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Pro-opiomelanocortin Gene Transfer to the NTS but not ARC Ameliorates Chronic Diet-Induced Obesity

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Abstract

Short-term pharmacological melanocortin activation deters diet-induced obesity (DIO) effectively in rodents. However, whether central pro-opiomelanocortin (POMC) gene transfer targeted to the hypothalamus or hindbrain nucleus of the solitary track (NTS) can combat chronic dietary obesity has not been investigated. Four-week-old Sprague Dawley rats were fed a high fat diet for five months, and then injected with either the POMC or control vector into the hypothalamus or NTS, and body weight and food intake recorded for 68 days. Insulin sensitivity, glucose metabolism and adrenal indicators of central sympathetic activation were measured, and voluntary wheel running (WR) assessed. Whereas the NTS POMC-treatment decreased cumulative food consumption and caused sustained weight reduction over 68 days, the hypothalamic POMC-treatment did not alter cumulative food intake and produced weight loss only in the first 25 days. At death, only the NTS-POMC rats had a significant decrease in fat mass. They also displayed enhanced glucose tolerance, lowered fasting insulin and increased QUICK value, and elevated adrenal indicators of central sympathetic activation. Moreover, the NTS-POMC animals exhibited a near 20% increase in distance ran relative to the respective controls, but the ARC-POMC rats did not. In conclusion, POMC gene transfer to the NTS caused modest anorexia, persistent weight loss, improved insulin sensitivity, and increased propensity for WR in DIO rats. These metabolic improvements may involve stimulation of energy expenditure via centrally regulated sympathetic outflow. The similar POMC treatment in the hypothalamus had minimal long-term physiological or metabolic impact. Thus, melanocortin activation in the brainstem NTS region effectively ameliorates chronic dietary obesity whilst that in the hypothalamus fails to do so.

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Keywords

ARC; NTS; dietary obesity; voluntary wheel running; melanocortin

Introduction

The brain melanocortin (MC) system has critical functions in the homeostatic regulation of body weight (Benoit et al., 2000, Ellacott and Cone, 2004). Pro-opiomelanocortin (POMC) is the precursor for alpha-melanocyte-stimulating hormone (α -MSH), the principal central melanocortin (Benoit et al., 2000, Ellacott and Cone, 2004). Rodents and humans with POMC deficiency or mutations are hyperphagic and obese (Krude et al., 1998, Yaswen et al., 1999). Alpha-MSH or its analog, melanotan II (MTII) inhibits food intake and enhances energy expenditure via activation of the two central MC receptors, MC3R and MC4R (Abdel-Malek, 2001, Cone, 2005). Although the hypothalamic MC system in the forebrain has received much attention, the role of the POMC network in energy homeostasis in the hindbrain has only been appreciated recently. In addition to the arcuate nucleus (ARC) of the hypothalamus, POMC and α -MSH are also found within the commissural region of the nucleus of the solitary tract (NTS) in the brainstem (Joseph et al., 1983, Palkovits et al., 1987) where the MC4R is also abundant (Mountjoy et al., 1994, Kishi et al., 2003). Both the MC agonist, MTII, and antagonist, SHU9119, evoke dose-dependent decreases or increases, respectively, in feeding and body weight upon either lateral or fourth intracerebroventricular (icv) administration, and the responses induced by hindbrain stimulation are as robust as those in the forebrain (Grill et al., 1998, Williams et al., 2000, Zheng et al., 2005). Since hypothalamic POMC neurons send direct projections to the NTS (Palkovits et al., 1987, Zheng et al., 2005), the NTS could serve as a site integrating the neuroendocrine and metabolic impact of POMC that is generated both within the NTS and the ARC.

Pharmacological activation of the central MC system is highly effective in reducing food intake and body weight in either lean or obese rodents including those of genetic and dietary origins (Hansen et al., 2001, Hwa et al., 2001, Scarpace et al., 2003, Ellacott and Cone, 2004, Li et al., 2004, Zhang et al., 2004). A transgenic mice model over-expressing the N-terminal POMC transgene also deters the development of dietary obesity (Savontaus et al., 2004, Lee M, 2007). However, both of these approaches would presumably evoke simultaneous MC activation in the forebrain hypothalamus and hindbrain NTS, and therefore, cannot dissect contributions from the individual brain regions to the metabolic outcomes. Our group has employed recombinant adeno-associated virus-mediated central POMC gene transfer (rAAV-POMC) to ameliorate pre-existing obesity of either genetic or dietary causes (Li et al., 2003, Li et al., 2005, Li et al., 2007). On a low fat chow diet, rAAV-POMC transfer to the ARC alone in adult-onset obese F344xBN rats resulted in a transient anorexia as opposed to a more persistent food reduction induced by rAAV-POMC transfer to the NTS (Li et al., 2003, Li et al., 2005, Li et al., 2007). On the other hand, the effectiveness of chronic central melanocortinergeric activation on weight regulation has not been evaluated in animals with established DIO and continued maintenance on the high fat (HF) diet. In particular, the separate activation of either the ARC or NTS by site-directed over-expression of POMC on the long-term metabolic consequences has not been examined.

In addition to affecting energy intake, melanocortins also impact physical activity. For example, elevated AgRP is one factor that can suppress physical activity (Tang-Christensen et al., 2004). The present study, thus, also aims to determine the potential role of POMC gene transfer in the ARC or NTS in enhancing physical activity, as assessed by voluntary WR.

To these ends, we employed dietary obese Sprague Dawley rats pre-treated with a 32% high fat diet for 5 months and delivered rAAV-POMC via microinjection to either the ARC or NTS. Food consumption, body weight, glucose and fat metabolism, adrenal sympathetic factors as well as the propensity for voluntary wheel running were assessed following 68 days of treatment.

Experimental Methods

Experimental animals

Five-month-old male Sprague Dawley rats were obtained from Charles River. The rats were fed a 32% HF diet (32% of the energy is provided from fat, 4.41 Kcal/g, RD12266B; Research Diets, New Brunswick, NJ) by the supplier from 4-week of age (HF-raised), and continued on the same HF diet throughout the study. Upon arrival, rats were examined and remained in quarantine for one week. Animals were cared for in accordance with the principles of the NIH Guide to the Care and Use of Experimental Animals and the protocols approved by the Malcom Randall Veterans Affairs Medical Center IACUC committee. Rats were housed individually with a 12:12 h light:dark cycle (07:00 to 19:00 hr).

Preparation and administration of rAAV-POMC

pTR-POMC encodes the full-length 935 base pair murine POMC cDNA (Uhler and Herbert, 1983) under the control of the hybrid cytomegalovirus immediate early enhancer/chicken β -actin promoter, and construction of this vector, designated as rAAV-POMC was described previously (Li et al., 2003). The control vector, rAAV-Control, is similar to rAAV-POMC except for the incorporation of the cDNA encoding an enhanced form of green fluorescent protein (GFP) in place of the POMC. Both vectors were packaged as Serotype 5 AAV, then purified, concentrated and tittered (Li et al., 2003). The titers for both vectors used in this study were 2.51×10^{13} physical particles/ml.

Rats, under isoflurane anaesthesia (5% induction, 2–2.5% for maintenance), were administered rAAV-POMC or rAAV-Control. Viral particles (containing 2.51×10^{10} in 1 μ l) were introduced into the NTS or basomedial hypothalamus by separate injections using coordinates established previously (Li et al., 2005, Li et al., 2007). A 10 μ l-capacity Hamilton syringe was used to deliver 1 μ l virus over 5 min to the target site. The needle remained in place at the injection site for a further 2 min before withdrawal. The analgesic, Buprenex (buprenorphine hydrochloride, 0.05 mg/kg; Reckitt and Colman, Richmond, VA), was provided to the animals before the surgery and 8–12hrs afterwards.

Histological analysis of GFP over-expression

Separate rats receiving rAAV-GFP injection to either the ARC or NTS were used for histological verification of GFP expression. The distribution of GFP immunofluorescence is used here as a marker for potential POMC transgene over-expression pattern. The rats were overdosed with sodium pentobarbital (100 mg/kg i.p.) one month after rAAV-GFP gene delivery (a duration of time to allow GFP protein expression to accumulate to maximal), and perfused at 65 ml/min through the left ventricle with 100 ml 0.1 M phosphate-buffered saline (PBS) pH 7.2–7.4 at ambient temperature, followed by 400 ml PBS containing 10% formalin. The intact rat bodies were refrigerated for 2 hours at 4°C prior to brain extraction and equilibrated in 30% sucrose PBS cryoprotectant. Following excising the whole brain, coronal blocks were frozen-sectioned at 50 μ m, and sections collected in 300 μ l PBS/well in 24-well polystyrene culture plates (Phoenix). Plates were incubated overnight on a rotating shaker in 200 μ l/well PBS plus 0.02% sodium azide, 0.1% Triton X-100, and 1 μ g/ml primary antibody recognizing GFP (Clontech). Following two 300 μ l PBS washes, sections were incubated overnight in biotinylated goat anti-rabbit IgG 1:1000, washed again, and

incubated overnight in extravidin peroxidase at 1:1000 in PBS. After two additional washes, sections were reacted 5 min in 0.5 mg/ml diaminobenzidine in 0.1 M sodium acetate with 1.2 to 5% hydrogen peroxide, then mounted on glass slides from 0.1 M saline and coverslipped with glycerol gelatin. All chemicals were from Sigma unless noted otherwise. Bright-field images were collected using an Olympus BH-2 microscope with an attached Hitachi KP-D581 color digital video camera equipped with ImagePro Plus software (Media Cybernetics).

RT-PCR

Expression levels of POMC in the NTS or ARC micropunch samples were identified by relative quantitative RT-PCR using the QuantumRNA 18S Internal Standards kit (Ambion, Austin, TX, USA). First-strand cDNA was generated from 1.5 µg RNA in a 20 µl volume using random primers containing 200 units of M-MLV reverse transcriptase (Life Technologies). RT-PCR was performed to co-amplify both sample signals and internal 18S standards by multiplexing specific primers, 18S primers, and competitors. The primer sequences employed for POMC are: sense, 5'-GCTTGCAAACCTCGACCTCTC-3', anti-sense, 5'-CTTGATGATG-GCGTTCCTGA-3'. Linearity for amplicons was determined to be 20–40 PCR cycles for POMC. The PCR products were electrophoresed on acrylamide gel and stained with Ethidium Bromide (Sigma, St. Louis, MO).

Northern analysis of adrenal TH, DβH and NPY mRNA levels

Adrenal glands were removed quickly and immediately frozen by immersion in liquid nitrogen. Tissues were stored at –80°C. At the time of the assay, adrenal glands were decapsulated, and the medullae were separated from the cortex. Adrenal medullae were weighed and homogenized in 100 µl of phosphate buffer (2 mM NaPO₄, 0.2% Triton, pH 7.0). TH, DβH and NPY mRNA levels were determined using Northern blot analysis as previously described (Tumer and Larochelle, 1995, Erdem et al., 2002). Briefly, total cellular RNA was extracted using Tri-reagent (Sigma, St. Louis, MO), and the isolated RNA quantified by spectrophotometry. Membranes were hybridized with ³²P random primer-generated probes. After hybridization, the membranes were washed and exposed to phospho-screen for 24 hours using Phospho Imager (Molecular Dynamics, Sunnyville, CA). Screens were scanned and analyzed using ImageQuant software (Molecular Dynamics).

Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed on days 63 and 65 after vector administration in the NTS and ARC animals, respectively. Rats were fasted overnight and injected intraperitoneally with glucose (2g/kg body weight) at 10:00 h. Tail blood was collected at 0, 15, 30, 60, 120 min after injection. Blood glucose was measured by One Touch SureStep glucometer (LifeScan, Milpitas, CA).

Wheel Running

Rats were housed in cages equipped with Nalgene Activity Wheels (1.081 meters circumference) obtained from Fisher Scientific (Pittsburgh, PA) and allowed free access to the wheel. Each wheel was equipped with a magnetic switch and a counter with liquid crystal display (LCD) that records the number of wheel revolutions. The numbers of revolutions were recorded daily for consecutive 5 days.

Grip Strength

Forelimb grip strength was determined using an automated grip strength meter (Columbus Instruments, Columbus Ohio). The rats were grasped by the base of the tail and suspended above a grip ring. After about 3 sec, the animal was gently lowered toward the grip ring and

allowed to grasp the ring with its forepaws. The experimenter then quickly lowered the remainder of the animal's body to a horizontal position and tugged the animal's tail until its grasp of the ring was broken. The mean force in grams was determined with a computerized electronic pull strain gauge that was fitted directly to the grasping ring, and the resulting value normalized to body weight. Average measurements from three successful trials were taken as the final outcome. Successful trials are defined as those in which the animal grasped the ring with both forepaws and pulled the ring without jerking (Carter et al., 2004).

Tissue harvesting and preparation

Rats were killed at day 86 under 5% isoflurane anaesthesia. Blood samples were collected by heart puncture, and serum was harvested by a 10-min centrifugation in serum separator tubes. The circulatory system was perfused with 40 ml of cold saline, and hypothalamus, perirenal and retroperitoneal white adipose tissues (PWAT and RTWAT, respectively), brown adipose tissue (BAT), and adrenal were excised. Protein concentrations of the tissue homogenates were determined using the DC protein assay kit (Bio-Rad, Hercules, CA).

Fasting serum insulin, triglyceride and glucose

Radio-immunoassay (RIA) kits from Linco Research were used to measure insulin (rat RIA). Triglyceride (TG) in serum was determined using enzymatic colorimetric kits from Wako Chemicals (Richmond, VA), and fasting glucose by Trinder agent (Sigma Diagnostics).

Statistical analysis

Data are presented as means $\pm$ SE. One-way ANOVA with POMC-treatment as the factor was employed for all data analyses except for the paired t-test used to draw comparisons between the Chow and DIO rats for specific parameters listed in Figure 1. When the main effect was significant ($p < 0.05$), a Bonferroni multiple comparison post-hoc test was applied to determine differences between means of the experimental groups.

Results

Metabolic impairment and physical function decline with high-fat feeding

The DIO rats had an average body weight of 700g after five months of HF feeding beginning at 4-week of age. Compared to a group of weight-matched and standard chow-raised rats, the DIO animals had more than 2-fold higher fasting serum leptin level (Fig 1a) and three times greater fasting serum triglycerides (Fig 1b). In addition, grip strength (Fig 1c) and voluntary wheel running activity (Fig 1d) were significantly decreased in DIO rats relative to their weight-matched, chow-fed counterparts.

POMC over-expression and immunohistological evaluation of GFP expression

The POMC transgene over-expression was verified by RT-PCR using brain micropunch samples. Eighty-six days after vector delivery, the ARC or NTS POMC mRNA levels were elevated in the POMC-treated rats compared with the respective controls (Fig 2E).

Because POMC is a precursor neuropeptide that gives rise to an array of bioactive products, it is not feasible to directly evaluate POMC peptide distribution at the brain targeting site(s) via imaging analysis. However, our control vector contains a GFP reporter gene, and GFP immuno-fluorescence can be used to identify cells transfected with rAAV-GFP and GFP expression pattern. This provides a representation of plausible rAAV-mediated POMC cell transduction and distribution of POMC expression. Strong and punctuated GFP immunoreactivity was visible in coronal sections through NTS including medial and

ventrolateral areas of the solitary tract (Fig. 2A), and dense GFP staining was observed along the 3rd ventricle of the basomedial posterior hypothalamus, especially in the region of the ARC of the hypothalamus (Fig. 2C). GFP expression in either the NTS or ARC was exclusively localized to neurons (Fig. 2B and 2D). GFP immunoreactivity was minimally seen in the contralateral side of vector delivery of either the NTS or hypothalamus (Fig. 2A and 2C).

Body weight and food consumption

POMC gene transfer into either ARC or NTS initiated body weight reductions that became statistically significant from respective controls by day 4 in the ARC-POMC rats (Fig 3a) and by day 6 in the NTS-POMC rats (Fig 3b). The ARC-Control and NTS-Control animals lost about 10 and 25g of body weight respectively in the first two days following surgery, but the ARC-Control group recovered this lost weight in 4 days (Fig 3a), whereas the NTS-Control group recuperated the weight reduction after 13 days (Fig 3b). The greater weight loss and longer duration for weight recovery with or without changes in food intake in the NTS control animals upon surgical manipulation has been observed in other studies (Li et al., 2005, Li et al., 2007). This phenomenon may be related to the importance of the NTS in the control of cardiovascular and respiratory responses, meal initiation and termination, and central modulation of energy expenditure. The body weight decrease with rAAV-POMC treatment in the ARC was transient, and by day 23, there was no longer a difference relative to controls (Fig 3a). On the contrary, the NTS-POMC rats maintained a lower body weight gain throughout the entire treatment period as compared to the NTS-Control animals (Fig 3b).

In contrast to the substantial reductions in body weight, decreases in food consumption were less apparent. The POMC treatment in the ARC, in fact, failed to either suppress daily food intake significantly over the entire experimental period (Fig 4a) or the cumulative food intake during the first 24 days or from days 25–56 (Fig 4c). The NTS-POMC rats displayed a decrease in food consumption over the first 24 days (Fig 4b), with cumulative food consumption significantly reduced during this period compared with the NTS-control animals (Fig. 4c). The cumulative food intake from days 25–56 in the NTS-POMC rats had a decreasing trend, but did not reach statistical significance relative to the controls (Fig 4c).

White, brown adipose tissues and serum parameters

Consistent with the body weight data, the individual white fat mass collected at the terminus of the study was not different between the ARC-Control and ARC-POMC animals, whereas the rAAV-POMC transfer to the NTS resulted in a significant loss of tissue mass in all three fat depots examined, including perirenal, retroperitoneal, and epididymal white adipose tissue (PWAT, RTWAT, and EWAT) (Table 1). Serum parameters, in general, paralleled the physiological data: only the NTS-POMC rats displayed lower fasting glucose, fasting insulin and enhanced QUICK value (the Quantitative Insulin Sensitivity Check Index, one indicator of insulin sensitivity) as compared to their respective controls (Table 1). On the other hand, the POMC delivery in either the ARC or NTS reduced serum triglycerides concentrations (Table 1).

Neither the POMC treatment in the ARC nor in the NTS resulted in any change in either the total brown fat mass or the uncoupling protein (UCP1) level (data not shown).

Glucose tolerance test

In addition to assessing fasting glucose and insulin values, we conducted a glucose tolerance test at day 63 to probe for a potential improvement in insulin sensitivity mediated by the POMC treatment. The POMC transfer into the NTS produced a significant increase in

glucose tolerance following a 2g/kg glucose loading compared to NTS-Control rats (expressed as area under the curve, 1269.6 + 64.9 for NTS-Control versus 958.2 + 88.1 for NTS-POMC, Fig 5), while it was without effect in the ARC POMC-treated animals (data not shown).

Wheel running

Physical activity constitutes one aspect of energy expenditure. One measure of volitional physical activity is voluntary wheel running. On day 45 (for the NTS group) and day 57 (for the ARC group) following POMC gene transfer, the home cages were equipped with running wheels and voluntary WR assessed for five consecutive days. During this time, the rats had access to food, *ad libitum*. The animals were not coerced in any way to run but had been subjected to a five-day WR assessment prior to rAAV-POMC gene therapy (Fig 1d, DIO). The ARC- and NTS-Controls ran to the same extent, and the POMC-treatment in the ARC did not impact WR activity. However, the NTS-POMC rats had a modest but significant 18% increase in the distance ran (Fig 6). The majority of the voluntary WR occurred at night (data not shown).

NPY, DBH and TH expression in adrenal

Central pharmacological melanocortin activation has been reported to increase sympathetic activity (Nogueiras et al., 2007). Therefore, we examined whether rAAV-POMC gene transfer into either the ARC or NTS stimulates the Hypothalamic-Pituitary-Adrenal axis, leading to an increase in catecholamine biosynthesis in the adrenal medulla. We measured three factors in adrenal medulla that are indicators for catecholamine biosynthesis, tyrosine hydroxylase, dopamine- β -hydroxylase, and neuropeptide Y mRNAs. Basal levels of TH, DBH and NPY mRNAs were similar in both ARC- and NTS-Control animals and were unchanged following POMC treatment in the ARC. In contrast, POMC gene transfer in the NTS significantly elevated all three indicators of catecholamine biosynthesis (Fig 7).

Discussion

HF-diet induced obese rodents represent a useful animal model to understand and investigate human dietary obesity resultant from an abundant supply of caloric dense, palatable foods. Chronic consumption of such HF diets not only creates dire metabolic consequences but also may adversely impact physical activity due to dietary weight gain. This aspect is demonstrated clearly in the present study, where wheel-running activity employed as one measure of physical activity, is diminished in dietary obese rats compared with standard chow-fed rats. In addition, the obese rats display impairment in muscle function manifested as decreased forelimb grip strength. It is uncertain currently if weakened muscle contributes to the decrease in WR activity in these animals. But in humans, obesity is a major contributor to disability; older persons with obesity spend two-times more years disabled than normal weight counterparts (Reynolds et al., 2005).

Combating DIO is a long-standing challenge. Typical leptin therapy has proven futile in obese humans and animals demonstrating both leptin and insulin resistance (Widdowson et al., 1997, Heymsfield et al., 1999, Levin and Dunn-Meynell, 2002). The central MC system, however, has emerged as an attractive therapy target for treating dietary obesity because melanocortin agonists evoke impressive weight loss and insulin sensitisation in obese rodents (Hansen et al., 2001, Li et al., 2004, Zhang et al., 2004). Mounting evidence underscores the fact that the MC systems in both the ARC of hypothalamus and NTS are important regulators of energy intake and expenditure (Grill et al., 1998, Ellacott and Cone, 2004, Zheng et al., 2005). Prominent POMC and AgRP neurons in the ARC project to other hypothalamic areas, and from there to the NTS and other places in the brainstem where the

descending hypothalamic inputs are integrated with peripheral afferent inputs from the liver and gastrointestinal tract (Ellacott and Cone, 2004). The caudal brainstem can carry out autonomic control of ingestion, digestion, and absorption of food separate from the forebrain influence (Gao and Horvath, 2008). Normal ingestive behaviour and energy balance cannot occur without the circuits of the brainstem (Gao and Horvath, 2008). Thus, to fully understand the critical functions of the central MC system in weight regulation, it is necessary to examine the contributions from the hypothalamus and brainstem separately.

We established, in this study, that the neuro-site directed rAAV-POMC gene transfer to either the NTS or ARC resulted in POMC over-expression in a reasonably region-specific manner in the obese rats. The POMC treatment in the NTS produced a mild and transient anorexia (for the first 24 days) whereas that in the ARC only generated a trend for reduction without affecting the cumulative food intake. The ARC-POMC rats displayed a lower body weight gain versus the respective controls up to day 24 post the POMC vector delivery, but their body weights merged with those of controls afterwards, matching the transient, 24-day food suppressive effect. The end-point adiposity levels and several serum parameters indicated no overall metabolic improvements after the duration of the POMC gene therapy in the ARC. In contrast, rAAV-POMC delivered to the NTS resulted in a weight reduction that was unabated throughout the entire experimental period despite the attenuation of the anorectic response after the initial 24 days. Additionally, POMC NTS treatment also decreased fat mass and enhanced glucose metabolism and insulin sensitivity.

Because the current study was long-term in nature and attenuation of the POMC anorectic effect (for treatment in either the ARC or NTS) occurred during the early phase of the experiment, we did not measure some important peptides/hormones involved in energy intake regulation such as NPY, ghrelin and cholecystokinin (CCK) at termination. Both ARC (with its location near the median eminence) and NTS (as recipient for afferent vagal neuron projections from the gastrointestinal tract) are sites for integration of neuronal and circulating signals pertaining to energy status, and gut hormones such as ghrelin and CCK can act on either region to affect food ingestion (Ellacott and Cone, 2004). It would be interesting to investigate, if the hypophagic response to the POMC transfer in the first 10 to 20 days or so involved these gut factors; and further, if the persistent weight reduction in the NTS-POMC rats caused any changes in circulating ghrelin concentration and/or its or perhaps CCK receptor levels in the CNS or gut.

There are two apparent implications from the current study. The experiment was conducted in rats with established dietary obesity, and the HF feeding regimen continued throughout the treatment period. Under these conditions, the POMC gene intervention was effective in ameliorating DIO and improving metabolism only when performed in the NTS as opposed to in the ARC. On the contrary, rats fed a standard chow diet for lifetime and possessing age-related obesity responded to the POMC gene therapy either in the ARC or NTS with comparable and sustained weight reduction and metabolic improvements (Li et al., 2005, Li et al., 2007). It may be reasonable to speculate that both the ARC and NTS are important brain sites for regulating caloric need (chow consumption), whereas the role of the ARC in hedonic feeding such as HF consumption could be limited compared to that of the NTS.

The second implication stems from the observation that the POMC treatment in the NTS resulted in a persistent weight reduction despite an attenuated anorectic response over time. This finding suggests stimulated energy expenditure contributes separately to weight regulation beyond the POMC effect on food consumption. Voluntary wheel running is one form of physical activity in the overall energy expenditure equation. Many factors can affect WR activity, including motivational, exploratory, muscular, age, and body size components (Skibicka and Grill, 2008). We used short-term WR activity over five consecutive days as an

assessment for physical activity. Whereas rAAV-POMC in the ARC did not alter WR activity, rAAV-POMC in the NTS increased WR by a moderate 18%. Despite the modesty, the small daily increase in physical activity could have a cumulative long-term impact on weight maintenance, as implicated by the sustained weight loss in the NTS-POMC animals. This increase in physical activity is particularly meaningful considering that dietary obese animals have already impaired WR activity relative to their non-obese chow-fed counterparts (the former only run half as much as the latter on a daily base). The POMC transfer to either the ARC or NTS did not affect brown fat tissue mass or UCP1 protein level, suggesting BAT thermogenesis is not a factor mediating POMC effect in the NTS. Because other assessments of energy expenditure were not performed, we cannot be certain that the increase in physical activity in the form of WR accounts for all the presumed elevation in energy expenditure with the NTS-POMC treatment.

Our data suggest that the mildly increased WR activity in the NTS-POMC rats might be related to central control of sympathetic outflow. Caudal brainstem MC receptor stimulation is known to trigger energetic responses via local sympathetic effector circuits (Skibicka and Grill, 2008). The increase in mRNA levels of the three adrenal factors assessed in response to the POMC NTS treatment indicates an augmented, brainstem-mediated sympathetic activity, and is in support of a possible connection between this increase and enhanced WR activity.

Improvement in insulin sensitivity induced by the NTS POMC-treatment may be another mechanism to deter fat deposition and weight gain besides augmented physical activity. Both the fasting glucose and insulin levels were substantially diminished in the NTS-POMC rats, and the QUICK value (Katz et al., 2000) indicated improved insulin sensitivity. Additionally, the NTS-POMC rats exhibited increased glucose disposal, which is consistent with prior knowledge that central melanocortin receptor activation inhibits insulin release from the pancreas (Fan et al., 2000) and enhances whole body glucose disposal (Heijboer et al., 2005) independent of feeding and fat mass. Worthy of note, the POMC-treatment in the ARC tended to lower serum glucose level, but the decrease was not significant and not accompanied by any change in fasting insulin or glucose tolerance. A recent study using a central melanocortin blockade revealed a direct, feeding-independent control of peripheral lipid metabolism by the central MC system (Nogueiras et al., 2007). Such a mechanism would explain the marked reduction of serum triglyceride concentrations following rAAV-POMC gene therapy in either the ARC or NTS of the brain.

This is the first study to examine and dissect the effects of long-term melanocortin activation in the forebrain ARC versus the hindbrain NTS in combating dietary obesity. Whilst the rAAV-POMC transfer to the NTS caused mild anorexia, persistent weight loss, improved insulin sensitivity, and increased propensity for voluntary wheel running in the dietary obese rats, the similar intervention in the ARC only had minimal physiological and metabolic impact. The NTS POMC treatment likely evokes several mechanisms involving modest and temporary anorexia, stimulation of energy expenditure via brainstem-regulated sympathetic outflow, increase in glucose disposal, and probably a direct suppression of insulin production. The findings from the current study implicate different roles that the hypothalamic or brainstem melanocortin system may play in long-term homeostatic and hedonic feeding regulation, and reveal the complexity of the neuroendocrine control of physiology and metabolism in obese rodents. Our data, in particular, highlight the effectiveness of the POMC treatment in the NTS in stimulating physical activity and enhancing insulin sensitivity. These results suggest the melanocortin activation of the NTS is an attractive strategy to provide long lasting weight control and metabolic benefits.

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Abbreviations

rAAV-POMC	recombinant adeno-associated virus-mediated POMC gene transfer
rAAV-Control	rAAV encoding GFP
HF	high fat
DIO	diet-induced obese or diet-induced obesity
WR	wheel running
ARC	arcuate nucleus
NTS	nucleus of the solitary track
MC	melanocortin
IPGTT	intraperitoneal glucose tolerance test
QUICK	the Quantitative Insulin Sensitivity Check Index
POMC	pro-opiomelanocortin
MC3R	melanocortin 3 receptor
MC4R	melanocortin 4 receptor
α-MSH	α -melanocyte stimulating hormone
MTH	melanotan II
AgRP	agouti-related protein
NPY	neuropeptide Y
DBH	dopamine-beta-hydroxylase
TH	tyrosine hydroxylase
UCP1	uncoupling protein 1
BAT	brown adipose tissue
WAT	white adipose tissue

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Figure 1.

Fasting leptin (a), fasting TGs (b), Grip strength (c) and voluntary wheel running (d) were measured in weight-matched Chow-raised (Chow) and HF-raised (DIO) rats. Data are means $\pm$ SE of 8–12 rats per group. DIO rats displayed significantly higher fasting leptin $*P < 0.0001$, fasting triglycerides $*P < 0.0001$, lower grip strength $*P = 0.0034$ and decreased daily wheel running activity $*P = 0.0012$ compared to weight-matched Chow animals.

Figure 2.

GFP immunoreactivity in coronal sections through nucleus tractus solitarius including medial and ventrolateral portions of the solitary tract (A) and along the 3rd ventricle in the area of the arcuate nucleus of the hypothalamus (C). GFP expression in both regions was exclusively localized to neurons (B, D). POMC mRNA levels were measured 86 after POMC gene delivery by relative quantitative RT-PCR with 18S rRNA as an internal standard. The representatives of POMC over-expression in either the ARC or NTS versus the respective controls (two samples from each group) were shown in E. Whereas rAAV-POMC gene delivery to either the ARC (ARC-POMC) or NTS (NTS-POMC) resulted in apparent elevation in POMC mRNA, only minimal levels of POMC were detectable in the ARC-GFP or NTS-GFP animals.

Figure 3.

Change in body weight (BW) following rAAV-POMC gene transfer in the ARC (a) or NTS (b). Data are means $\pm$ SE of 5–6 rats per group. According to ANOVA followed by post-hoc analysis at the individual time point, the ARC-POMC rats had significantly lower body weight than that of ARC-Control rats only from days 4 through 22 (a), whereas the NTS-POMC rats displayed significantly reduced change in body weight relative to the NTS-Control rats from day 6 until termination (b).

Figure 4.

Daily food intake (FI) following in rAAV-POMC gene transfer in the ARC (a) or NTS (b), and the accumulative FI from days 1–24 and 25–56 for either treatment (c). Data are means $\pm$ SE of 5–6 rats per group. The ARC-Control and ARC-POMC rats had similar daily food intake during the entire experimental period (a) whilst the NTS-POMC rats exhibited lower food intake than the NTS-Control rats at certain days during the first 24 days after POMC gene transfer (b). The accumulative FI from days 1–24 was significantly decreased in the NTS-POMC animals versus either the NTS-Control, or ARC-POMC or ARC-Control rats. $P = 0.0012$ for difference with one-way ANOVA, and *** $P < 0.05$ by post-hoc analysis (c). The trend of reduction in accumulative FI remained in the NTS-POMC rats from days 25–56 without reaching a statistical significance.

Figure 5.

Levels of blood glucose following intraperitoneal administration of glucose (2 g/kg body weight) on day 63 after rAAV-POMC transfer to the NTS. Data are means $\pm$ SE of 5–6 rats per group. * $P < 0.05$ for difference in blood glucose in the NTS-POMC vs NTS-Control rats by unpaired t -test following repeated measures ANOVA.

Figure 6.

Daily voluntary wheel running over a consecutive five-day period commencing at day 45 for POMC transfer to the NTS or day 57 to the ARC. Data are means $\pm$ SE of 5–6 rats per group. $P = 0.0003$ for difference by one-way ANOVA. *** $P < 0.05$ for difference between the NTS-POMC rats relative to NTS-Control, ARC-Control or ARC-POMC animals, respectively, by post-hoc analysis.

Figure 7.

Adrenal tyrosine hydroxylase (a), neuropeptide Y (b) and dopamine-β-hydroxylase (c) mRNAs levels. Data are means $\pm$ SE of 5–6 rats per group. POMC gene transfer in the NTS significantly elevated all three indicators of catecholamine biosynthesis relative to NTS-Control, ARC-Control and ARC-POMC rats, respectively (***) $P < 0.05$, by one-way ANOVA and post-hoc analysis.

Table 1

Adiposity and serum parameters.

	Treatment			
	ARC-Control	ARC-POMC	NTS-Control	NTS-POMC
PWAT (g)	4.4 ± 0.3	4.7 ± 0.5	4.8 ± 0.6	3.4 ± 0.3 ^{a,b}
RTWAT (g)	29.2 ± 2.3	28.1 ± 1.8	32.8 ± 3.4	21.8 ± 1.6 ^{a,b}
EWAT (g)	18.1 ± 1.0	18.5 ± 1.4	17.3 ± 1.8	13.9 ± 0.7 ^b
Fasting Glucose (mg/dL)	87.4 ± 5.2	77.6 ± 3.0	86.8 ± 3.3	73.0 ± 2.8 ^a
Fasting Insulin (ng/ml)	0.62 ± 0.06	0.65 ± 0.06	0.66 ± 0.13	0.36 ± 0.06 ^{a,b}
QUICK	0.58 ± 0.02	0.59 ± 0.02	0.55 ± 0.01	0.73 ± 0.04 ^{a,b}
Fasting Triglycerides (mg/dL)	412.2 ± 27.6	256.6 ± 39.9 ^c	435.4 ± 84.5	239.4 ± 17.6 ^{a,b}

Data are means ± SE of 5–6 rats per group. One-way ANOVA analysis yielded significant *p* value for each of the seven parameters listed in Table 1. By post-hoc analysis,

^a *p* < 0.05 or 0.01 for difference between NTS-POMC vs. NTS-Control;

^b *p* < 0.05 or 0.01 for difference between ARC-POMC vs. NTS-POMC;

^c *p* < 0.05 for ARC-POMC vs. ARC-Control.