and energy expenditure was measured by indirect calorimetry at 18–20 wpi. Hyperphagia is the predominant cause of obesity for Mc4r patients as well as mice.33 Recording weekly food intake, by 28 wpi, BDNF mice were observed to be consuming 18.13 ± 0.11 g/mouse/week of normal chow pellets, much less than that of YFP mice (28.08 ± 0.29 g/mouse/week) (Figure 3A). Likewise, male mice in the autoBDNF group appeared to consume less normal chow per week than their YFP counterparts (Figure S2A). Regarding energy expenditure, indirect calorimetry was measured at 18–20 wpi over a 24-hr period after habituation. Interpreting the results of oxygen consumption can be problematic in obesity models because adipose tissue is considered to be metabolically less active than lean tissues,34 and autoBDNF-treated mice were almost half the body weight of YFP mice (Figure 3B, 23.92 ± 1.85 g versus 40.03 ± 3.23 g, respectively) or two-thirds the body weight of YFP mice in males (Figure S2B). Therefore, the oxygen consumption data were calibrated normalized to body weight, and energy expenditure was individually plotted according to lean mass (Figures 3D, 3E, S2D, and S2E). Oxygen consumption in BDNF-treated mice was significantly increased in both dark and light phases compared to YFP mice (Figures 3D and S2D). Nonlinear regression analysis of energy expenditure versus lean mass in each group demonstrated increased slope in the autoBDNF mice relative to controls (Figure 3E and S3E). The respiratory exchange ratio (RER) was increased in the BDNF group during the dark phase, suggesting increased carbohydrate oxidation as opposed to lipid oxidation (Figures 3F and S2F). Physical activity was elevated in both groups during the dark hours and diminished during the light phase, with no difference observed between the two groups (Figures 3G and S2G). Food intake was also not different during this period in the metabolic chambers (Figures 3C and S2C). The absence of changes in physical activity and food consumption indicated that BDNF treatment elevated the resting metabolic rate. Rectal temperatures measured at 15 wpi revealed no significant differences between the two groups (Figures 3H and S2H).

**BDNF Treatment Reduces Adiposity and Alleviates Hepatic Steatosis**

We next wondered what tissues may have been affected by this net negative energy balance. To measure adiposity, echoMRI analysis was performed at 28 wpi. Absolute fat mass was 6.73 ± 2.28 g in the BDNF mice compared to 24.14 ± 3.68 g in the YFP control group (Figure 4A). For males, fat mass was 3.77 ± 2.11 g in the autoBDNF cohort compared to 14.99 ± 1.93 g in the YFP control group (Figure S3A). BDNF treatment led to over 50% reduction of adiposity: fat mass, relative to body weight, was 21.95% ± 4.53% in BDNF mice in contrast to 47.95% ± 3.09% in YFP mice (Figure 4B). This magnitude of reduction in adiposity was also observed in BDNF males (Figure S3B). Conversely, proportionate lean mass was significantly higher in BDNF mice compared to controls (71.99% ± 4.28% versus 52.00% ± 2.63%, respectively). During sacrifice at 30 wpi, along with pancreas and liver, four adipose tissue depots were weighed: brown adipose tissue (BAT), inguinal white adipose tissue (IWAT), gonadal white adipose tissue (GWAT), and retroperitoneal white adipose tissue (RWAT). Significant reductions in absolute mass were confirmed across all four fat deposits in the BDNF-treated mice (Figures 4C and S3C). The weight of the pancreas or liver did not differ significantly among the two treatment groups when normalized to body weight (Figures 4D and S3D). Histologically, H&E sections showed decreased the size of white adipocyte and lack of white adipocyte infiltration in
BAT in BDNF mice (Figure 4E). Additionally, BDNF treatment alleviated liver steatosis, as shown by oil red O staining (Figure 4F). Observations of other fat deposition in the body revealed qualitatively smaller mesenteric, perirenal, and pericardial adipose tissue (Figure 4G).

BDNF Treatment Improves Glycemic Control and Reverses Hyperleptinemia and Hyperinsulinemia

Symptoms of Mc4r mutant mice include hyperleptinemia, hyperinsulinemia, and slight hyperglycemia. To address whether these symptoms were alleviated, a glucose tolerance test was first performed at 16 wpi. BDNF-treated mice displayed lower fasting blood glucose (82.50 ± 6.41 mg/dL) compared to controls (158.67 ± 9.98 mg/dL) and significantly improved glucose tolerance (Figures 3A and S3E). In addition, an insulin tolerance test was performed at 30 wpi. Control mice failed to respond to the insulin challenge, indicating severe insulin resistance (Figures 5B and S3F). In contrast, BDNF-treated mice displayed substantial improvement in insulin tolerance (Figures 5B and S3F). Serum was harvested at sacrifice to examine metabolic parameters. BDNF treatment robustly alleviated hyperleptinemia (Figure 5C) and hyperinsulinemia (Figure 5D) of Mc4r-deficient mice. No significant differences were observed in adiponectin, insulin-like growth factor 1 (IGF-1), glucose, or triglycerides in fed mice (Figure 5E).

Behavioral Assessments Reveal No Adverse Psychological Effects

Besides evaluating metabolic efficacy, we were equally if not more interested in the long-term safety of this approach to mitigating Mc4r obesity. Therefore, we performed various assays to screen for changes in anxiety- or depression-like behavior from 20 to 22 wpi. The open field (OF) test is classically used to assess exploratory behavior, general locomotion, and anxiety. OF draws on the natural conflict between the tendency to explore a new environment and avoid an exposed open area. An increase in time spent in the center of the open field is considered to reflect reduced anxiety level. The BDNF-treated mice spent more time (Figure 6A) and significantly traveled proportionally more distance in the center of the arena (Figure 6B), suggesting an anxiolytic effect. The BDNF-treated mice additionally exhibited more locomotion (Figure 6C). The elevated T-maze is an ethologically based approach-avoidance conflict test targeting the natural conflict between the tendency of mice to explore a novel environment and the tendency to avoid a brightly lit open area. Unlike the OF test, the number of open arm entries observed during the elevated plus maze was unchanged for the BDNF treatment group.
(Figure 6D). Likewise, the time spent in the open arms did not differ between the two cohorts (Figure 6E). In another anxiety behavior test, cold-induced defecation (CID), BDNF mice showed no difference in fecal boli compared to YFP mice (Figure 6F). Additionally, the novelty suppressed feeding (NSF) test assesses hyponeophagia, in which exposure to a novel environment suppresses feeding behavior. NSF has been used to study anxiety- and depression-related behaviors because it is sensitive to anxiolytic and chronic antidepressant treatments. BDNF treatment did not change the latency to feed or the total amount of food consumed in the 10 min testing period (Figures 6G and 6H). With respect to assays for depression, the tail suspension and forced swim tests (FST) were employed. For the tail suspension test, the time being immobile was significantly diminished in the first 2 min of the 6-min test for BDNF-expressing mice (Figure 6I). The total amount of time immobile, though, was not significantly changed in the BDNF mice (Figure 6J). In parallel, the FST is one of the most commonly used rodent behavioral tests for screening antidepressant drugs. No significant effect was observed in FST (Figures 6K and 6L). Lastly, home cage activity was monitored with night vision recordings, and no differences were observed (data not shown).

Blood Pressure

Peripherally, obese MC4R-deficient mice and patients exhibit decreased blood pressure relative to control subjects. Activation of arcuate neurons and the sympathetic nervous system, a consequence of our BDNF gene therapy, may potentially increase heart rate and blood pressure. Tail cuff manometry demonstrated no significant differences in either systolic or diastolic blood pressure between the groups (Figures 7A and 7B). Moreover, pulse was not altered between groups (Figure 7C).

Hypothalamic Gene Expression

Finally, to verify the expression of our vector, sustained transgene expression was confirmed by immunohistochemistry and qRT-PCR by the end of the study. Representative brain sections were analyzed...
for YFP fluorescence in the control group, along with immunofluorescent staining of hemagglutinin (HA) tag in the autoBDNF group. In the hypothalamus, expression of either vector was observed in the ARC and VMH nuclei of the hypothalamus (Figures 8A and 8B). Further expression profiling of the whole hypothalamus confirmed autoregulation at the mRNA level (Figure 8C). BDNF and Agrp were both significantly increased 18.05-fold (±4.48) and 1.85-fold (±6.41), respectively, along with Crh (1.86-fold ±0.26) in the autoBDNF mice. No significant changes in inflammatory markers were observed (Figure 8C).

**DISCUSSION**

Recommendations for current primary care practices in the management of obesity involve a combination of behavioral counseling,6 pharmaceuticals,8 lifestyle modification,7 or bariatric surgery in extreme circumstances.46,47 Applied to obese MC4R-deficient patients, these lifestyle interventions were ineffective in children48 and adults49 because MC4R may be required for sustained weight loss.49 Bariatric surgery has had conflicting results. Gastric banding efficacy may depend on the number of functional MC4R alleles50,51 or the specific mutation within the MC4R gene.52 Roux-en-Y gastric bypass (RYGB) may be more effective because it leads to similar weight loss in heterozygous MC4R patients versus controls,53,54 but patient samples in these studies were small. Bariatric surgery is also disadvantageous due to a complication rate of 17%, including nutrient deficiency, and post-operative mortality, which could impact patients’ quality of life.55,56 Altogether, these treatments target the periphery, but the primary defect in energy homeostasis originates within the brain. Deep brain stimulation studies are underway but have surgical complications as well.56,57 Because many treatment options fail or are contraindicated for this patient cohort, there is an unmet need for novel therapeutics for MC4R-deficient obesity.

Regarding MC4R, the main shortcomings of current studies with small molecule agonists for MC4R are that either the patient sample size is small,58 the human patients recruited are deficient in genes besides MC4R, or some being tested raise the concern of hypertension following treatment.59 MC4R is a G protein coupled receptor (GPCR) that can bind agonists as well as antagonists and can couple to the three major classes of G proteins, stimulating multiple transduction pathways.60 Moreover, MC4R has a potential role in sexual function.61 Consequently, pharmacological modulation of feeding circuitry at the level of this GPCR may be challenging, given the existence of competing endogenous ligands and potential unwanted systemic side effects.

Gene therapy of genetic diseases provides a significant advantage over infusion of proteins because it provides a potentially long-term and stable delivery method. Moreover, the therapeutic vector needs to be only given once and the limited experience of stereotactic delivery of AAV vector in the human brain has shown excellent safety and tolerability.62–65 There have been several studies on hypothalamic gene therapy for obesity.66–72 The majority of these studies used viral vectors to overexpress leptin or the leptin receptor in order to avoid the problems of peripheral leptin delivery. However, in DIO rats, an AAV vector expressing leptin failed to decrease body weight, consistent with an obesity-induced leptin resistance at the
hypothesis because leptin gene therapy showed a robust effect in animals of normal weight.\textsuperscript{73} An alternative therapeutic gene is POMC. The hypothalamic injection of an AAV vector expressing POMC led to significant weight loss and improved insulin sensitivity in obese rats.\textsuperscript{74,75} However, the same authors reported that AAV-POMC predisposed the lean rats to increased weight gain when the animals were switched to a high-fat diet.\textsuperscript{76} The authors concluded that the hypothalamic overexpression of POMC led to downregulation of MC4R and the adaptive response countered the anorexigenic activity.

In a variety of animal studies in lean and diet-induced or genetic models of obesity, we have shown that BDNF gene transfer in the hypothalamus efficiently reduced adiposity, elevated energy expenditure, and alleviated obesity and diabetes.\textsuperscript{30,77} Here, in the first gene therapy study of MC4R obesity, we applied the autoregulatory BDNF vector to the \textit{Mc4r} heterozygous mice that best recapitulated the human MC4R-deficient patients. Long-term BDNF gene therapy to \textit{Mc4r}-deficient obese mice led to a persistent alleviation of hyperphagia, along with a concomitant increase in energy expenditure. The suppression of food intake was more pronounced than that observed in \textit{db/db} mice, whereas no change or increased food intake was found in BDNF-treated wild-type mice of normal weight, suggesting that \textit{Mc4r}-deficient mice were more sensitive to BDNF treatment. In addition to the complete correction of the hyperphagia, hyperleptinemia, and hyperinsulinemia of this monogenic disease, BDNF gene therapy also increased the basal metabolic rate. Weight loss through dieting and exercise could result in a compensatory decrease in resting metabolic rate\textsuperscript{78} in order to achieve homeostasis, which partially explains the difficulty of maintaining weight loss. Thus, the elevation of basal metabolic rate by BDNF gene therapy could aid in resetting the homeostatic set point for body weight to a lower energy level.

With respect to the safety of BDNF levels on behavior, most studies associate a reduction in the neurotrophin with cognitive deficits. Post-natal deletion of \textit{Bdnf} resulted in increased anxiety along with obesity,\textsuperscript{21} whereas forebrain-specific deletion led to impairments in spatial learning and certain pattern discrimination tasks.\textsuperscript{79} Clinically, low serum levels of BDNF were correlated with depression in patients.\textsuperscript{80,81} In one instance, excess BDNF increased seizure activity, although expression of \textit{Bdnf} was widespread among a variety of tissues.\textsuperscript{82} In this study, our battery of behavioral assessments demonstrated the absence of adverse behavioral effects associated with BDNF gene therapy.

Titrating the gene dosage for every patient is challenging. The ideal goal would be to deliver in only one surgery one viral vector that can be intrinsically regulated by the host’s physiological needs. This study demonstrated that a physiological autoregulatory vector of BDNF achieved a sustainable plateau of weight loss in \textit{Mc4r}-deficient mice. Future studies on dosing and unilateral versus bilateral injections in a larger animal model will reinforce the safety of this molecular therapy.
Besides MC4R obesity, AAV-Bdnf gene therapy is broadly applicable toward mitigating other forms of obesity. This includes not only monogenic obesity syndromes with defects in leptin-melanocortin signaling, but also genetic diseases with obesity symptoms due to dysregulated BDNF signaling, such as Prader-Willi (PWS) or WAGR syndrome.53,83 This is especially true for Prader-Willi syndrome, in which intense binge feeding would be a contraindication for bariatric surgery.53 Risk of obesity-associated diseases like diabetes and cancer are expected to decrease once obesity is treated, thus compounding the implications of this study.

In conclusion, hypothalamic BDNF gene therapy with an autoregulatory AAV vector corrects obesity and metabolic syndromes of MC4R-deficient obesity in a clinically relevant mouse model. Energy homeostasis is reset by both a reduction of food intake and increase of energy expenditure, with no adverse effects at the behavioral and cardiovascular level. This preclinical study provides efficacy and safety evidence indicating a promising targeted molecular therapy for monogenic obesity.

MATERIALS AND METHODS

Animals

Mc4rloxTB/+ mouse breeders were purchased from Jackson Laboratory (Stock No. 006414, Bar Harbor, ME). Mouse litters were group housed (no more than five per cage) in a 12:12 light:dark cycle, with ad libitum access to standard rodent chow and water in a humidity- and temperature-controlled environment. All animal experiments were carried out in compliance and conform to the regulatory standards of the Ohio State University Institutional Animal Care and Use Committee.

rAAV Vector Constructs

The recombinant AAV (rAAV) vector plasmid contains the following expression cassette flanked by inverted terminal repeats (ITRs): cytomegalovirus enhancer plus chicken β-actin promoter (CBA), woodchuck posttranscriptional regulatory element (WPRE), and bovine growth hormone polyadenosine (BGH polyA) tail. Between the CBA and polyA is a multiple cloning site (MCS), in which HA-tagged human Bdnf (AAV-HA-Bdnf, referred to simply as Bdnf) or destabilized YFP control (AAV-dsYFP, referred to as YFP) is inserted. The second cassette with Agrp minimal promoter driving miR-Bdnf expression is cloned after the first cassette—containing Bdnf—within the ITRs. All vectors were packaged into serotype 1 capsids and purified by iodixanol gradient centrifugation.

Stereotoxic Surgery

3-month-old Mc4rloxTB/+ mice were randomly assigned to receive AAV-Bdnf-miR-Bdnf or AAV-YFP. Mice were anaesthetized with a single intraperitoneal dose of ketamine/xylazine (100 mg/kg and 20 mg/kg) and secured via ear bars and incisor bar on a Kopf stereotoxic frame (Tujunga, CA). A single midline incision was made through the scalp to expose the skull, and two small holes were made with a dental drill above the injection sites. rAAV vectors were adjusted to $1.0 \times 10^{10}$ genomic particles per μL, and then administered bilaterally by a 10-μL Hamilton syringe (Reno, NV) attached to a Micro4 Micro Syringe Pump Controller (World Precision Instruments, Sarasota, FL) at a rate of 100 nL/min for a total of 0.5 μL into the arcuate/ventromedial hypothalamus (anteroposterior [AP]: $-1.20$ mm; medial lateral [ML]: ± 0.50 mm; dorsal ventral [DV]: $-6.20$). When the infusion was finished, the syringe was slowly retracted from the brain, the scalp was sutured, and mice were administered buprenorphine for pain relief (0.5 mg/kg). Animals were returned to clean cages—with HydroGel (ClearH2O, Westbrook, ME) provided for supplemental hydration—resting atop a 37°C heating pad and carefully monitored post-surgery until fully recovered.

Food Intake and Body Weight

Following surgeries, body weight and food intake—on normal chow diet—were recorded every 5–7 days. Animals injected with the same vector remained housed together, whereas animals injected with different vectors were separated into different cages post-surgery. Food intake was averaged per mouse per week in each cage. Animals were monitored up to 30 wpi.

Body Temperature

At 15 wpi, rectal temperature was measured at 2 PM for all mice after 5 min sedation with 2.5% isoflurane. The Physitemp BAT —12 rectal
thermometer (Clifton, NJ) remained in place for 30 s to allow temperature to stabilize before being recorded. Mice were then returned to their home cages to recover.

Glucose Tolerance Test
Glucose tolerance test was performed at 16 wpi. After an overnight fast (>16 hr), mice were injected intraperitoneally with 20% glucose solution (1 mg/kg body weight) at 10 AM. Tail blood was collected at 0, 15, 30, 60, 90, and 120 min post-injection. Blood glucose concentrations were measured with a portable glucometer (Bayer Contour Next, Parsippany, NJ).

Energy Expenditure
At 18–20 wpi, mice underwent indirect calorimetry using the Oxymax Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH). Mice, singly housed with access to ample food and H2O, acclimated to the metabolic chambers for 1 day, and then behavioral and physiological parameters (O2 consumption, CO2 production, respiratory exchange ratio, food intake, H2O consumption, temperature, and physical activity) were recorded at room temperature for up to 2 days.

echoMRI
Mice at 29 wpi were subjected to echoMRI scanning in order to measure body composition of fat, lean, free water, and total water masses in live, awake mice. Mice were placed—and free to move—in a specially sized, clear plastic holder without sedation or anesthesia. The holder was inserted into a tubular space inside an EchoMRI 3-in-1 (EchoMRI, Houston, TX) machine. Each scan took less than 5 min.

Insulin Tolerance Test
Insulin tolerance test was performed at 30 wpi. Non-fasted mice were injected intraperitoneally with insulin (0.75 U/kg body weight) at 2 PM. Tail blood was obtained at 0, 15, 30, 60, 90, and 120 min post-injection, and blood glucose levels were measured as described for the glucose tolerance test.

Open Field Test
To assess exploration, general motor activity, and anxiety, mice were placed individually into the center of an open square arena (60 cm × 60 cm, enclosed by walls of 48 cm) for 10 min. Activity was recorded and analyzed by TopScan software (Clever Sys, Reston, VA). The parameters measured include distance traveled in the periphery, distance traveled in the center of the arena (36 cm × 36 cm), and total distance traveled in the arena. The ratios of distance traveled in the periphery and center areas provide an indication of anxiety, whereas the total distance traveled provides a measure of exploratory and general motor activity. The arena was cleaned with 70% ethanol between trials to remove odor cues.

Elevated Plus Maze
To assess exploration, general motor activity, and anxiety, mice were placed individually into the center of an elevated plus maze with two open and closed arms (50 cm height, 52 × 10 cm open arms, 52 × 10 × 38 cm3 closed arms) for 5 min. Activity was recorded and analyzed by TopScan software (Clever Sys, Reston, VA). The parameters measured include entry to the open arms, time in the open arms, and total distance traveled in the arena. The entry and duration in the open arms provide an indication of anxiety, whereas the total distance traveled provides a measure of exploratory and general motor activity. The arena was cleaned with 70% ethanol between trials to remove odor cues.
NSF
Mice were fasted overnight with food removed at 1730 hr. The testing phase was conducted the next day at 1400 hr. Mice were individually placed into a brightly lit novel open cage (38 cm × 24 cm × 22 cm). A round piece of white filter paper (7-cm diameter) was placed in the center of the cage with a single pre-weighed food pellet. The latency to consumption (first bite of the food pellet) was recorded as a measure of anxiety-like behavior. The cut-off time was 10 min. To assess if there was any difference in consumptive drive, each mouse was placed in a standard cage with the pre-weighed food pellet after its first bite or at cut-off time if it failed to eat within 10 min. The amount of food consumed in 5 min was measured.

CID
A large container was filled halfway with ice. Small novel cages (28 × 16 × 12 cm³) were placed on top of the ice. Mice were placed individually into the cages, and then lids were placed on top. After 20 min, mice were removed and the number of fecal matter was counted as a measure of anxiety-like behavior. Mice were allowed to recover in a cage partially on a heating pad for 1 hr prior to returning to its home cage. All cages used were cleaned with 70% ethanol between trials.

Tail Suspension Test
A small cylindrical tube (Becton Dickinson, Franklin Lakes, NJ) was slipped over the mouse tail to prevent climbing motion and escape from the test. Mice were suspended in air individually by tape attached to a shelf (64-cm height) for 6 min. Trials were video-recorded and a blinded experimenter scored the amount of time mice remained immobile as a measure of depressive-like behavior.

FST
Mice were placed individually in a transparent cylinder (21 cm in diameter, 24 cm in height) containing water (25 ± 2°C) to a depth of 15 cm for 6 min. At the end of each trial, mice were dried and returned to their home cage. Trials were video-recorded, and a blinded experimenter scored the amount of time mice remained immobile as a measure of depressive-like behavior.

Home Cage Activity
Before the initiation of the dark phase of the mouse circadian cycle, a night view camera was set up to record home cage activity for 24 hr.

Tail Cuff Manometry
At 28 wpi, awake mice were placed into a restrictive plastic tube at 2 PM for blood pressure recordings with the Kent Scientific CODA High Throughput tail-cuff blood pressure system (Torrington, CT). Mice were subjected to the tail cuff for 20 recording cycles. After the first 10 cycles for habituation, if at least 5 stable recordings were measured in the next 10 cycles, stable values in those 10 cycles were averaged for that mouse. Otherwise, up to two more attempts of 20 cycles were made the same day to obtain reliable measurements. Upon completion of recording data, the mouse was returned to its home cage.

Serum Harvest and Analysis
Truncal blood was collected at 10 AM following decapitation at sacrifice. Serum was allowed to clot on ice for at least 30 min before centrifugation at 10,000 rpm for 10 min at 4°C. Serum was collected and stored at −20°C until further analysis. Biomarkers were analyzed with the following kits: glucose, triglyceride, and cholesterol with Cayman Colorimetric Assay kits (Ann Arbor, MI), leptin, IGF-1, and adiponectin/Acrp30 with R&D DuoSet ELISA Development Systems (Minneapolis, MN) and insulin with Alpco Mouse Ultrasensitive Insulin ELISA (Salem, NH).

Adipose Tissue Histology
Adipose tissue depots (brown, inguinal, gonadal, and retroperitoneal) were fixed in 10% formalin (w/v), then transferred to 70% ethanol, embedded in paraffin, and sectioned at 4 μm thickness. H&E staining was then performed, and stained sections were imaged on a Zeiss Axioskop 40 light microscope (Goettingen, Germany) to assess fat cell morphology and size.

Liver Histology
Liver was dissected at sacrifice, snap frozen on dry ice, and stored at −80°C. For sectioning, liver tissue was embedded in O.C.T. (Sakura Finetek, Torrance, CA) before being sectioned into 10-μm slices on a Leica cryostat. Lipids in frozen liver sections were then stained by using an oil red O solution (Sigma, St. Louis, MO).

Perfusion
At 30 wpi, mice were intracardially perfused with 4% paraformaldehyde (PFA) (Sigma, St. Louis, MO) in PBS, and fixed brains were incubated in 4% PFA on a rocker overnight at 4°C. The next day, brains were rinsed in PBS three times before being submerged in 30% sucrose in PBS and 0.03% sodium azide for at least 3 days on a rocker at 4°C. Brain tissues were then embedded in O.C.T. (Sakura Finetek, Torrance, CA) before being sectioned into 30-μm slices on a Leica cryostat.

Immunohistochemistry
Slides with cryosections were dried, washed in PBS, and blocked in 5% normal goat serum, 1% bovine serum albumin, 0.3% Triton TX-100, 0.3M glycine, and 0.03% sodium azide in PBS for 1 hr. Rabbit anti-HA (Cell Signaling, Cat. No. 2250, 1:1,600, Danvers, MA) was diluted in blocking buffer and applied onto slides overnight at 4°C. The following day, slides were washed three times in PBS before incubation with Alexa Fluor secondary antibody (Invitrogen, Carlsbad, CA) and nuclear counterstain DAPI in blocking buffer at room temperature for 1 hr. After three washes in PBS, slides were mounted using Aqua-Poly/Mount (Polysciences, Warrenton, PA) and coverslipped. Fluorescence microscopy was performed on a Zeiss microscope (Thornwood, NY), and images were captured with Zen Pro software. Confocal microscopy was performed on a FluoView FV1000 microscope (Zeiss, Oberkochen, Germany).
qRT-PCR

Hypothalamus was block dissected from mouse brains at sacrifice 30 wpi. Tissue was sonicated, and RNA was isolated using the QIAGEN RNeasy Mini kit with RNase-free DNase treatment (Germantown, MD). Next, cDNA was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Finally, qPCR was carried out on an ABI PRISM 7000 Sequence Detection System using the Power SYBR Green PCR Master Mix. Primers designed to detect mouse mRNA include Bdnf, Agrp, Npy, Cartpt, Pomc, Insr, Mc4r, Obrb, Ntrk2, Crh, Il1b, Nkbia, and Socs3. Primer sequences are available on request.

Statistical Analysis

Data are expressed as mean ± SEM. We used Prism Mac version 6.0f software (GraphPad, La Jolla, CA) and SPSS Statistics v24.0.0.0 (IBM, Armonk, NY) to analyze the following: Student’s t test for body weight or food intake at single time points, adiposity, body temperature, organ weights, serum ELISAs, behavior, blood pressure, and qRT-PCR data. Mixed analysis of variance was performed on time course measurements (body weight, VO2, RER, physical activity, glucose tolerance test [GTT]), and insulin tolerance test [ITT]).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at https://doi.org/10.1016/j.omtm.2017.09.005.

AUTHOR CONTRIBUTIONS

J.I.S. and N.J.Q. designed the studies, carried out the research, interpreted the results, and wrote the manuscript. X.L., W.H., and T.M. carried out the research. L.C. conceived the concept, designed the studies, interpreted the results, and revised the manuscript. All authors approved the manuscript.

CONFLICTS OF INTEREST

L.C. is an inventor of US patent 9,265,843 B2 on the autoregulatory BDNF vector. All the other authors declare no conflict of interest.

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