Identification of hypothalamic Neuron-derived Neurotrophic Factor (NENF) as a novel factor modulating appetite

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ABSTRACT

Disruption of finely-coordinated neuropeptide signals in the hypothalamus can result in altered food intake and body weight. We identified neuron-derived neurotrophic factor (Nenf) as a novel secreted protein through a large scale screen aimed at identifying novel secreted hypothalamic proteins that regulate food intake. We observed robust Nenf expression in hypothalamic nuclei known to regulate food intake, and its expression was altered under the diet-induced obese (DIO) condition relative to the fed state. Hypothalamic Nenf mRNA was regulated by brain-derived neurotrophic factor (BDNF) signaling, itself an important regulator of appetite. Delivery of purified recombinant BDNF into the lateral cerebral ventricle decreased hypothalamic Nenf expression, while pharmacological inhibition of trkB signaling increased Nenf mRNA expression. Furthermore, recombinant NENF administered via an intracerebroventricular cannula decreased food intake and body weight and increased hypothalamic Pomc and Mc4r mRNA expression. Importantly, the appetite-suppressing effect of NENF was abrogated in obese mice fed a high-fat diet, demonstrating a diet-dependent modulation of NENF function. We propose the existence of a regulatory circuit involving BDNF, NENF, and melanocortin signaling. Our study validates the power of using an integrated experimental and bioinformatic approach to identify novel CNS-derived proteins with appetite-modulating function and reveals NENF as an important central modulator of food intake.
INTRODUCTION

Energy homeostasis involves finely-tuned coordination of signals arising from both peripheral sources and the central nervous system (CNS) to establish a well-balanced homeostatic feedback loop. Hypothalamic neuropeptides and other secreted proteins play a critical role in modulating body weight and food intake. Lesions of the lateral nuclei of the hypothalamus result in anorexia, and while lesions of both the ventromedial (VMH) or paraventricular nuclei (PVN) result in excessive food intake and body weight gain (3, 22). Further, disruption of hypothalamic neuropeptide signals (e.g., brain-derived neurotrophic factor; Bdnf, Agouti-related protein; Agrp) in loss-of-function mouse models can lead to metabolic diseases and obesity (48, 63).

Neurotrophic factors act as neuromodulators to maintain growth, survival, and differentiation of neurons (24, 49). Genetic evidence implicates the BDNF signaling pathway in regulating feeding and body weight. A missense mutation in the BDNF receptor, Tyrosine kinase receptor B (TRKB), has been identified in severely obese children (19, 59). Additionally, postnatal Bdnf deletion and haploinsufficiency both produce hyperphagia and obesity in mice (17, 46). Further, mice lacking the melanocortin-4 receptor (Mc4r) exhibit excessive weight gain, and Bdnf has been shown to be a downstream effector by which Mc4r signaling modulates energy balance (6, 58).

Thus, the Bdnf/trkB signaling pathway contributes toward maintaining energy homeostasis in the CNS, and its disruption results in increased susceptibility to obesity.

To discover novel regulators of feeding and body weight, we conducted a large-scale experimental and bioinformatic screen for hypothalamic factors that may signal
downstream of Bdnf/trkB to regulate food intake and body weight. Several characteristics prompted us to select one candidate protein from this screen for in-depth characterization, Neuron-derived Neurotrophic Factor (Nenf or neudesin). Mouse Nenf contains 171 amino acids, is 91% similar to human NENF, is primarily expressed in neurons, but is absent from glia (31). In addition, it is expressed in white adipose tissue (28). Nenf is a secreted heme-binding protein with neurotrophic and neuroprotective activities, and possesses the ability to suppress adipocyte differentiation (28-31). However, a role for NENF in energy balance has not yet been explored.

We show here that BDNF signaling inversely regulates expression of NENF in the CNS, and that melanocortin signaling also regulates Nenf mRNA expression in response to food intake. Since increased levels of hypothalamic BDNF suppress food intake and body weight and alter gene expression (13, 55), our findings suggest that NENF may function as a downstream effector of the Bdnf/trkB pathway to influence melanocortin signaling and appetite, and that this relationship is disrupted in DIO mice. Indeed, central administration of recombinant NENF significantly increased Pomc and Mcr4 mRNA expression. This phenomenon was not observed in DIO mice, suggesting that overfeeding results in potential resistance to NENF signaling. These findings demonstrate the power of the integrated approach used here to identify novel hypothalamic regulators of ingestive behavior.

METHODS

Animals and housing
C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME) were used for all studies, and all mice were fed their respective diets from weaning. Wild-type mice were fed a standard rodent chow diet (2018, Harland-Teklad). Diet-induced obese (DIO) mice were fed a high-fat diet (60% kcal derived from fat content; Research Diets, Inc., D12492i). TrkB<sup>F616A</sup> knock-in mice (14) were backcrossed to C57BL/6J, and these mice were also fed a standard rodent chow (2018, Harland-Teklad). All mice were individually housed in clear plexiglass cages, with a solid bottom that contained alpha dry or corn cob bedding. Before the start of a feeding experiment, mice were adapted to wire mesh floors for 3 days to allow for collection of food spillage throughout the study. The housing environment was regulated to maintain a constant temperature (72 °F), humidity (45% ± 5) and a 12 h: 12 h light-dark cycle. All animals had access to water *ad libitum* and their respective diets were restricted only during indicated fasting times. All animal experiments were approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine and were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

**Experiment 1:** Screenng strategy identified novel hypothalamic secreted proteins that may regulate neural pathways associated with food intake.

In silico analysis

Publically available *in situ* hybridization (ISH) data from adult male C57BL/6J mice was obtained Allen Brain Atlas (5) and cross-referenced against *in situ* hybridization data generated in our own lab in the course of a large-scale microarray and ISH-based screen aimed at identifying genes that regulate mouse hypothalamic
development (9, 50). Genes that appear to show stronger expression in hypothalamus relative to other brain regions were then cross-checked against the SAGE Genie database (http://cgap.nci.nih.gov/SAGE) to confirm enrichment in hypothalamus relative to both other neuronal and non-neuronal tissues. Sequences of genes that showed prominent and selective expression in adult hypothalamus were then screened for the presence of a signal peptide (http://uswest.ensembl.org/index.html). The chromosomal locations of putative secreted proteins were then checked against the human obesity gene map (41) to ascertain whether they mapped to genomic regions that contain a QTL implicated in the regulation of body composition or food intake. Potential function of the candidate protein was checked against the PubMed database (http://www.ncbi.nlm.nih.gov) to ensure that no previous study implicated the candidate proteins in appetite control.

The following five criteria were applied to further narrow candidates for functional characterization: 1) enriched for expression in hypothalamic nuclei known to regulate body composition, hunger, satiety, or the endocrine axis; 2) maps to the genomic region containing a QTL implicated in the regulation of body composition or food intake; 3) contains a signal peptide sequence in the N-terminus for protein secretion, as our focus is on identification of novel secreted neural proteins that regulate food intake; 4) shows altered gene expression under fed, fasted, and diet-induced obese (DIO) conditions; and 5) no previous study implicates the candidate protein in appetite or weight control.

Experiment 2: Changes in physiological state of energy balance regulate Nenf gene expression.
“Fed” control mice (8 weeks of age, n=4) were given *ad libitum* access to water and standard rodent chow. “Fasted” mice (8 weeks of age, n=4) were raised on a diet similar to fed controls, but given access only to water for 24 hours prior to euthanasia. Diet-induced obese (DIO) mice (8 weeks of age, n=4) were provided a high-fat diet. The Fed and DIO mice received no food for 2 hrs to prevent random consumption of a meal prior to euthanasia, but were allowed access to water.

In Situ Hybridization

To generate an Nenf riboprobe for *in situ* hybridization (ISH), plasmid encoding the Nenf cDNA was purified using a DNA miniprep kit according to manufacturer’s protocol (K2100-10, Invitrogen, Carlsbad, CA) and used as a template for *in vitro* transcription. The Nenf riboprobe was synthesized with T3 RNA polymerase (Roche) for 2 hrs at 37 ºC and purified by LiCl precipitation. Brains were collected from *ad libitum* fed mice, with food removed 2 hrs before tissue collection. Freshly-isolated brain tissues were immediately frozen on dry ice in OCT compound, and then stored at -80 ºC. Frozen tissue was cut into sections (25 µm thickness) using a cryostat (Microm, model HM550; Thermo Scientific, Waltham, MA) and mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). ISH was performed as previously described with the following modifications (10, 12).

**Experiments 2-6: Quantitative real-time PCR**

As previously described (12), the anterior commissure and the oculomotor nerve were used as neuroanatomical markers to dissect hypothalami in phosphate-
buffered saline solution. RNA was extracted and quantified using real-time PCR analysis as previously described (12, 13). Briefly, RNeasy Midi kits (Qiagen, Valencia, CA) were used to extract RNA. Superscript II reverse transcriptase (Invitrogen) and random primers were used to create cDNA from 1 µg of RNA. SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Carlsbad, CA) was used in quantitative real-time PCR analysis, which generated a Ct value for the threshold cycle of each sample. The $\Delta$Ct value was generated by normalizing the data to 18 S rRNA, or $\Delta\Delta$Ct generated by normalizing the data to the control treatment. The primers used for qPCR are listed in Table 1.

Experiments 3-5: Recombinant protein production

A full-length open reading frame (ORF) encoding human NENF was obtained (Invitrogen, CA), and the region of the ORF lacking the NENF signal peptide sequence was amplified by PCR. Gateway cloning was used to insert NENF cDNA (lacking the signal peptide) into the pDEST17 vector encoding an N-terminal GST followed by a 3C2 protease cleavage site. Optimal bacterial culture conditions for the expression of NENF-GST fusion protein were determined. Bacteria were harvested, lysed, and the soluble recombinant GST fusion protein was purified according to standard protocols. The purified protein was verified via immunoblotting for GST (Invitrogen, according to manufacturer’s protocol). The N-terminal GST tag was removed by 3C2 protease, and the purity (>95%) of the resulting untagged NENF was verified by silver staining. Protein concentration was determined by reference to a BSA standard curve and was measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Concentration of
purified NENF was determined by fitting the value to the known standard using the equation $y=mx+b$. Full-length recombinant NENF or BDNF with a C-terminal FLAG epitope tag (DYKDDDDK) was expressed in mammalian GripTite™ HEK 293 cells (Invitrogen). Serum-free Opti-MEM media (Invitrogen) containing secreted NENF or BDNF was purified as previously described (40). Purified proteins were dialyzed against 20 mM HEPES buffer (pH 8.0) containing 135 mM NaCl in a 10 kDa cut-off Slide-A-Lyzer dialysis cassette (Pierce). After protein concentration was determined using Coomassie Plus protein assay reagent (Thermo Scientific), samples were aliquoted and stored at -80 °C.

**Experiment 3:** **NENF levels are modulated by the BDNF signaling pathway.**

**TrkB Inhibition**

Bdnf is expressed in the hypothalamus of the adult mouse brain (23), primarily in the VMH with low expression levels in the dorsomedial hypothalamus (DMH), in the arcuate nucleus (ARC), and at very low levels in the lateral hypothalamus (LH) (51, 55, 58). Therefore, a knock-in allele containing a point mutation (F616A) in the trkB gene was used to pharmacologically and temporally inhibit trkB signaling in mice via a highly permeable and selective small molecule inhibitor, 1NMPP1(14). The treated group received 1NMPP1 (80 µM) for 14 days via drinking water starting at 2 months of age. 1NMPP1 was subsequently removed to allow for reactivation of trkB singling after chemical inhibition. ISH was conducted four months after treatment, as previously described, to measure Nenf mRNA levels in the ARC of the hypothalamus; semi-quantitative differences were analyzed using densitometry with ImageJ.
(http://rsbweb.nih.gov/ij/), which was normalized to the background. Mice were fasted 2-16 hrs prior to collection of brain samples.

**Stereotaxic Cannulation**

Unilateral cannulas for infusion of proteins were implanted into the lateral cerebral ventricle of C57BL/6J male mice (8 weeks of age, n=10), as described previously (2). One week after surgery, the correct placement of cannula was verified as previously described, with the following minor modifications: intracerebroventricular (i.c.v) injection of 1 nmol neuropeptide Y (NPY; American Peptide Company, Inc.), during the light cycle, was used to elicit a ~100% increase in food intake relative to vehicle (saline), during a 1-hr food intake test relative to saline injection (2). Utilizing the dose of the purified BDNF (130 ng) or HEPES buffer (25 mM HEPES pH 8.0 and 135 mM NaCl) as a vehicle, since the protein was suspended in this buffer, food intake and body weight were measured for 24-hours after i.c.v. injection. The i.c.v. injection was administered just prior to the start of the dark cycle. The spillage of food was accounted for and subtracted from total food intake.

**Experiment 4:** NENF administration inhibited food intake and decreased body weight.

C57BL/6J male mice (8 weeks of age) were cannulated and placement of the cannula was verified as described previously (2). Two weeks after recovering from surgery, baseline food intake was measured at the start of the dark cycle at 1, 2, 4, and 24
hrs. Saline (0.9%) was used as a vehicle for the recombinant NENF purified from bacterial cells and HEPES buffer was used as a vehicle control for the recombinant NENF purified in mammalian cells, since the proteins were suspended in these buffers after purification. Central administration of saline or 0.1, 1, 10, and 100 nmol of NENF (made in bacteria) (n=10), or HEPES or 12 nmol (200 ng) of NENF-FLAG (made in mammalian cells) (n=11), was given to the mice via i.c.v cannulation. These doses were selected as an initial dose-response curve since other neuropeptides known to modulate food intake have been administered via i.c.v cannulation within this range of doses (39, 61). For studies involving an i.c.v NENF injection, NENF was injected just before onset of the dark cycle, and food intake measurements began after completion of the injections, which approximately coincided with the start of the dark cycle. Before the NENF injection, mice were not food restricted, although mice do tend to decrease food intake naturally during the light cycle. Food intake was measured after subtracting spillage from the total food consumed. Body weights were taken at the time of injection and 24 hrs after injection.

**Kaolin test**

C57BL/6J male mice (4 months of age, n=12) were cannulated and placement of the cannula was verified as previously described (2). An *ad libitum* amount of kaolin pellets (Research Diets, Inc., New Brunswick, NJ), along with *ad libitum* access to chow, was provided for one week to acclimatize the mice to the novelty of the kaolin. Food intake measurements were recorded at the start of the dark cycle. After acclimatization, an i.c.v injection of vehicle (HEPES buffer) was administered just prior to the start of the
dark cycle, and baseline food intake measurements were recorded for both the Kaolin and chow diets, with spillage of each diet subtracted, at 2 and 24 hrs. An i.c.v injection of the recombinant NENF protein (12 nmol) produced in mammalian cells was delivered via i.c.v, and food intake measurements for both the Kaolin and chow diets were recorded at 2 and 24 hrs, with spillage of each deducted.

Experiment 5: NENF administration increased Pome and Mc4r gene expression, and high-fat diet abrogated the appetite-suppressing effect of NENF.

Mice were cannulated (C57BL/6J, n=10) to compare the central effects of NENF on food intake in animals consuming a chow versus high-fat diet. We compared the intakes of mice that were approximately 1 year old and that consumed only a chow diet with the intakes of body weight matched DIO mice (6 months old). Weight matching provided a control for potential physiological changes arising from the phenotype induced by body weight. All mice were fed their respective diets since weaning. At the start of the dark cycle, baseline food intake was measured two weeks after surgery. Recombinant mammalian NENF was injected at 12 nmol, and food intake was measured at 1, 2, 4, and 24 hrs after injection of the vehicle at the start of the dark cycle, subtracting spillage from the total amount of food consumed. One week after the food intake test, a second injection of the mammalian recombinant NENF was administered via i.c.v, 6 hrs before the dark cycle. Mice were not allowed access to food after this injection, and hypothalami were collected three hours later.
Experiment 6: Inhibition of melanocortin signaling increased food intake, which subsequently increased Nenf gene expression.

C57BL/6J male mice were cannulated (n=14), and placement of the cannula was verified as above. AGRP (61) and SHU-9119 (20) were obtained from Phoenix Pharmaceuticals, Inc. One nmol AGRP, an inverse agonist for MC4R, 1 nmol SHU-9119, an antagonist for MC4R, or vehicle (saline) were injected via i.c.v in a 2 µL volume, 8 hrs before start of the dark cycle. Mice were allowed to consume food for the first 2 hrs, then food was removed for 2 hrs. Hypothalami were collected 4 hrs after initial i.c.v injection, and gene expression was measured using quantitative real-time PCR, as previously described.

Statistical Analysis

All comparisons were made using a one-way ANOVA. A two-way ANOVA for main effects between treatment groups was also performed where appropriate. A Student’s *t*-test was utilized to analyze the semi-quantitative values generated from the ISH data and qPCR. All statistical analyses were conducted using Statistica (v.8.0, Tulsa, OK). Values reported are means ± standard error of the means (SEM), and for all statistical tests *P* < 0.05 was considered significant.

RESULTS
Screening strategy identified novel hypothalamic secreted proteins that may regulate neural pathways associated with food intake.

We recently conducted a microarray-based screen to identify genes that are dynamically and/or selectively expressed in the developing and adult mouse hypothalamus from embryonic (E) 10 to postnatal day (P) 42, and expression patterns for 1,200 genes were verified by ISH (50). This data set was used as a starting point to identify secreted hypothalamic proteins that may play novel roles in regulating neural pathways involved in control of food intake and metabolism. Further data were obtained by analyzing the publically available gene expression data generated by the Allen Brain Atlas consortium (38). In total, we identified 250 candidate genes that were prominently and selectively expressed in adult hypothalamic nuclei known to regulate food intake, body weight, and expression of relevant neuropeptide hormones. One candidate, NENF, met all 5 criteria for candidates of interest and was explored in detail.

Changes in physiological state of energy balance regulate Nenf gene expression.

ISH indicated that Nenf mRNA is expressed primarily in the PVN of the hypothalamus, with lower levels of expression in the hypothalamic ARC (Figure 1 and 2E). We examined whether metabolic state affects expression levels of Nenf in the hypothalamus. Indeed, Nenf, Bdnf and Pomc mRNA expression in the hypothalamus was suppressed in the DIO, but not the fasted state (Figure 1B, 1C and 1D: Nenf- $F_{1,8}=5.63$, $P=0.04$, Bdnf- $F_{1,8}=10.64$, $P=0.013$, Pomc- $F_{1,8}=17.68$, $P=0.005$). As expected, fasting upregulated mRNA for the orexigenic hypothalamic neuropeptide, Agrp (Figure 1E: Agrp- $F_{1,8}=15.49$, $P=0.005$). Interestingly, the changes in expression patterns of Nenf in
response to diet-induced obesity closely resembled changes seen for Bdnf. Since BDNF signaling has been associated with the development of both obesity and anorexia (1, 4, 15, 18, 21, 26, 36, 37, 42-45, 47, 51-53), we determined whether BDNF signaling modulates Nenf mRNA expression.

**Nenf mRNA levels are modulated by the BDNF signaling pathway.**

To determine whether Nenf mRNA levels are regulated by the BDNF/trkB signaling pathway, we administered recombinant BDNF protein through i.c.v injection just before onset of the dark cycle. Importantly, central administration of BDNF does not cause a conditioned taste aversion (55). An acute increase in BDNF levels in the brain resulted in robust decrease in food intake just 1 hr later, and body weight decreased the day after injection, relative to vehicle (Figure 2A and B, food intake: 0-1 hr - $F_{1,9}=9.07$, $P=0.016$, 1-2 hrs - $F_{1,9}=10.81$, $P=0.01$, 2-4 hrs - $F_{1,9}=6.77$, $P=0.03$; body weight: $F_{1,9}=12.33$, $P=0.007$). No difference in food intake between groups was observed at 24 hrs post-injection (data not shown). However, Nenf mRNA expression, detected by quantitative real-time PCR, was reduced in the hypothalamus 3 hrs after BDNF injection (Figure 2C, $F_{1,9}=6.40$, $P=0.03$).

We next sought to test whether transient inhibition of BDNF activity affects hypothalamic Nenf mRNA levels. Mice homozygous for a targeted mutation of trkB, which alters the highly-conserved phenylalanine at position 616 within the catalytic kinase domain to alanine (F616A), enabled us examine the effects of selective and reversible inhibition of trkB signaling on Nenf mRNA levels by using the synthetic compound 1NMPP1 (8, 14). 1NMPP1 is a highly membrane-permeable compound that
can be conveniently delivered in drinking water and readily crosses both the placental and
blood-brain barriers to reversibly regulate the activity of neuronal-expressed mutant trkB
(56). Therefore, 1NMPP1, or vehicle solution without 1NMPP1, was administered via
drinking water to 2-month-old mice for 14 days. Hypothalamic Nenf mRNA levels were
then characterized in both treatment groups at 4 months of age. Following inhibition of
trkB signaling at 2 months of age, mice at 4 months of age displayed a robust increase in
ARC Nenf mRNA levels via ISH (Figures 2D and E, $F_{1,11}=29.43$, $P=0.0002$).

**NENF administration inhibited food intake and decreased body weight.**

To directly determine whether NENF could affect food intake and body weight,
we bacterially-produced recombinant NENF or two purified control proteins (CNTF and
GST) in mice weighing, on average, 25 g. In addition, recombinant NENF produced in
mammalian cells was injected as an additional control for bacterially-produced NENF. A
single dose of recombinant NENF protein, with the GST tag removed, was injected.

Central administration of 0.1, 1, 10, and 100 nmol doses of NENF significantly decreased
food intake (Figure 3A-E, food intake: 0.1 nmol: 0-1 hrs, $F_{1,20}=2.92$, $P=0.09$; 1-2 hrs,
$F_{1,20}=7.00$, $P=0.013$; 2-4 hrs, $F_{1,20}=12.56$, $P=0.001$; 4-22 hrs, $F_{1,20}=13.36$, $P=0.001$; 1
nmol: 0-1 hrs, $F_{1,20}=32.67$, $P=0.00001$; 1-2 hrs, $F_{1,20}=26.32$, $P=0.00005$; 2-4 hrs,
$F_{1,20}=15.49$, $P=0.0008$; 4-22 hrs, $F_{1,20}=63.53$, $P=0.0000001$; 10 nmol: 0-1 hrs,
$F_{1,20}=0.01$, $P=0.98$; 1-2 hrs, $F_{1,20}=11.22$, $P=0.002$; 2-4 hrs, $F_{1,20}=9.22$, $P=0.005$; 4-22 hrs,
$F_{1,20}=19.00$, $P=0.0001$; 100 nmol: 0-1 hrs, $F_{1,20}=8.98$, $P=0.006$; 1-2 hrs, $F_{1,20}=16.98$, $P=0.0004$; 2-4 hrs, $F_{1,20}=9.39$, $P=0.005$; 4-22 hrs, $F_{1,20}=18.48$, $P=0.0002$) and body
weight (Figure 3F: body weight - 0.1 nmol: $F_{1,20}=8.75$, $P=0.007$; 1 nmol: $F_{1,20}=5.87$,}
The 100 nmol dose was most efficacious, with decreased food intake at 0-1 hr (Figure 3A); the 1, 10, and 100 nmol doses decreased food intake at 1-2 hrs, and all doses decreased food intake at 2-4 and 4-22 hrs (Figure 3B-D, respectively). Cumulative food intake over 24-hrs was significantly reduced for all doses of recombinant NENF (Figure 3E: cumulative food intake, vehicle: 11.02 kcal ± 0.45, 0.1 nmol: 5.77 kcal ± 1.13, $F_{1,20}=27.11$, $P=0.00002$; 1 nmol: 9.86 kcal ± 0.55, $F_{1,20}=8.77$, $P=0.02$; 10 nmol: 6.34 kcal ± 1.27, $F_{1,20}=18.27$, $P=0.0002$; 100 nmol: 6.05 kcal ± 1.12, $F_{1,20}=27.91$, $P=0.00001$). Differences in food intake were not significant between groups at 48 hrs (vehicle: 13.43 kcal ± 0.6, NENF: 9.67 kcal ± 0.5). In contrast, central administration of 30 nmol GST elicited no changes in food intake or body weight (data not shown). As expected, our positive control, CNTF, decreased food intake following i.c.v injection (data not shown). To ensure that the appetite-suppressing effect of recombinant NENF was not due to bacterial contaminants found in the purified protein, we confirmed our results with recombinant protein produced in a serum-free mammalian expression system. Indeed, 12 nmol (200 ng) of recombinant NENF produced in mammalian HEK 293 cells also decreased food intake in mice when given by i.c.v injection (Figure 3G, 2-4 hrs - $F_{1,11}=6.74$, $P=0.02$, 4-22 hrs - $F_{1,11}=5.79$, $P=0.03$). In addition, recombinant NENF produced in mammalian cells was able to reduce body weight 24 hrs after one i.c.v injection (Figure 3H, $F_{1,11}=12.74$, $P=0.003$).

Central BDNF administration does not cause a conditioned taste aversion (55); we tested whether NENF administration produced a kaolin response to determine if the animals had decreased food intake that was attributable to visceral illness and,
consequently, an aversion to food. Kaolin and chow food intake were measured at 2 and 24 hrs after i.c.v injection of either vehicle or the mammalian recombinant NENF. No significant difference in Kaolin intake was observed between the two groups at the 2- and 24-hr time points (Kaolin 2 hrs: vehicle = 0.02g ± 0.01, NENF = 0.01g ± 0.01; 24 hrs: vehicle = 0.2g ± 0.1, NENF = 0.13g ± 0.06), but chow intake was decreased in the group injected with NENF (Chow 2 hrs: vehicle = 0.36g ± 0.06, NENF = 0.14g ± 0.08; Chow 24 hrs: vehicle = 3.89g ± 0.26, NENF = 1.64g ± 0.6).

Central NENF administration increased Pomc and Mcr4 mRNA expression, and high-fat diet abrogated the appetite-suppressing effect of NENF.

In DIO mice, the expression of Pomc is profoundly suppressed as a direct result of obesity-induced physiological changes (25), which alters central melanocortin secretion and signal activation in response to satiety signals, such as leptin (16). To investigate whether NENF might decrease food intake by modulating melanocortin mRNA levels, we injected recombinant NENF via i.c.v in DIO mice (51.5±2.5 g) and body-weight matched (BWM) mice that had consumed only chow (BWM; 46.6±1.6 g). Because DIO mice, consuming a high-fat diet then switched to a chow diet, exhibit restored secretion of melanocortin neuropeptides (16), the BWM group is an appropriate control for the DIO group to investigate the role of melanocortin effects downstream of NENF. Given that NENF is downstream of BDNF, whose expression is modulated by melanocortin signaling, we hypothesized that NENF administration might be much less effective at reducing food intake and body weight in DIO mice. Indeed, we failed to observe any
changes in food intake or body weight relative to vehicle-injected DIO mice when the mammalian recombinant NENF was injected (Figures 4A-C), with an overall main effect of group (BWM vs DIO: $F_{1,26}=5.09, P=0.02$), time ($F_{1,26}=6.01, P=0.02$), but no overall interaction between group and time ($F_{1,26}=1.40, P=0.27$). Cumulative food intake (Day 1) was not different between the DIO mice treated with NENF or vehicle (DIO vehicle: $13.73 \pm 3.37$ kcal; DIO NENF = $15.04 \pm 5.98$ kcal, $F_{1,11}=0.01, P=0.90$). However, similar to Figure 4B and 4C, the BWM mice injected with NENF via i.c.v. showed suppressed cumulative food intake (Day 1), relative to the vehicle group (BWM vehicle: $19.60 \pm 0.86$ kcal; BWM NENF = $15.29 \pm 1.19$ kcal, $F_{1,9}=12.74, P=0.03$). There was no overall difference in the calories consumed between the DIO vehicle and BWM vehicle groups (BWM vehicle: $19.60 \pm 0.86$ kcal ; DIO vehicle = $13.73 \pm 3.37$ kcal, $F_{1,11}=1.11, P=0.31$).

Next, we used qPCR to analyze changes in gene expression induced by central administration of recombinant NENF in the hypothalamus of BWM and DIO mice (Figures 4D-F). Mice receiving a single i.c.v injection of mammalian recombinant NENF exhibited increased hypothalamic Nenf, Pomc, and Mc4r gene expression in BWM mice. Interestingly, recombinant NENF injected i.c.v in DIO mice increased hypothalamic Nenf mRNA levels, but did not alter Pomc or Mcr4 mRNA levels (Figures 4D-F, respectively, BWM: Nenf- $F_{1,10}=5.06, P=0.054$, Pomc- $F_{1,10}=5.36, P=0.04$, Mc4r- $F_{1,10}=14.16, P=0.004$; DIO: Nenf- $F_{1,16}=6.03, P=0.03$, Pomc- $F_{1,16}=0.11, P=0.73$, Mc4r- $F_{1,16}=1.06, P=0.32$).

Inhibition of melanocortin signaling increased food intake, which subsequently increased Nenf gene expression.
To determine whether and where NENF may function with the BDNF and melanocortin signaling pathways, we used an experimental paradigm based on studies of Boghassian et al. (11), who demonstrated that AGRP delivery to the amygdala increases Bdnf mRNA levels after allowing animals 2 hrs of food access following injection. We hypothesized that central delivery of AGRP or SHU-9119 would induce increased food intake, and this in turn would induce hypothalamic NENF gene expression as a signal to reduce food intake, likely in response to satiety factors.

We injected mice with either 0.1 nmol AGRP (n=6), 1 nmol SHU-9119 (n=6), or vehicle (saline) control (n=6). Mice injected with AGRP or SHU-9119 had increased food intake relative to control mice, verifying the accuracy of cannulations and the efficacy of drugs injected (Figure 5A: control vs AGRP: \(F_{1,12}=7.00, P=0.03\), control vs SHU-9119: \(F_{1,12}=6.66, P=0.03\)). As expected (11), Bdnf mRNA levels were increased in the hypothalamus after i.c.v injection of either AGRP or SHU-9119 (Figure 5B: control vs AGRP: \(F_{1,12}=6.39, P=0.03\), control vs SHU-9119: \(F_{1,12}=10.94, P=0.01\)). Importantly, Nenf mRNA levels were increased as well (Figure 5C: control vs AGRP: \(F_{1,12}=8.85, P=0.02\), control vs SHU-9119: \(F_{1,12}=6.00, P=0.03\)).

**DISCUSSION**

Hypothalamic anorexigenic and orexigenic neurotrophic factors and neuropeptides change expression patterns in response to dietary signals to modulate overall energy balance. In response to a long- or short-term fast, expression of orexigenic
neuropeptides increases to drive food intake until satiety is reached. In contrast, expression of anorexigenic neuropeptides increases to suppress food intake and utilizes energy resources stored in adipose tissues, maintaining the body’s equilibrium for energy balance. The proportion of orexigenic to anorexigenic expression levels alternate in response to central and peripheral signals known to modulate energy balance (63). We propose that hypothalamic NENF interacts with BDNF and melanocortin signaling to modulate the production of either positive or negative energy balance, which may contribute toward long-term whole-body energy balance.

The mature form of BDNF regulates food intake and body weight primarily by activating trkB receptor signaling. Genetic loss-of-function of Bdnf, either during development or in postnatal animals, results in increased food intake and obesity (17, 27, 46, 51, 58). A similar phenotype is seen following chemical-genetic mediated inhibition of trkB during development (Byerly, unpublished data), suggesting that rewiring central neural circuitry regulating feeding during the pre- or postnatal period can result in permanent changes in the adult. Previous studies have reported that high-fat/high-sugar diets decrease hypothalamic expression of both Bdnf and trkB (34, 35, 57, 60), and we have confirmed that Bdnf mRNA levels were also suppressed in DIO mice. In a state of positive energy balance, short-term changes (14 days) in trkB inhibition resulted in upregulation of Nenf mRNA levels up to 4 months later. In a state of i.c.v. induced negative energy balance, created by central administration of BDNF to reduce food intake, rapid decreases in hypothalamic Nenf mRNA levels were observed. However, changes in Bdnf, Nenf and Pomc mRNA levels were not observed after a 24-hour fast,
suggesting that the duration of the fast may not have been the optimal duration to observe
gene expression changes (e.g. the fast was too long or not long enough).

Nenf is expressed in nuclei of the hypothalamus known to be involved in energy
regulation, and changes in diet (DIO mice) altered Nenf gene expression in the same
direction as Bdnf gene expression; thus, we predicted that NENF would inhibit food
intake. Indeed, central delivery of recombinant NENF decreased food intake and body
weight, suggesting that it is a novel anorexigenic neurotrophic factor. The dose of NENF
delivered via i.c.v. altered the duration of time until decreased food intake was observed,
with the highest dose (100 nmol) suppressing food intake by 1 hour and the lowest dose
(0.1 nmol) by 4 hours, relative to vehicle injected mice. However, the lower limit of
physiological detection for NENF did not fall into the range of doses delivered, and
remains to be determined.

Food intake is controlled by hypothalamic melanocortins acting on the Mc4r, with
Agrp acting as an orexigenic inverse agonist of α-melanocyte-stimulating-hormone (α-
msh), a peptide product of proopiomelanocortin (Pomc), which acts as an anorexigenic
receptor agonist. We have shown that NENF is also a catabolic neurotrophic factor
expressed in the PVN and ARC nuclei, with expression only observed in the ARC after
long-term trkB inhibition. Nenf mRNA is modulated by melanocortin signals, a pathway
also known to modulate Bdnf expression (6, 58). However, under conditions in which
hypothalamic expression of Pomc is profoundly suppressed, such as is seen in DIO mice,
central administration of NENF failed to elicit changes in food intake, and did not
increase Pomc and Mcr4 mRNA levels. The lack of effect in the DIO mice does not
appear to be secondary to body weight, since chow fed mice that had reached comparable
body weight still responded. DIO mice have been shown to have only one-third of the
activation of the Mc4r relative to control, resulting in decreased central melanocortin
activation (16). Therefore, these data support the hypothesis that NENF acts as an
anorexigenic neurotrophic factor that contributes to energy balance, and DIO mice may
be resistant to NENF signals as a result of altered central melanocortin signaling.

The DIO diet is high in fat and dense in energy, which may result in it being a
more palatable diet given the increased fat content relative to a standard chow diet.
Therefore, the high-fat diet fed to DIO mice may have altered reward cues associated
with food intake, relative to BWM mice fed a standard chow diet. Environmental factors
such as energy-density of the diet, palatability/taste, or large quantities of available food,
provide reward cues to reinforce and drive food intake behavior (7, 33, 62). These reward
cues have been shown to weaken feedback signals, such as leptin, that serve to decrease
food intake and modulate whole-body energy balance (7, 62). Similar quantities of food
were provided to DIO and BWM mice daily. Therefore, the quantities of available food
most likely did not influence the reward cues between the two groups. However, the
energy density of the diet as well as palatability and/or taste differences between the diets
may have influenced the amount consumed between the two groups (DIO vs. BWM),
overriding the signals triggered by centrally administered recombinant NENF. Thus, it is
possible that the DIO mice have alterations in reward-driven food intake behavior (62). It
remains to be determined if NENF is less effective in altering energy balance when
reward-driven food intake is reinforced by components of the high-fat diet fed to the DIO
mice, relative to the standard chow fed to the BWM mice.
The percent reduction of food intake for the mammalian recombinant NENF treatment group, relative to saline, resembled the percent reduction observed for the 1 nmol dose of the bacterially-produced recombinant NENF at 2-4 and 4-22 hrs. The dose injected for the mammalian recombinant NENF was 12 nmol, suggesting that the mammalian recombinant protein was 10-fold less effective than the bacterially-produced recombinant NENF protein. This difference between the mammalian and bacterially-derived NENF may be attributable to residual contaminants, such as lipopolysaccharides (LPS) or muramyl dipeptide (MDP), present in bacterially-produced recombinant protein even after LPS removal by a commercially available kit. LPS and MDP have been shown to reduce food intake via intraperitoneal injection (32) and i.c.v injection (54). In addition, the recombinant NENF produced in mammalian cells is an ideal control for the recombinant NENF produced in bacteria, because the mammalian expression system allows for native protein modifications that would not be found in bacteria, such as glycosylation. Our findings suggest that the purified mammalian recombinant NENF is indeed effective in regulating food intake when produced in a system that does not contain LPS and possesses endogenous protein modifications found in the mouse. Given that we used the mammalian protein in DIO and BWM mice to show alterations in hypothalamic neuropeptide gene expression, we confirm that NENF is an anorexigenic neurotrophic factor capable of inhibiting food intake and regulating expression of genes.

Perspectives and Significance
Many gene have been identified to be associated with obesity (41), however the function of proteins produced by these genes have not yet been characterized in regards to food intake or whole-body energy balance. An integrated approach, combining experimental and bioinformatic techniques, led to the identification of candidate hypothalamic neuropeptides with novel appetite-modulating function. One such candidate, NENF, when centrally administered to mice, decreased food intake and body weight, thus validating the general approach used to identify functionally-relevant secretory proteins that regulate neuronal pathways controlling ingestive behaviour.

Alterations to BDNF/trkB or melanocortin signaling led to a compensatory change in hypothalamic Nenf mRNA levels, suggesting that NENF gene expression is responsive to both trkB and melanocortin signaling to regulate food intake and energy balance. Our results indicate that DIO mice may be resistant to NENF signaling, since DIO mice have weakened central melanocortin activation (16) and do not show upregulated melanocortin signaling following i.c.v. administration of recombinant NENF. We propose that this regulatory circuit contributes to the maintenance of normal energy homeostasis and is likely to be disrupted in obesity. We demonstrated that hypothalamic signalling pathways in the mouse respond to the human NENF protein. Physiological processes activated by NENF signalling may be evolutionarily conserved since the human and mouse protein have 91% similarity (31), which suggests that NENF may be a novel candidate biomarker for disease susceptibility or development of novel drug therapeutics in humans.

GRANTS
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25. Huang XF, Han M, South T, and Storlien L. Altered levels of POMC, AgRP and MC4-R mRNA expression in the hypothalamus and other parts of the limbic system of mice prone or resistant to chronic high-energy diet-induced obesity. *Brain Res* 992: 9-19, 2003.


Table 1: qPCR primers used to measure hypothalamic gene expression.

Figure 1:
Nenf and neuropeptide gene expression in the hypothalamus. A) *In situ* hybridization (ISH) for Nenf in the paraventricular nucleus (PVN) and the arcuate nucleus (ARC) of the hypothalamus. B-E) Neuropeptide and neurotrophin gene expression during fed, fasted, and DIO food intake states (n=4 per group). B) Nenf, C) Pomc, and D) Bdnf were decreased in both the DIO state relative to the fed state. E) Agrp was increased in the fasted state relative to the fed state. Data are means ± SEM, *P*<0.05.

Figure 2:
Changes in BDNF signaling alter Nenf gene expression. A-C) BDNF delivered i.c.v to mice decreased A) food intake, B) body weight, and C) hypothalamic Nenf mRNA levels after a single injection relative to vehicle (BDNF: n=5; vehicle: n=6). D-E) TrkB signaling was inhibited in adult mice for 14 days. The control group received no 1NMPP1 (n=5-6/group). TrkB<sup>F616A</sup> mice administered 1NMPP1 to inhibit trkB signaling demonstrated increased Nenf gene expression in the ARC at 4 months of age by both D) qPCR and E) ISH. Data are means ± SEM, *P*<0.05.

Figure 3:
Intracerebroventricular recombinant NENF injection decreased food intake and body weight. A-D) 0.1, 1, 10, and 100 nmol doses of bacterially-derived NENF were injected into mice (n=10 per dose) and food intake was measured at 0-1, 1-2, 2-4, and 4-22 hrs
after injection and compared to mice injected with vehicle (saline). E) Cumulative food intake and F) body weight were decreased 24 hrs after a single injection of NENF for all doses. G-H) NENF protein purified from HEK 293 cells was injected as a control for the bacterially-expressed purified protein. Protein derived from mammalian cells (12 nmol dose) decreased G) food intake and H) body weight relative to vehicle (HEPES buffer) injected mice, after 24-hours, in a similar manner as bacterially-expressed NENF. Data are means ± SEM, *P<0.05.

Figure 4:
Intracerebroventricular NENF injection in age and body-weight matched control or DIO mice. A) Recombinant NENF injection decreased body weight in control mice (body-weight matched were used for control; BWM) consuming chow, but not in DIO mice. B-C) BWM mice had decreased food intake after 1 hr and 2 hrs following i.c.v NENF injection relative to vehicle injection (BWM, n=4-6/group), but DIO mice (n=8) had no change in food intake relative to saline-injected mice. D-F) Neuropeptide gene expression levels were altered after NENF injection. D) Nenf mRNA levels were increased following injection in both BWM and DIO mice. E) Pomc and F) Mc4r gene expression increased in BWM mice, but not in DIO mice, 3 hrs after NENF injection. *P<0.05 vs. vehicle. #P=0.054 vs. vehicle.

Figure 5:
Effect of AGRP and SHU-9119 on food intake and hypothalamic gene expression. Mice received AGRP (0.1 nmol), SHU-9119 (1 nmol), or saline (control) via i.c.v injection. A)
After injection, food was provided and intake was measured from hours 0-2, then no food was provided hours 2-4. B-C) Hypothalamic Nenf and Bdnf gene expression was increased 4 hrs after initial injection, following access to food and increased food intake. Values are shown as means ± SEM. *P<0.05 vs. saline.
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<td>5’ GGCC TCACTACATCCAA 3’</td>
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A. Food Intake

B. BDNF injection

Body Weight

% relative to baseline

C. BDNF injection

Nenf mRNA

relative mRNA

D. Nenf mRNA

mRNA (arbitrary units)

E. Nenf mRNA levels

Control

1NMPP1 treated
A) Body weight (24-hr)

B) 1 hr Food Intake

C) 2 hrs Food Intake

D) Nenf

E) Pomc

F) Mc4r