Transgenic overexpression of neuromedin U promotes leanness and hypophagia in mice

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Abstract

Recent work has shown that neuromedin U (NmU), a peptide initially identified as a smooth muscle contractor, may play a role in regulating food intake and energy homeostasis. To further evaluate this putative function, we measured food intake, body weight, energy expenditure and glucose homeostasis in transgenic mice that ubiquitously overexpress murine proNmU. NmU transgenic mice were lighter and had less somatic and liver fat, were hypophagic, and had improved insulin sensitivity as judged by an intraperitoneal insulin tolerance test. Transgenic mice had higher levels of hypothalamic NPY, POMC and MCH mRNA. There was no difference in O₂ consumption between genotypes; however, NmU transgenic mice displayed a modest increase in respiratory quotient during food deprivation and refeeding. There were no behavioral disturbances in the NmU transgenic mice that could account for the results (e.g. changes in locomotor activity). When placed on a high-fat diet, transgenic mice remained lighter than wild-type mice and ate less, but gained weight at a rate similar to wild-type mice. Despite the increased weight gain with high-fat feeding, glucose tolerance was significantly improved in the transgenic mice. These findings support the hypothesized role of NmU as an endogenous anorexigenic peptide.

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Introduction

Neuromedin U (NmU) is a peptide that was first isolated from porcine spinal cord (Minamino et al. 1985a,b). Two active peptides were identified in this work: an amidated 25-amino-acid peptide (NmU-25) that is generated by enzymatic cleavage at the C-terminal region of a 174-amino-acid precursor (Lo et al. 1992) and an 8-amino-acid cleavage product of NmU-25 (NmU-8) (Minamino et al. 1985b), both of which possess smooth muscle contractile activity (Minamino et al. 1985a,b). NmU cDNAs from dog, rabbit, frog, rat and human have been cloned (Domin et al. 1989, Kage et al. 1991, O’Harte et al. 1991, Lo et al. 1992, Austin et al. 1995) and demonstrate a strong amino-acid sequence homology. In particular, it has been shown that the C-terminal heptapeptide core of NmU (FLFRPRN-NH₂) constitutes a conserved domain that is sufficient for biologic activity (Hashimoto et al. 1991).

Expression analysis in mammalian species has shown that NmU precursor mRNA is widely distributed but is particularly high in the upper gastrointestinal (GI) tract and the central nervous system (CNS). In human tissue, NmU mRNA is found in the intestine, stomach, pituitary gland and several brain regions, including hypothalamus, locus coeruleus, thalamus, medulla and substantia nigra; it is also found in lymphoid cells (Sumi et al. 1987, Austin et al. 1995, Howard et al. 2000, Raddatz et al. 2000). A similar expression pattern is observed in rat (Domin et al. 1987, Fujii et al. 2000) and mouse (Funes et al. 2002). Immunohistochemical analysis of rat tissue displays a protein pattern consistent with mRNA expression, with NmU-like immunoreactivity found in the submucosal and myenteric ganglion cells of the small intestine as well as several brain regions (Honzawa et al. 1987, 1990, Augood et al. 1988).

Two human NmU receptors have been identified (Hedrick et al. 2000, Shan et al. 2000). One receptor is expressed predominantly in the periphery (NmU-R1), notably in the small intestine, pancreas, stomach, testis, liver, kidney and immune cells (Hedrick et al. 2000, Howard et al. 2000, Raddatz et al. 2000), with little to no expression in the CNS. The second receptor (NmU-R2) is expressed predominantly in the CNS, including the paraventricular nucleus (PVN) of the hypothalamus, the
wall of the third ventricle in the hypothalamus, the CA1 region of the hippocampus and the spinal cord, but it is also detected in testis, lung, kidney and thyroid (Howard et al. 2000, Raddatz et al. 2000, Shan et al. 2000). Likewise, two NmU receptors have been identified in the mouse and display an expression pattern and pharmacology similar to the human receptors (Funes et al. 2002).

In addition to the effects on smooth muscle contraction, several biologic activities have since been reported for NmU (reviewed in (Brighton et al. 2004). These include regulation of blood pressure, ion transport in the gut, splanchnic blood flow and adrenocortical function (Minamino et al. 1985b, Brown & Quito 1988, Gardiner et al. 1990, Malendowicz et al. 1994). Recently, NmU has been implicated in the control of energy homeostasis. Specifically, intracerebroventricular (i.c.v.) administration of NmU to rats and mice suppresses starved-induced feeding, and increases locomotor activity, grooming and rectal temperature (Howard et al. 2000, Kojima et al. 2000, Nakazato et al. 2000). Similar effects have been seen with intra-PVN administration (Wren et al. 2002). This is accompanied by increased c-Fos immunoreactivity in the PVN, and supraoptic and parabrachial nuclei (Niimi et al. 2001). Furthermore, i.c.v. administration of anti-NmU antiserum increases feeding in rats (Kojima et al. 2000). NmU is expressed in the ventromedial hypothalamic area and the nucleus tractis solitarii of the rat, and its expression is decreased with starvation or leptin receptor deficiency (Ivanov et al. 2002), consistent with its postulated role as an endogenous anorexigenic neuropeptide. Recent work has shown that mice lacking the gene encoding NmU develop an obese phenotype characterized by hyperphagia, hyperactivity, hypothermia and decreased energy expenditure (Hanada et al. 2004), consistent with pharmacologic data.

To further explore the role of NmU in body weight regulation, we evaluated body composition, food intake, energy expenditure and glucose homeostasis in mice that broadly express a transgene encoding murine proNmU. The results of this analysis show that NmU transgenic (Tg) mice maintained on a chow diet are leaner than wild-type mice and hypophagic, and have increased insulin sensitivity, while Tg mice maintained on a high-fat diet are leaner and have an improved glucose tolerance.

Materials and Methods

Animal care and maintenance

Mice were group housed in ventilated cages and given chow (PicoLab; 11% of calories from fat) and water ad libitum. Prior to study at 10–24 weeks of age, mice were individually housed in polycarbonate cages and fed chow, a semipurified low-fat diet (10% of calories from fat), or a semipurified high-fat diet (45% of calories from fat; D12450B and D12451 respectively; Research Diets, New Brunswick, NJ, USA), and had ad libitum access to water. They were maintained under a 12 h/12 h light/dark cycle at a temperature of 22 °C.

Three separate cohorts of male mice were used for the feeding and indirect calorimetry studies reported. For experiments in which animals were studied under chow-fed conditions, daily food intake was measured for 24 consecutive days in 24–32-week-old mice (n=17–20 per group). Animals used in studies examining diet-induced obesity (n=7–8 per group) were transferred from chow to a low-fat diet at 10–13 weeks of age, and food intake and body weight were monitored weekly for 5 weeks, after which time the mice were transferred to a high-fat diet. Daily food intake and body weight was monitored for 20 consecutive days and periodically thereafter for 11 weeks. Mice used for indirect calorimetry studies were maintained on chow and studied at 27–28 weeks of age (n=8 per group).

At the end of each study, animals were killed by CO2 asphyxiation, and tissues for RNA isolation were dissected and placed into RNA Later (Ambion, Austin, TX, USA) for 48–96 h at 4 °C and frozen at −80 °C until analysis. All studies were conducted in an American Association for Laboratory Animal Care accredited facility, according to protocols approved by the Schering-Plough Research Institute Animal Care and Use Committee. The procedures were performed in accordance with the principles and guidelines established by the National Institutes of Health for the care and use of laboratory animals.

Transgene construction and generation of Tg mice

Generation of Tg mice The entire coding sequence of the mouse NmU gene (GenBank AF203444) was amplified by PCR primers containing a Kozak consensus sequence and subcloned into the EcoRI site of a vector containing the human CMV enhancer/chicken β-actin promoter expression cassette graciously provided by Dr M. Okabe, Osaka University (Okabe et al. 1997). The transgene was isolated from the plasmid by restriction digest with BamHI and SalI and further purified from the vector DNA by sucrose gradient centrifugation, as previously described (Mann & McMahon 1993). Fractions containing the transgene were pooled, concentrated by Microcon-100 filters (Millipore, Billerica, MA, USA), and washed five times with microinjection buffer (5 mM Tris–HCl (pH 7.4), 5 mM NaCl and 0.1 mM EDTA). Transgene DNA was re suspended in microinjection buffer to a final concentration of 1–5 ng/μl, microinjected into eggs ([C57BL/6] x DBA/2] F2; Jackson Laboratory, Bar Harbor, ME, USA), and transferred into oviducts of ICR foster mothers (Charles River Laboratories, Wilmington, MA, USA), according to published procedures (Hogan 1986). At 10 days after birth, a piece of tail from the resulting animals was clipped for DNA analysis. Identification of Tg mice was accomplished by PCR amplification

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of mouse tail DNA (Lira et al. 1990), using the following recognition primers for the transgene (polyA): forward, 5′-agtttgccaggtctctatgagt-3′; reverse, 5′-ctcttgatgtttaaaa tggattgccctcc-3′. The endogenous LDL gene, used as an internal control, was amplified with the following primers: 5′-accccaagacgtgcctccaggatga-3′ (forward) and 5′-gcagct cctcttctgaccttg-3′ (reverse). PCR conditions were as follows: 94 °C, 30 s; 60 °C, 30 s; 70 °C, 60 s for 30 cycles. Tg animals were kept under pathogen-free conditions.

Quantitation of gene expression

**Tissue preparation** Mice were killed with CO2, and tissues removed and placed in RINAlater (Ambion) until processing. The static levels of NmU and the two NmU receptors were examined in 8-week-old mice under chow-fed conditions, with a total of 10 tissues taken from two NmU Tg mice and two wild-type litters. To determine the effect of the transgene on NmU and other hypothalamic obesity-related genes, we collected hypothalamic tissue from eight Tg and nine wild-type, chow-fed, 30-week-old mice and used them for quantitative PCR analysis. Total RNA was isolated from the tissues with TRI-reagent (MRC, Cincinnati, OH, USA), or a commercial kit (RNeasy Lipid Kit; Qiagen) and tested for quality and quantity with an Agilent 2100 Bioanalyzer (Waldbrun, Germany). In addition, three mice of each genotype were perfused with 4% paraformaldehyde; their brains were removed and stored in 30% sucrose to be used in situ hybridization studies described below.

**Quantitative PCR** Taqman primers and probes (Table 1) were designed with Primer Express software (ABI, Foster City, CA, USA), and purchased from ABI. The quantitative PCR was carried out with an ABI Prism 5700 sequence detection system, using the components from the Platinum Quantitative RT-PCR One-Step Kit (Invitrogen, Carlsbad, CA, USA). The final concentrations of the primers and probe in the PCR reactions were 200 and 100 nM respectively, and each 12·5 µl PCR reaction contained 5·0 µl (5 ng) total RNA prepared as described above. The RT-PCR reactions for all genes were performed according to the following protocol: one 30-min cycle at 48 °C, followed by one 20-min cycle at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Separate plates of the same RNAs were used to quantify 18S RNA as an internal control for RNA quality. The PCR data were quantitated on standard curves generated with serial dilutions of gene-specific PCR products encompassing the amplicons generated by the quantitative PCR primers. The RNAs from the four mice (two Tg, two wild-type) used to assess NmU expression in several tissues were run separately, in duplicate, and the mean value for each mouse was calculated. The RNAs from the hypothalamus of the chow-fed mice of each genotype were run separately, in quadruplicate, for each neuropeptide, and results were expressed as the mean ± s.e. of fg/reaction.

**In situ hybridization** Forty-five-mer sense and antisense oligonucleotide probes were designed, and the sequences used were as follows: sense, 5′-TCA AAG CAG AAT ACC AGA GTC CTT CCG TGG TGC AAA GTA AGG GAT-3′; antisense, 5′-ATC CCT TAC TTT GAC CCA CGG AAG GAC TCT GGT ATT CTG CTT TGA-3′. The probes were 3′-end labeled with 35S-dATP, using TdT (Stratagene, La Jolla, CA, USA). Coronal sections through the mouse brain were cut at 40 µm with a cryostat, and the tissue sections hybridized, washed and exposed to Kodak Biomax autoradiographic film according to previously published protocols (Gustafson et al. 1997, Kowalski et al. 1998).

**Assessment of NmU peptide production** Spleens from an 8-week-old NmU Tg mouse and a wild-type littermate were aseptically harvested, finely minced, and placed into culture for 5 days (0·5 ml serum-free RPMI). Conditioned medium was subsequently collected, clarified by centrifugation, and assayed for bioactivity against NmU-R2-transfected HEK293 cells with the fluorescent imaging plate reader (Molecular Devices, Sunnyvale, CA, USA), as previously described (Shan et al. 2000). Spleens harvested from non-Tg littersmates provided media for a negative control, as did nonconditioned medium.

**Tissue histology** A wild-type and an NmU Tg mouse were killed by CO2 asphyxiation, and several tissues were dissected and placed into 10% neutral buffered formalin. Representative sections of each tissue were embedded in paraffin and sectioned on a Zeiss HM 330 microtome at 5 µM and stained with standard hematoxylin and eosin. Tissue histology was assessed by an experienced pathologist blinded to the genotype of the animals under examination.

**Measurement of adiposity and body composition** At the end of the chow and high-fat feeding experiments, animals were killed by CO2 asphyxiation and bled by cardiac puncture for measurement of plasma metabolites and hormones (see below). The nasoanal length was measured, and the right epididymal, retroperitoneal and inguinal adipose depots and the mesenteric adipose depot were dissected and weighed. The total dissectible adipose tissue weight of each animal was defined as the sum of [(right epididymal, retroperitoneal and inguinal depots) 2] + (mesenteric depot). In a separate cohort of mice used for indirect calorimetry (see below), a nuclear magnetic resonance
Table 1 Primer and probe sequences used in the quantitative PCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Taqman probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>NmU (AF203444)</td>
<td>CTGCATGAGAGAAGAATGAAGAGA</td>
<td>TGGCCTGAATAAAAGATATCCC</td>
<td>CCAGAGTCCCTCCGTTGGGTCAA</td>
</tr>
<tr>
<td>NMUR1 (AF044602)</td>
<td>TGTATCTGCTCATTGGGCTGC</td>
<td>TCTCCGTCCTCTATTTGCA</td>
<td>TCAAGGGCAGAAAACCCGACG</td>
</tr>
<tr>
<td>NmUR2 (NM 153079)</td>
<td>AGGTCTACGATTTGGCACA</td>
<td>CTGAGATGGAAGGCAAAGACAC</td>
<td>TTTGGCCGGTGTTGGGATGCTACCTC</td>
</tr>
<tr>
<td>AGRP (NM 007427)</td>
<td>GGCTCCACTGAAAGGGCATC</td>
<td>CTGCTGGATGCTGCTGATTC</td>
<td>CGTTCCTCAAGTTCTAGTCTGAAT</td>
</tr>
<tr>
<td>CRH (AY128673)</td>
<td>TGGCGGAAGTCTTGGAAATG</td>
<td>AATCTCAGTCTTGTGCTCGT</td>
<td>CCGGCGAGACGATGCTACGCAA</td>
</tr>
<tr>
<td>CART (NM 013732)</td>
<td>CTACTCTGCGGCTGATGATGC</td>
<td>GACCTTTGCAACGCTTCG</td>
<td>AACGCGGCAACTTCCGCGCTC</td>
</tr>
<tr>
<td>IRS (X01317)</td>
<td>TACCACTCAGGAAAGGGCA</td>
<td>TGAATTACCCGCGGCTGC</td>
<td>CGAGGCCCTGTTAATGGGATGCTC</td>
</tr>
<tr>
<td>MCH (AK02072)</td>
<td>CAAACAAAGGAGGAGGACG</td>
<td>AGTGCGAAGCCGTTGAGTTAC</td>
<td>ATGACAAATAAAGATCTCAAGAAACCAAAC</td>
</tr>
<tr>
<td>NPY (NM 023456)</td>
<td>TATGCGAAGAGATCCAGCCC</td>
<td>CACATGGAAGGGTCTTCAAGC</td>
<td>TCGAGCCTCTAATGGAAGGACAGAAACCG</td>
</tr>
</tbody>
</table>
resonance (NMR)-based method (EchoMRI; Echo Medical, Houston, TX, USA (Tinsley et al. 2004)) was used to measure body fat and muscle mass.

Liver lipid determination
Triglyceride, free cholesterol and cholesterol esters were determined in a 100–200 mg aliquot of liver. The liver lipids were extracted by the method of Folch et al. (1957) and triglyceride, cholesterol ester and free cholesterol content were determined by high-performance liquid chromatography analysis, as previously described (Burrier et al. 1995).

Plasma measurements
Plasma insulin and leptin were measured with ELISA kits (Alpco, Windham, NH, USA and Crystal Chem, Chicago, IL, USA), and triglycerides and free fatty acids were measured by enzymatic colorimetric assays (Sigma and Wako Inc., Richmond, VA, USA). Plasma corticosterone was measured by RIA (ICN, Irvine, CA, USA). Serum NmU concentrations from one male NmU Tg and one male wild-type littermate were determined with an NmU dot blot kit according to manufacturer’s protocol (Phoenix Pharmaceuticals, Belmont, CA, USA) with the exception that mouse NmU was used as a standard in place of rat NmU.

Glucose tolerance tests
Tail blood was collected after 24 h of food deprivation and basal glucose levels were measured by the glucose oxidase method (Glucometer Elite, Bayer, Elkhart, IN, USA). After this measure, glucose (1 mg/g body weight) was administered intraperitoneally, and tail blood was collected at 20, 40, 60, and 90 min post-dose for glucose determination.

Insulin tolerance tests
Tail blood was collected after 24 h of food deprivation and basal glucose levels were measured using a glucose oxidase method (Glucometer Elite). After this measure, insulin (0.75 mU/g body weight) was administered intraperitoneally, and tail blood was collected at 20, 40, 60, 90, and 120 min post-dose for glucose determination.

Indirect calorimetry
Oxygen consumption (V\textsubscript{O2}) and carbon dioxide production (V\textsubscript{CO2}) were measured in mice every 30 min (settle time: 155 s; measure time: 45 s) for 23 h with the use of an indirect calorimeter (Oxymax, Columbus Instruments, Columbus, OH, USA). Measurements were taken in an airtight container (2-5 l) with an air flow rate of 0·5 l/min; 27–28-week-old wild-type (n=8) and Tg (n=8) mice were acclimated to the chambers for 4 days prior to measurement, and were monitored in the fasted state, during 24-h food deprivation, and during 24 h of refeeding. Respiratory quotient (RQ) was calculated as the molar ratio of V\textsubscript{CO2}/V\textsubscript{O2}. The V\textsubscript{O2} and RQ values from four consecutive readings, encompassing a 2-h period, were averaged for analysis.

Behavioral studies
Two cohorts of NmU Tg mice (n=11 and 14) and wild-type littermates (n=14 and 15) were maintained on chow and tested in a behavioral battery including the following: the primary observational screen from the SHIRPA protocol (Rogers et al. 1999), locomotor activity (LMA; Coulbourn Tru-Scan, 60 min), elevated plus maze (transparent closed arms, 5 min), accelerating rotarod (Accuscan SmartRod, eight trials, acceleration rate: 20 r.p.m./60 s), spatial Y-maze (Dellu et al. 2000), motor coordination battery (Lalonde et al. 1994, 1997, Klein et al. 1996, Carter et al. 1999, Contet et al. 2001), hotplate (Schreiber et al. 1996), tail suspension (Steru et al. 1985), forced swim (Redrobe & Bourin 1998), warm water tail flick (Janssen 1963), auditory startle threshold (Varty et al. 2001), prepulse inhibition of auditory startle (Varty et al. 2001), passive avoidance (one-trial step-through, 2 s 0·6 mA, 24-h retention, 300 s maximum) and pentylenetetrazole-induced seizure threshold (Nutt et al. 1986). The group housed mice began testing when they were 12–24 weeks and ended when they were 20–31 weeks of age.

GI transit studies
Chow-fed mice were starved for 24 h, after which time a bolus dose (0·4 ml) of a charcoal mixture (1% methylcellulose: 5% acacia: 10% charcoal) was orally administered. After 20 min, the animals were killed by CO\textsubscript{2} asphyxiation, the GI tract from the stomach to cecum was removed, and the distance traveled by the charcoal mixture was measured and expressed as percentage of the total intestinal length.

Statistical analysis
Values are reported as means ± s.e.m. except where indicated. Statistical significance of body weight, daily food intake, plasma measures and neuropeptide expression between genotypes was determined by Student’s t-test. Differences in V\textsubscript{O2} and RQ during the dark and light phases of the fed, fasted and refed states were analyzed by repeated measures analysis of variance, followed by Fisher’s PLSD where appropriate. A P value of <0·05 was considered significant.
Results

Generation of Tg mice

Mice overexpressing murine NmU were generated in the C57BL/6 DBA2/J background (B6D2) using the CMV-NmU transgene. In this transgene, NmU is expressed from a human CMV enhancer-chicken β-actin promoter cassette which directs expression to virtually all tissues (Okabe et al. 1997). A total of two Tg founders were generated as determined by PCR genotyping. The line that displayed the highest level of transgene expression was used in all studies reported. The mice developed and reproduced normally and did not show any visible pathologic abnormalities. Overall, there were no robust or reproducible differences between NmU Tg mice and wild-type mice in any of the behavioral measures including LMA (data not shown).

Expression analysis

As shown in Table 2, the NmU transgene was expressed at high levels in all tissues examined, including throughout the CNS. Despite this high level of NmU transgene expression, there was no apparent up- or downregulation of NmU receptor mRNA (Table 2). Localization of the NmU transgene expression in the brain indicated that overexpression was ubiquitous but particularly apparent in the paraventricular, supraoptic, suprachiasmatic and arcuate nuclei of the hypothalamus, the piriform cortex and the CA3 field of the hippocampus (Fig. 1). This pattern of brain NmU overexpression was similar in all three Tg mice studied. Because transcriptional regulation of the NmU transgene was under the control of the human CMV enhancer/chicken β-actin promoter, and not the endogenous promoter, it is unlikely that nutritional state or other physiologic alterations affected NmU transgene expression to any appreciable extent.

In order to detect active peptide, spleen cells (total spleen) from a Tg mouse and a non-Tg littermate were cultured in serum-free RPMI (without phenol red) for 5 days, and the conditioned medium was subsequently assayed for NmU activity, using NmU-R2-expressing HEK293 cells (Shan et al. 2000). A calcium flux was detected in response to the addition of medium from the Tg spleen cells (Fig. 2) while a much smaller response was detected using medium from wild-type spleen cells. These data suggest that bioactive NmU is produced from the Tg proNmU gene. The serum NmU concentration determined with a dot blot kit was between 50 and 100 nM in an NmU Tg mouse and below the level of detection in a wild-type littermate, indicating that the bioactive NmU is secreted into the blood.

The effect of the NmU transgene on food intake and body composition

The body weight of 24–30-week-old chow-fed NmU Tg mice was significantly less (~14%) than wild-type mice (Table 3). NmU Tg mice had significantly smaller epididymal, inguinal, retroperitoneal and mesenteric adipose depots (Fig. 3A), and 33% less total dissectible fat (10.9 ± 0.8 vs 8.4 ± 0.6% of body weight for wild-type and NmU Tg mice respectively; P<0.05). Consistent with these findings, NmU Tg mice had significantly lower plasma leptin levels (Table 3). There was no significant difference in nasoanal length or rectal temperature between genotypes (data not shown). The liver weight of NmU Tg mice was significantly less than wild-type mice, even when expressed as a function of body weight. This was accompanied by significantly less triglyceride (Table 4). There were no significant differences in plasma triglycerides, free fatty acids or corticosterone between genotypes (Table 3). In a separate cohort of 28-week-old, chow-fed mice, NMR analysis confirmed the lower

Table 2 Expression of neuromedin U (NmU) and the neuromedin U receptors (NmUR1 and NmUR2) in tissues from 8-week-old wild-type (wt) mice and NmU-overexpressing mice (NmU Tg). Values are expressed as fg/reaction for each mouse (n=2 per genotype)

<table>
<thead>
<tr>
<th>Tissue/genotype</th>
<th>NmU WT</th>
<th>NmU Tg</th>
<th>NmUR1 WT</th>
<th>NmUR1 Tg</th>
<th>NmUR2 WT</th>
<th>NmUR2 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>0.09, 0.72</td>
<td>504.5, 1161</td>
<td>467, 1.55</td>
<td>1.41, 1.36</td>
<td>1.80, 0.33</td>
<td>0.22, 0.24</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.06, 0.06</td>
<td>16950, 6750</td>
<td>0.65, 0.62</td>
<td>2.02, 0.68</td>
<td>0.34, 1.36</td>
<td>0.21, 0.47</td>
</tr>
<tr>
<td>Stomach</td>
<td>67.3, 17.85</td>
<td>21400, 12050</td>
<td>0.02, 0.02</td>
<td>0.06, 0.32</td>
<td>0.28, 0.10</td>
<td>0.13, 0.10</td>
</tr>
<tr>
<td>Intestine</td>
<td>3.23, 3.23</td>
<td>11210, 6510</td>
<td>3.23, 4.84</td>
<td>5.24, 3.11</td>
<td>7.85, 4.15</td>
<td>3.73, 2.50</td>
</tr>
<tr>
<td>Testis</td>
<td>0.09, 0.13</td>
<td>1525, 3025</td>
<td>5.35, 12.20</td>
<td>8.00, 4.96</td>
<td>2.16, 0.32</td>
<td>0.59, 1.03</td>
</tr>
<tr>
<td>Forebrain</td>
<td>0.14, 0.09</td>
<td>539, 105</td>
<td>0.94, 0.74</td>
<td>0.39, 0.95</td>
<td>2.02, 1.29</td>
<td>0.56, 0.96</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.22, 0.10</td>
<td>448, 276</td>
<td>0.41, 0.69</td>
<td>0.59, 0.68</td>
<td>4.28, 4.28</td>
<td>3.52, 2.41</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.04, 0.04</td>
<td>292, 589</td>
<td>0.17, 0.35</td>
<td>0.72, 0.29</td>
<td>0.51, 0.47</td>
<td>0.85, 0.38</td>
</tr>
<tr>
<td>Brainstem</td>
<td>1.05, 0.15</td>
<td>1222, 993</td>
<td>2.05, 4.70</td>
<td>3.00, 4.40</td>
<td>8.09, 6.82</td>
<td>3.90, 5.10</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>1.01, 0.53</td>
<td>564, 565</td>
<td>0.75, 1.04</td>
<td>0.47, 0.51</td>
<td>14.3, 9.21</td>
<td>5.11, 5.66</td>
</tr>
</tbody>
</table>
adiposity of NmU Tg mice (fat mass of 12.0 ± 1.5 g vs 6.9 ± 1.4 g (P<0.05) and lean mass of 21.4 ± 0.6 g vs 21.3 ± 0.9 (n.s.) for wild-type and Tg mice respectively).

NmU Tg mice were hypophagic, with average daily intake (calculated from 24 consecutive days of food intake measures) 20% less per day than wild-type animals (Fig. 4A). This difference remained when intake was expressed as a function of metabolic mass (kcal/(g body weight)0.75), with NmU Tg mice eating significantly less than wild-type mice (Fig. 4B). The hypophagic phenotype of NmU Tg mice was accompanied by significant differences in anorexigenic and orexigenic hypothalamic neuropeptides (Table 5). Specifically, NmU Tg mice had higher levels of NPY, POMC and MCH expression. No differences in CART, AgRP and corticotropin-releasing hormone (CRH) were observed between genotypes. Additionally, the GI transit time was measured in the NmU Tg and wild-type mice, and no significant effect was found (charcoal distance was 57.5 ± 1.2 vs 51.6 ± 3.3% of intestinal length for NmU Tg (n=14) and wild-type (n=13) mice respectively; n.s.).

The effect of the NmU transgene on glucose homeostasis

There was no significant effect of the NmU transgene on fasting glucose levels in chow-fed animals (Table 3). Insulin levels, however, were significantly lower in Tg mice (Table 3). Glucose tolerance, judged by an intraperitoneal glucose tolerance test (ipGTT), was similar between wild-type and Tg mice (data not shown); however, the Tg mice had improved insulin sensitivity (intraperitoneal insulin tolerance test (ipITT); Fig. 5).

Figure 1 Localization of NmU mRNA in mouse brain by in situ hybridization. Autoradiograms showing the overexpression of NmU in coronal sections of a Tg mouse at the level of the hypothalamic paraventricular (A) and arcuate (B) nuclei (bregma -0.82 and -1.82 for A and B respectively), and a wild-type mouse at the level of the hypothalamic paraventricular nuclei (C). While the expression appears to be ubiquitous relative to the wild-type brain, the overexpression is particularly apparent in the paraventricular (PVH), suprachiasmatic (SCN), supraoptic (SON), dorsomedial (DMN) and arcuate (ARC) nuclei of the hypothalamus. CA3: field CA3 of the hippocampus; Pir: piriform cortex.

Figure 2 NmU activity is detected in culture supernatant from Tg spleen cells. Spleen cells from an 8-week-old wild-type or NmU Tg mouse were cultured in serum-free medium for 5 days and then assayed for the ability to flux calcium in HEK293 cells stably expressing human NmU-R2. Human NmU-25 was used at the indicated concentrations as a positive control while unconditioned medium was used as a negative control. Data points are the mean of at least triplicate determinations with standard deviation shown.
Energy expenditure

There was a significant effect of genotype on body weight in the cohort of mice used in these studies (33·4 ± 1·1 vs 39·6 ± 1·7 g for NmU Tg and wild-type respectively (P < 0·01)). There was no significant effect of genotype on VO₂ (ml/min per kg⁰·⁷⁵) during ad libitum feeding, a 24-h fast, or the subsequent 24 h of refeeding (Fig. 6A). The RQ of Tg mice was significantly higher during the dark phase (F1,14= 2·67; P < 0·05) and the light phase (F1,14= 12·55; P < 0·005) of a 24-h fast, and in the dark phase of the refeeding period (F1,14= 10·3; P < 0·01) (Fig. 6B), indicating that the transition toward fat oxidation with fasting was attenuated in the Tg mice. The higher RQ with refeeding was not due to alterations in the rate of eating when food was presented after a 24-h fast (data not shown).

The effect of the Nmu transgene on diet-induced obesity

At 15–18 weeks of age, wild-type (n=8) and Nmu Tg mice (n=8) were placed on a high-fat diet. Initial body weights of Nmu Tg mice in this cohort were significantly lower than wild-type mice (29·7 ± 1·3 vs 36·3 ± 0·8 g; P < 0·005), similar to the findings in mice reported above. Food intake and body weight gain were monitored daily for 20 days. The Nmu Tg mice ate significantly less than wild-type mice (cumulative intake of 321 ± 11 vs 390 ± 9 kcal/mouse for Nmu Tg and wild-type mice respectively; P < 0·05); however, there was no statistical difference when daily intake was expressed as a function of metabolic mass (1·21 ± 0·03 vs 1·14 ± 0·03 kcal/(g body weight)⁰·⁷⁵ for wild-type and Nmu Tg mice respectively). Furthermore, there was no significant difference in feed efficiency during this 20-day period (11·8 ± 0·6 vs. 10·3 ± 2·0 kcal/g body-weight gain for wild-type and Nmu Tg, mice respectively).

After 11 weeks on the high-fat diet, the Nmu Tg mice weighed significantly less than wild-type mice; however, there was no significant effect of the transgene on percent body-weight gain over this time (Table 6). Nmu Tg mice had significantly smaller retroperitoneal, mesenteric and inguinal fat pad weights (Fig. 3B), with a trend toward less total dissectible fat content (18·6 ± 0·4 vs 16·7 ± 1·3% of body weight for wild-type and Nmu Tg mice respectively; P=0·089). Consistent with the lower fat mass, Nmu Tg mice had significantly lower plasma leptin levels (Table 6).

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**Table 3** Body weight, plasma leptin, insulin, glucose, corticosterone, triglycerides and free fatty acids of 24–32-week-old wild-type and Nmu Tg mice maintained on a chow diet (n=17–20 animals per group)

<table>
<thead>
<tr>
<th>Measure</th>
<th>Wild-type</th>
<th>Nmu Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>39·8 ± 1·3</td>
<td>34·4 ± 1·1*</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>19·4 ± 3·27</td>
<td>5·29 ± 1·28*</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>4·59 ± 0·94</td>
<td>1·96 ± 0·69*</td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>4·6 ± 0·2</td>
<td>4·4 ± 0·2</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>53·1 ± 8·6</td>
<td>76·9 ± 12·4</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1·99 ± 0·2</td>
<td>1·96 ± 0·2</td>
</tr>
<tr>
<td>Free fatty acids (mM)</td>
<td>0·559 ± 0·035</td>
<td>0·552 ± 0·044</td>
</tr>
</tbody>
</table>

*, significantly different from wild-type animals.

---

**Figure 3** Epididymal (Epi), retroperitoneal (RP), inguinal (Ing) and mesenteric (Mes) adipose depot weights of (A) 24–32-week-old wild-type and Nmu Tg mice that were maintained on chow (n=17–20 per group) or (B) 20–24-week-old wild-type and Nmu Tg mice maintained on chow and then switched to a high-fat diet for 11 weeks (n=7–8 per group). Black bars: wild-type; gray bars: Nmu Tg. *Significantly different from wild-type animals (P<0·05).
The liver weights of NmU Tg mice were significantly less than wild-type mice when expressed as absolute weight or as percentage of body weight (Table 7). This smaller liver weight in NmU Tg mice was accompanied by significantly less triglyceride and cholesteryl ester (Table 7).

Glucose tolerance after high-fat feeding

After 8 weeks on the high-fat diet, plasma insulin levels in NmU Tg mice were approximately 30% of those in wild-type mice (Table 6), and there was a trend toward a significant effect of the transgene on fasting glucose concentrations (114±3±7·0 vs 98·6±4·1 mg/dl for wild-type and NmU Tg mice respectively; \( P=0·076 \)). Consistent with the attenuated hyperinsulinemia, the transgene had a significant effect on glucose tolerance (Fig. 7), indicating that the negative impact of high-fat feeding on glucose homeostasis was partially corrected by the presence of the NmU transgene.

Discussion

NmU has been implicated in the control of food intake and body weight regulation. We show that mice possessing a human CMV enhancer/chicken β-actin-pro-NmU transgene broadly overexpress murine pro-NmU, and that bioactive peptide can be produced from the transgene. NmU Tg mice maintained on a chow diet are hypophagic and lean relative to wild-type mice, and display improved insulin sensitivity. There is no significant effect of NmU overexpression on \( V_{O2} \) or locomotor activity. Furthermore, when fed a high-fat diet, NmU Tg mice remain leaner than wild-type mice, although they are not refractory to an increase in adiposity with high-fat feeding. Despite the weight gain, high-fat-fed NmU Tg mice display improved glucose tolerance. Collectively, these findings support the hypothesized role of NmU as an anorectic peptide involved in regulation of energy homeostasis.

Table 4  Liver weight and lipids of 24–30-week-old wild-type and NmU Tg mice maintained on a chow diet (\( n=17–20 \) animals per group)

<table>
<thead>
<tr>
<th>Measure</th>
<th>Wild-type</th>
<th>NmU Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>1·94±0·13</td>
<td>1·43±0·05*</td>
</tr>
<tr>
<td>Liver (% body weight)</td>
<td>4·82±0·2</td>
<td>4·17±0·10*</td>
</tr>
<tr>
<td>Liver lipids (mg/g liver)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>77·5±10·9</td>
<td>50·7±5·8*</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>3·70±0·13</td>
<td>3·59±0·24</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>0·74±0·07</td>
<td>0·85±0·11</td>
</tr>
</tbody>
</table>

*  , significantly different from wild-type animals.

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Because NmU expression (and presumably synthesis and release) in hypothalamic and hindbrain neurons is regulated by nutritional state (Ivanov et al. 2002), and i.c.v. or intra-PVN administration of NmU elicits robust anorexigenic effects (Howard et al. 2000, Kojima et al. 2000, Nakazato et al. 2000, Wren et al. 2002), NmU probably exerts its effects on food intake and energy expenditure within the CNS, perhaps through NmU-R2 (Shan et al. 2000). Consistent with this, we show that the transgene is expressed throughout the CNS and that its presence

Figure 4  Effect of the NmU transgene on average daily food intake in 24–32-week-old chow-fed mice. Food intake, expressed as grams per day (A) or as kcal/(g body weight (BW))\( ^{0.75} \) per day (B), is an average over a 24-day period. \( n=17–20 \) per group.

*Significantly different from wild-type controls (\( P<0·05 \)).
Table 5 Expression of neuropeptide mRNAs in the hypothalamus of 30-week-old wild-type (n=9) mice and mice overexpressing NmU (NmU Tg; n=8) maintained on a chow diet. Units are fg/reaction except for 18S, which is expressed as pg/reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Wild-type</th>
<th>NmU Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>2748 ± 116</td>
<td>2732 ± 88</td>
</tr>
<tr>
<td>NmU</td>
<td>0.063 ± 0.008</td>
<td>0.369 ± 0.21*</td>
</tr>
<tr>
<td>NPY</td>
<td>3050 ± 260</td>
<td>4570 ± 670*</td>
</tr>
<tr>
<td>POMC</td>
<td>7960 ± 530</td>
<td>13550 ± 1900*</td>
</tr>
<tr>
<td>CART</td>
<td>390 ± 50</td>
<td>380 ± 30</td>
</tr>
<tr>
<td>MCH</td>
<td>1450 ± 100</td>
<td>2070 ± 180*</td>
</tr>
<tr>
<td>AgRP</td>
<td>138 ± 14</td>
<td>130 ± 19</td>
</tr>
<tr>
<td>CRH</td>
<td>0.84 ± 0.1</td>
<td>0.85 ± 0.15</td>
</tr>
</tbody>
</table>

*, significantly different from wild-type animals.

confers effects on feeding and body weight that are consistent with the actions produced by exogenous central administration of NmU.

The overexpression of NmU significantly altered the expression of several hypothalamic neuropeptides implicated in the regulation of food intake and energy expenditure. Chow-fed NmU Tg mice had levels of NPY, POMC and MCH mRNA that were higher than those of wild-type mice. Higher expression of POMC may result in an increased production and release of α-MSH, a potent anorexigenic peptide acting as an agonist at melanocortin 4 receptors, thus providing a possible mechanism driving the hypophagia in response to NmU overexpression. Because NPY and MCH are orexigenic, the higher expression level of these may be compensatory to the lower body weight, lower circulating leptin, or hypophagia. The hypophagia in Tg mice that occurs despite elevated NPY and MCH expression suggests that NmU may be involved in the downstream effects of these two neuropeptides. Further work examining the responsiveness of these animals to exogenous administration of orexigenic and anorexigenic peptides, as well as deprivation-induced changes in expression of these neuropeptides, may provide more insight.

The lean phenotype of the NmU-overexpressing mice appears to be predominantly due to hypophagia, as it occurs in the absence of behavioral changes that might otherwise contribute to reduced body weight (e.g. increased activity) or an increased oxygen consumption indicating hypermetabolism. The failure to detect hyperactivity or hyperthermia in NmU Tg mice was surprising in light of the reported increase in locomotor activity and rectal temperature after i.c.v. NmU administration (Howard et al. 2000, Nakazato et al. 2000, Hanada et al. 2001). One possible reason for this discrepancy is that an acute increase in NmU signaling, as occurs with i.c.v. administration, promotes a response that is different from that seen with chronic NmU signaling. Because the relative expression of the transgene within the hypothalamus is variable between nuclei, it is also possible that NmU is not elevated in all the relevant neurons required to elicit an effect on locomotor activity and body temperature. Lastly, the expression level or localization of central NmU receptors may be altered by the chronic NmU signaling in Tg mice. Our data do not support the latter possibility as evidenced by no gross difference in NmU-R1 and -R2 mRNA in all tissues studied; however, subtle changes in expression or differences in neural distribution of receptors may be present.

Prior reports have shown that central administration of NmU increases hypothalamic CRH expression, and that central or peripheral NmU administration increases plasma adrenocorticotropic hormone (ACTH) and corticosterone (Malendowicz et al. 1994, Wren et al. 2002). Furthermore, the hyperactivity associated with central NmU administration is abolished in CRH knockout mice or after treatment with a CRH-R1 antagonist (Hanada et al. 2001). Interestingly, we found no significant difference in hypothalamic CRH expression or plasma corticosterone levels between chow-fed, wild-type and NmU Tg mice, despite the elevated NmU expression in the PVN of Tg mice. These findings indicate that the hypophagia observed in the NmU Tg mice is unlikely to be a result of a heightened CRH tone; however, measures of CRH protein and pharmacologic responses to CRH receptor antagonists are required to fully evaluate this.

Evaluation of energy expenditure by indirect calorimetry showed no effect of genotype on $V_{O2}$, observations that are not consistent with those demonstrating a ~20% increase in oxygen consumption with i.c.v. NmU administration (Hanada et al. 2003). It is possible that the reported increase in $V_{O2}$ after i.c.v. NmU administration is due to an increase in locomotor activity and grooming behavior, and not to increased thermogenesis.
Figure 6  Effect of the NmU transgene on (A) VO$_2$ and (B) RQ during the fed state, a 24-h fast and 24 h of refeeding in 27–28-week-old mice. Filled circles: wild-type; open circles: NmU Tg. n=8 per group. *Significantly different from wild-type animals (P<0·05).
Table 6 Terminal body weight and weight gain, plasma leptin, insulin, triglycerides and free fatty acids of 27–30-week-old wild-type (n=8) and NmU Tg (n=7) mice after 11 weeks of high-fat feeding

<table>
<thead>
<tr>
<th>Measure</th>
<th>Wild-type</th>
<th>NmU Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>49.7 ± 0.9</td>
<td>40.7 ± 2.7*</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>13.5 ± 0.7</td>
<td>10.9 ± 1.4</td>
</tr>
<tr>
<td>Body weight gain (%)</td>
<td>37.9 ± 2.5</td>
<td>36.2 ± 3.6</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>71.1 ± 3.1</td>
<td>47.5 ± 10.7*</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>57.1 ± 1.26</td>
<td>15.7 ± 9.3*</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.89 ± 0.1</td>
<td>0.89 ± 0.2</td>
</tr>
<tr>
<td>Free fatty acids (mM)</td>
<td>1.17 ± 0.09</td>
<td>1.31 ± 0.10</td>
</tr>
</tbody>
</table>

*, significantly different from wild-type animals.

(Howard et al. 2000, Ivanov et al. 2002, Wren et al. 2002, Hanada et al. 2003). The lack of behavioral differences between wild-type and NmU Tg mice is consistent with this. Interestingly, depriving the mice of food for 24 h revealed a modest but significantly higher RQ that was also apparent during the early refeeding phase. Specifically, NmU Tg mice showed an attenuated shift in the transition toward fat oxidation with fasting. Although the mechanism(s) responsible for this shift in substrate oxidation are not known, it is possible that NmU overexpression provoked subtle changes in circadian oscillations during food deprivation and refeeding. Supporting this hypothesis are studies in rats and mice showing that NmU is expressed in the SCN (Howard et al. 2000, Nakahara et al. 2004), and that i.c.v. injections of NmU to rats during the subjective light phase induces a phase shift in the circadian rhythm (Nakahara et al. 2004).

We observed no difference in the intestinal transit of a charcoal-based marker between wild-type and NmU Tg mice. Although NmU stimulates ileal smooth muscle contraction in dogs and humans in vitro (Maggi et al. 1990, Westfall et al. 2002), it failed to stimulate porcine jejenum, guinea pig ileal smooth muscle, and rat small and large intestinal longitudinal smooth muscle in vitro (Minamino et al. 1985a, Brown & Quito 1988, Benito-Orfíla et al. 1991). The absence of a difference between wild-type and NmU Tg mice in this regard is therefore not entirely surprising, and indicates that changes in intestinal motility are probably not involved in the phenotype observed. Recent work, however, has shown that central administration of NmU decreases gastric acid secretion and slows gastric emptying in rats (Mondal et al. 2003), potentially affecting meal size and frequency. This contrasts with studies showing that NmU directly stimulates contraction of rat fundic circular smooth muscle in vitro (Benito-Orfíla et al. 1991). Given that there are species differences in smooth muscle contractility in response to NmU in vitro, studies examining gastric emptying and nutrient absorption are required to fully assess whether changes in GI smooth muscle physiology play a role in the phenotype of these Tg mice.

The exact mechanism by which the NmU transgene confers improved glucose homeostasis is not known. There is a strong positive correlation between visceral fat and insulin resistance (Bjorntorp 1991), while reductions in visceral fat mass, either through lipectomy or caloric restriction, have a pronounced effect on hepatic insulin sensitivity in rodents (Barzilai et al. 1999a,b). The reduction in hepatic triglyceride and somatic adipose tissue content associated with the improved glucose homeostasis in Tg mice is consistent with these observations, suggesting that the lower fat mass of NmU Tg mice is driving this aspect of the phenotype. It is not known, however, whether this is due to alterations in hepatic insulin sensitivity, peripheral insulin sensitivity, or both. Whether or not the change in glucose homeostasis is due to a fat-mass-independent mechanism remains to be determined.

In summary, we have shown that ubiquitous expression of a transgene encoding murine NmU in mice produces a lean, hypophagic phenotype with improved glucose...
homeostasis. Based on prior pharmacologic reports, we hypothesize that this phenotype is the result of increased bioactive Nmu peptide and signaling within the CNS. These results suggest that Nmu receptors, particularly Nmu-R2, may be a target for the treatment of obesity and eating disorders. Future studies characterizing mice null for the Nmu1, Nmur1 and Nmur2 genes will extend these findings, and better define the role of Nmu, and the relevant receptors, in feeding behavior and energy homeostasis.

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