Brain stem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections

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Zheng, Huiyuan, Laurel M. Patterson, Curtis B. Phifer, and Hans-Rudolf Berthoud. Brain stem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections. Am J Physiol Regul Integr Comp Physiol 289: R247–R258, 2005. First published March 3, 2005; doi:10.1152/ajpregu.00869.2004.—Melanotan II (MTII), a stable melanocortin 4 receptor (MC4R) and melanocortin 3 receptor (MC3R) agonist injected into the fourth ventricle near the dorsal vagal complex, potently inhibited 14-h food intake by decreasing meal size but not meal frequency; SHU9119, an antagonist, increased food intake by selectively increasing meal size. Furthermore, MTII injected into the fourth ventricle increased and SHU9119 tended to decrease heart rate and body temperature measured telemetrically in freely moving rats. Numerous α-melanocyte-stimulating hormone-immunoreactive axons were in close anatomical apposition to nucleus tractus solitarius neurons showing c-Fos in response to gastric distension, expressing neurochemical phenotypes implicated in ingestive control, and projecting to brown adipose tissue. In retrograde tracing experiments, a small percentage of arcuate nucleus POMC neurons was found to project to the dorsal vagal complex. Thus melanocortin signaling in the brain stem is sufficient to alter food intake via changing the potency of satiety signals and to alter sympathetic outflow. Although the anatomical findings support the involvement of hypothalamomedullary POMC projections in mediating part of the descending, indirect signal, they do not rule out involvement of POMC neurons in the nucleus tractus solitarius in mediating part of the direct signal. Satiation; obesity; α-melanocyte-stimulating hormone; MC4R; nucleus tractus solitarius

Since the discovery of leptin, significant progress has been made in the identification of transmitters and peptides expressed by the hypothalamic neural circuitry that act as downstream mediators for leptin and other internal state factors involved in the control of food intake and energy homeostasis. Proopiomelanocortin (POMC) and neuropeptide Y (NPY) neurons located in the arcuate nucleus have been recognized as some of the key mediators of leptin’s central effects on food intake and energy balance. Release of α-melanocyte-stimulating hormone (α-MSH) and agouti-related protein (AgRP) from terminals of POMC and NPY/AgRP neurons, respectively, modulate food intake and energy balance very strongly through their actions on the melanocortin-3 and -4 receptors (MC3R and MC4R). Activation of MC4R suppresses food intake and increases energy expenditure. AgRP acts as an endogenous antagonist for MC3R and MC4R and potently stimulates food intake.

One population of leptin receptor-bearing arcuate nucleus neurons (15) coexpresses POMC (44, 49) and cocaine- and amphetamine-related transcript (CART) (12, 21, 50, 72). These neurons project heavily to most periventricular zone nuclei, including the paraventricular nucleus (PVN), to the lateral hypothalamic area (LH), and to many other brain areas, including the dorsal vagal complex (DVC) and spinal cord (21, 22). The POMC-derived peptide α-MSH [or its longer-lasting analog melanotan II (MTII)] and the CART peptide potently suppress food intake when injected intracerebroventricularly (1, 42).

Another population of leptin receptor-bearing neurons contains mRNA for NPY, often coexpresses AgRP (13), and projects in parallel to most of the targets of POMC/CART neurons (5, 8, 19, 20, 22, 24). NPY and AgRP have strong orexigenic effects when injected into the lateral or third cerebral ventricles, directly into the perifornical hypothalamus (37, 68), or near the caudal brain stem (16, 78).

Because both arcuate nucleus POMC/CART and AgRP/NPY neurons have extensive projections throughout most of the brain, one of the open questions is which projections are particularly relevant for the modulation of food intake and energy balance. The brain stem harbors the neurons and circuits directly involved in ingestion, digestion, and absorption of food, as well as in utilization of metabolites and fuels (9). The neural circuits controlling most of these tasks are complete within the brain stem and do not require the forebrain for their execution (35). From experiments with the supracollicular decerebrate rat, it appears that the isolated brain stem is capable of terminating a meal and thus exhibiting the basic behavior of satiety (36). However, the brain stem circuitry cannot translate signals related to longer-term energy status. Lack of appropriately increased meal size in response to food deprivation in the decerebrate rat suggests that the brain stem in isolation is not primarily involved in homeostatic control of energy balance and body weight. Furthermore, the brain stem circuitry cannot respond directly to cognitive, rewarding, emotional, and social influences and translate them into adaptive behavior. These signals (also referred to as indirect controls; see Ref. 66) originate from the hypothalamus and above.

Hypothalamic POMC and AgRP neurons might thus exert some of their effects on food intake and energy balance through their caudal brain stem projections. Evidence for this...
has been presented by the observations that melanocortin receptor ligands injected into the fourth ventricle or directly into the DVC modulate food intake as efficiently as when injected into the third ventricle (34, 77, 78). However, because POMC is expressed in a small population of commissural nucleus tractus solitarius (NTS) neurons, it is not clear whether, under physiological conditions, α-MSH originating from this local population or from the hypothalamus is activating medullary MC4R to decrease food intake. Although it has been shown that both of the POMC neuron populations contribute to the α-MSH-immunoreactive (ir) fiber innervation of the caudal medulla, (57), medulla-projecting POMC neurons have not been directly identified and quantified. One of our aims was thus to identify hypothalamic POMC neurons with projections to the DVC using a retrograde tracing strategy. In addition, we wanted to analyze the anatomical relationship between α-MSH-ir fibers in the DVC and neurons expressing specific neurochemical markers or expressing c-Fos in response to gastric nutrient infusion.

Pharmacological stimulation of MC3R and MC4R by means of third ventricular MTII injections has been shown to decrease food intake by selectively decreasing meal size (4). Although a site of action of MTII in the hypothalamus cannot be ruled out, this finding strongly implicates a direct role for medullary melanocortin receptors because the caudal brain stem is thought to be most directly involved in the control of meal size (66). Williams et al. (77) showed that fourth ventricular MTII injection dose dependently decreased consumption of glucose solution during a 30-min scheduled meal, suggesting amplification of postigestive feedback inhibition (77). Thus our second aim was to probe the effects of melanocortin receptor agonists and antagonists injected near the dorsal medulla on meal structure and food intake during a normal dark-phase feeding period, using an automated feeding monitor.

Finally, intracerebroventricular administration of MC4R ligands has also been shown to modulate energy expenditure (76). Thus our third aim was to measure locomotor activity as well as indicators of sympathetic activity, such as body temperature and heart rate. As an anatomical counterpart to this functional assessment, we also analyzed the relationship between α-MSH fibers and sympathetic premotor neurons innervating interscapular brown fat tissue.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 200–320 g were housed in groups in shoebox cages or individually in hanging wire mesh cages in a climate-controlled room (22 ± 2°C) on a 12:12-h light-dark cycle with lights on at 0600 and off at 1800. Food and water were available ad libitum except as specified below. Eight-week-old green fluorescent protein (GFP)-labeled GABA male mice were obtained from Jackson Laboratory [FVB-Tg (Gad GFP) 45704 Swind; Bar Harbor, ME] and were perfused on arrival at the animal facility. All experimental protocols were approved by the Institutional Animal Care and Use Committee (Pennington Biomedical Research Center) and were conducted in compliance with USDA regulations and American Physiological Society principles for research involving animals.

Chronic brain cannula implantation and microinjections. Animals were anesthetized with ketamine-acepromazine-xylazine (80:1.6:4.0 mg/kg sc) and given atropine (1 mg/kg ip). Guide cannulas (24 gauge for the fourth ventricle and 22 gauge for the NTS) were aimed 2.0 mm above the intended sites, cemented with four screws to the skull, and occluded with stainless steel obturators. Beveled stainless steel injectors (30 gauge for the fourth ventricle and 33 gauge mounted onto a 26-gauge sleeve for the NTS) extending 2.0 mm from the tip of the guide cannulas were used for injections. Coordinates were as follows: 2.5 mm from the posterior occipital suture, 0.0 mm from the midline, and 6.0 mm below the skull for fourth ventricular infusions and 0.0 mm from posterior occipital suture, 0.5 mm lateral, and 6.0 mm below the skull for direct NTS injections (with the tooth bar 4 mm below interaural zero). Seven to ten days were allowed for recovery, during which the animals were adapted to the injection procedure with two or three mock injections. We conducted injections using a hand-held Hamilton microsyringe attached to a piece of PE20 tubing in volumes of 3 µl for the fourth ventricle and 0.3 µl per side for the NTS. Cannula placement and patency were verified at the end of the experiment by checking the diffusion of Chicago Sky Blue (Sigma, St. Louis, MO; 3 µl, 2% in 0.5% sodium acetate) for 5 min in the fourth ventricle or diffusion of rhodamine-labeled MTII (Phoenix Pharmaceuticals, Belmont, CA; 0.3 µl, 1.67 nmol in saline) for 30 min in the NTS.

Food intake. In experiment 1, the effects on meal structure of the MC3R and MC4R agonist MTII and the antagonist SHU9119 (Phoenix Pharmaceuticals) administered near the DVC via fourth ventricular injections were tested. Rats bearing chronic guide cannulas aimed at the fourth ventricle overlying the dorsal medulla were adapted to an automated feeding system with granulated diet available from a jar sitting on a balance that could be reached through a narrow alcove attached to a standard hanging wire cage. Animals were adapted to a granulated high-fat diet with 56% energy from fat (51). This was done to increase caloric intake in the feeding monitor because daily caloric intake of powdered chow was relatively low.

In a pilot study with six rats, MTII was injected at a dose of 0.1 nmol. This dose produced a 60% suppression of overnight food intake by decreasing meal size but also marginally decreasing meal frequency. We believe that this decrease in meal frequency was due to the large suppression of total intake, and we therefore used a lower dose (0.05 nmol) that should result in less suppression of total intake and no suppression of meal frequency for the main experiment.

In a first group of 12 rats, MTII (0.05 nmol in 3 µl of sterile saline) or saline was injected over 2 min into the fourth ventricle in counterbalanced order at 1700 h. In a second group of 10 rats (4 rats that had MTII injections and 6 naïve rats), SHU9119 (0.1 nmol in 3 µl of saline) or saline was injected in the same way. This dose of SHU9119 was based on an earlier study that compared lateral ventricular with fourth ventricular injection (34). Rats were put back into the cage, and food intake was monitored for the entire dark period until 0900 the next day. Injections were separated by 4 days to make sure the relatively long-lasting effects of these MC4R ligands were not carrying over to the next test. We analyzed the feeding records using custom-made software. A feeding bout was defined as a meal if ≥0.3 g of food was ingested and if it was separated from another meal by ≥10 min.

The purpose of experiment 2 was to determine whether the SHU9119 administered directly into the DVC would increase food intake by mainly increasing meal size. In experiment 1, we found that SHU9119 not only increased meal size but also meal frequency, suggesting that this might be a ceiling effect of meal size produced by the higher palatability of the high-fat diet. Therefore, we used regular powdered chow (Purina 5001) in this experiment.

Six rats bearing chronic guide cannulas aimed at the DVC were adapted to an automated feeding system with powdered chow available from a jar sitting on a balance that could be reached through a narrow alcove attached to a standard hanging wire cage. After a stable baseline intake was reached, the MC3R and MC4R antagonist SHU9119 (0.065 nmol in 300 nl of sterile saline) or 300 nl of saline were injected over 2 min unilaterally into the DVC at 1600, 2 h before dark onset. This dose was similar to the one used by Williams et al. (78). The injector was left in place for another 1 min and then...
removed, and the cannula was closed. The rat was returned to the feeding monitor and left undisturbed through the entire dark period (from 1800 to 0600) until 0800 the next day. SHU9119 and control saline injections were given in a counterbalanced order 5 days apart.

Measurement of body temperature, heart rate, and locomotor activity. In 10 rats, 6 with heart leads, core body temperature, physical activity, and heart rate were simultaneously registered telemetrically via a preimplanted transponder with the VitalView data-acquisition system (Mini-Mitter). An E-Mitter transponder and a fourth ventricular cannula were implanted during the same surgery. Briefly, after implantation of the fourth ventricular cannula, a small midline abdominal incision was made below the diaphragm. The transponder was slipped into the abdominal cavity along the sagittal plane, with the leads tunneled under the skin and secured to the chest muscles with metal sutures. The abdominal opening was closed after verification that the heart beat could be detected. The animals were allowed to recover for 7–10 days. On the test days, rats were briefly (<2 h) deprived of food and water, injected with MTH (0.5 nmol in 3 μl of saline), SHU9119 (0.5 nmol), or saline alone into the fourth ventricle, and placed back into their home cages. Preweighed normal rat chow was made available immediately. Heart rate, core temperature, and physical activity were continuously monitored for 20 h at 1-min intervals. Chow intake was also recorded after 2 h.

Retrograde tracing. To identify hypothalamic POMC neurons with projections to the DVC, the retrograde tracer cholera toxin B-subunit (CTB; List Biological Laboratories, Campbell, CA), rhodamine-labeled fluorescent latex microspheres (retrobeads; Lumafuor, Naples, FL), or Fluorogold (2% in sterile saline; Fluorochrome, Denver, CO) was injected into the DVC. Although all three tracers worked equally well, CTB produced the most robust labeling and was chosen for the quantitative analysis. The dorsal surface of the medulla was exposed with the head fixed in a stereotactic apparatus. A glass micropipette with a tip diameter of ~10 μm was filled with 0.5% CTB in sterile saline, the retrobeads, or Fluorogold, and pressure injections (100–200 nl total) were placed at three different rostrocaudal levels to blanket the entire DVC unilaterally. Animals were allowed 3–5 days for retrograde transport, at which time they were euthanized; the medulla (injection site) and the hypothalamus were then processed for CTB immunohistochemistry or directly visualized (retrobeads and Fluorogold).

Retrogradely labeled neurons in the rat medulla projecting to brown adipose tissue (BAT) were labeled using red fluorescent protein-expressing pseudorabies virus [PRV 614, a gift from Drs. Bruce Banfield and Roger Tsien (7)]. The interscapular BAT pads were exposed, and three sites per pad were injected with 0.5 μl of 8.4 × 10⁹ plaque-forming units/ml, using a 2-μl Hamilton syringe fitted with a 26-gauge needle. After 4–5 days of survival, the rats were perfused for histological processing.

Tissue processing and immunohistochemistry. Rats or mice were deeply anesthetized with pentobarbital sodium (90 mg/kg ip) and transcardially perfused with heparinized saline (20 U/ml) followed by ice-cold 4% phosphate-buffered (pH 7.4) paraformaldehyde. Brains were extracted, blocked, and postfixed in the same fixative overnight. Tissue was immersed for 24 h in 18% sucrose and 0.05% sodium azide in 0.1 M PBS. Coronal sections of forebrain or medulla (20–30 μm) were cut in a cryostat and separated into five series. For immediate processing, sections were held in PBS (4°C), whereas for long-term storage (~20°C) a cryoprotectant solution (50% PBS, 30% ethylene glycol, 20% glycerol) was used. Free-floating sections were pretreated with 0.5% sodium borohydride in PBS to minimize aldehyde cross-linking of the fixative. Appropriate washing in PBS followed incubations. For immunoperoxidase methods, sections were treated with 1.5% hydrogen peroxide, 20% methanol, and 0.25% Triton X-100 in PBS to quench endogenous peroxidases. Blocking in normal sera against the host of the secondary antibodies preceded the primary antibody incubations for both immunoperoxidase and fluorescent labeling. Primary antibodies were diluted in 0.1% gelatin-0.05% sodium azide in PBS with 0.5% Triton X-100 and incubated for 20 h at room temperature or for 48 h at +7°C. Secondary antibodies were diluted in PBS-Triton X-100 and incubated at room temperature for 2 h. Biotinylated secondary antibodies (1:500, Jackson ImmunoResearch, West Grove, PA), incubated in avidin-biotin complex (1:500, Vectastain ABC elite kit, Vector Labs, Burlingame, CA) and visualized with appropriate chromagens, were used for immunoperoxidase labeling, whereas simple fluorescent-conjugated secondaries were used in the fluorescent labeling. Negative controls (leaving out the primary antibody) or preadsorbed controls were used to establish the lack of nonspecific staining.

For CTB, Fluorogold, and retrobead labeling, injection sites were verified in the medulla by directly mounting the sections on charged slides. The hypothalamus was processed free-floating. To visualize CTB, sections were incubated in goat antiserum to CTB (anti-cholera, 1:20,000; List) followed by Cy2-conjugated secondary antibody (1:200, Jackson). For double-labeling hypothalamic neurons, sections were incubated in rabbit anti-α-MSH (1:10,000, ImmunoStar, Hudson, WI) or rabbit anti-CART (1:10,000; Phoenix Pharmaceutica), followed by Cy3-conjugated secondary antibody (1:600; Jackson) in combination with CTB or Fluorogold tracing or Cy2 in combination with retrobeads. Fluorescent-labeled sections were mounted in 100% glycerol with 5% n-propyl galle at as an antifade agent, except for the retrobead-traced tissue, which was mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL).

For mapping α-MSH-ir axon profiles in the DVC, the primary antibody used was a sheep polyclonal diluted to 1:50,000 (Chemicon International, Temecula, CA). Sections were labeled with an immunoperoxidase method using Vector SG (Vector Labs) as the substrate. Vector SG-labeled sections were mounted on clean slides, dried overnight, dehydrated, and coverslipped with Entellan (Electron Microscopy Sciences, Hatfield, PA).

Brain stem α-MSH fibers and tyrosine hydroxylase neurons were double labeled by simultaneous incubation in the primary antibodies (sheep α-MSH at 1:25,000 and monoclonal mouse anti-tyrosine hydroxylase at 1:400; ImmunoStar), followed by Cy3 (α-MSH) and Cy2 (tyrosine hydroxylase) secondary antibodies. Similarly, monoclonal mouse anti-glucagon-like peptide-1 (GLP-1, 1:2,000, gift from Dr. D. D’Alessio, University of Cincinnati, Cincinnati, OH), with Cy3 as the fluorophore, was used to double label the sheep α-MSH (1:25,000) coupled to Cy2. α-MSH fibers (sheep antibody, 1:50,000) and nitric oxide synthase (monoclonal bNOS, 1:2,000; Sigma-Aldrich, St. Louis, MO) neurons were stained by using the immunoperoxidase method with Vector SG as the α-MSH chromagen and nickel/cobalt-enhanced diaminobenzidine (Pierce, Rockford, IL) labeling the bone NOS. Glutamic acid decarboxylase-GFP mouse brain stem sections were labeled for α-MSH fibers (rabbit primary antibody, 1:10,000) using goat anti-rabbit Alexa 594 (1:2,000; Molecular Probes, Eugene, OR) as the fluorescent tag.

In the gastric infusion model, immunohistochemistry for c-Fos included an Oncogene/Calbiochem primary antibody (c-Fos AB-5, 1:30,000) with a DAB chromagen from Sigma. Vector SG was the substrate for the α-MSH (sheep primary antibody, 1:50,000) double label in the DVC.

Double labeling of α-MSH axon profiles and retrogradely traced neurons from BAT employed a Cy2 fluorescent conjugate for the α-MSH (sheep) to contrast with the red fluorescent protein of the pseudorabies virus.

Quantification of labeled neurons. Quantitative analysis of the number of α-MSH neurons projecting to the DVC was based on averages of 13–23 coronal hypothalamic sections from three rats with successful retrograde tracer injections. The sections spanned most of the arcuate nucleus and retrochiasmatic area. From each side of each section, retrogradely labeled neurons in green and α-MSH-ir neurons in red were observed through fluorescent filters using a ×20 Fluor objective of a Zeiss Axioplan microscope. Neurons were considered labeled when their respective staining was clearly above background.

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and when the cell nucleus was in the plane of the image. Colocalization of labels in a given neuron (double-labeled neurons) was determined by switching back and forth between red and green filters.

**Activation of gastrointestinal signals to the NTS.** To activate visceral satiety mechanisms known to result in increased c-Fos expression in brain stem neurons, rats were fitted with chronic gastric fistulas for the infusion of nutrients as described earlier (79). After recovery from surgery, rats were adapted to overnight food deprivation and the gastric infusion procedure in special cylindrical acrylic test cages. On test days, the gastric fistula was hooked up with an infusion line connected to a syringe pump containing a complete mixed liquid diet (vanilla-flavored Ensure, 1 kcal/ml). Thirty minutes later, the diet was infused at a rate of 1.6 ml/min for 10 min to mimic rate of ingestion, as determined in separate tests. At the end of infusion, the infusion catheter was capped without disturbing the animal; 90 min after the start of infusion, animals were euthanized with an overdose of pentobarbital sodium and then transcardially perfused with 100 ml of cold saline, followed by 500 ml of phosphate-buffered (pH 7.4) paraformaldehyde. Brains were extracted and post-fixed for an additional 2–4 h.

**Statistical analysis.** Meal parameters were analyzed with separate one-way ANOVAs. Body temperature, heart rate, and locomotor activity were originally sampled every 60 s. Floating averages were calculated for 15-min intervals, and post hoc comparisons were made for specific time periods using repeated-measures ANOVAs. The numbers of POMC, CTB, and double-labeled neurons, as well as the percentages of double-labeled neurons that expressed POMC or that were retrogradely labeled, were analyzed with ANOVA (PROC MIXED in SAS 8.2), followed by Bonferroni-adjusted comparisons.

**RESULTS**

**Modulation of meal size by brain stem administration of melanocortin receptor ligands.** Administration of the MC3R and MC4R agonist MTII (0.05 nmol) into the fourth ventricle decreased dark-phase intake of high-fat diet by 49% compared with saline control \([F(1, 10) = 53.8, P < 0.01]\) (Fig. 1). Meal pattern analysis showed that meal size was significantly decreased by 42% in MTII-treated rats \([F(1, 10) = 26.0, P < 0.01]\), but meal frequency was not significantly decreased by 19% \([F(1, 10) = 2.9, P = 0.12]\). Meal duration was decreased by 22%, almost reaching significance \([F(1, 10) = 4.03, P = 0.072]\). MTII treatment also significantly increased the satiety ratio by 110% \([F(1, 10) = 58.1, P < 0.01]\). In contrast, administration of the MC3R and MC4R antagonist SHU9119 (0.1 nmol) into the fourth ventricle significantly increased overnight intake of the high-fat diet by 61% \([F(1, 9) = 25.7, P < 0.01]\). This was accomplished by selectively increasing meal size by 63% \([F(1, 9) = 13.6, P < 0.01]\) but not meal frequency (Fig. 1). Meal duration was increased by 30%; however, because of the large variation, this increase was not statistically significant \([F(1, 9) = 0.47, P = 0.51]\). The satiety ratio was significantly decreased by 45% \([F(1, 9) = 18.0, P < 0.01]\). Similar effects were obtained when normal chow was used as diet (data not shown).

In **experiment 2**, administration of SHU9119 (0.065 nmol) directly into the DVC significantly increased overnight intake of regular chow by 51% \([F(1, 5) = 9.99, P < 0.05]\) compared with saline control (Fig. 2). This was achieved by selectively increasing meal size by 36% \([F(1, 5) = 26.0, P < 0.01]\) but not meal frequency \([F(1, 5) = 0.79, P = 0.42]\). As with fourth ventricular administration, meal duration was not significantly changed \([F(1, 5) = 0.41, P = 0.55]\), but the satiety ratio significantly decreased by 37% \([F(1, 5) = 67.7, P < 0.01]\).

Histological verification showed that the cannula tips were located in the NTS and that the injected solution diffused throughout the DVC (Fig. 3).

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![Fig. 1](http://ajpregu.physiology.org/)

**Fig. 1.** Effect of fourth ventricular SHU9119 and melanotan II (MTII) injection on meal structure as determined in an automatic feeding system. MTII (0.05 nmol) or saline (Sal) and SHU9119 (SHU; 0.1 nmol) or saline were injected in separate groups of rats 1 h before dark onset, and total intake of high-fat diet (A), meal size (B), meal frequency (C), meal duration (D), and satiety ratio (E) were analyzed over a 16-h period of free feeding. Bars represent means ± SE of 12 rats (MTII) and 10 rats (SHU9119). **P < 0.01, based on separate ANOVAs.
Effects on body temperature, heart rate, and locomotor activity. Indexes of autonomic functions and locomotor activity following fourth ventricular administration of the MC4R ligands were measured telemetrically. Immediately after saline or drug injection, there was an initial increase in all measures due to the arousal associated with the injection. Injection of MTII (0.5 nmol) increased heart rate for ~8 h after the injection, compared with saline control (Fig. 4). A post hoc comparison for the time points 30–300 min after injection revealed a significant effect \( F(1, 1, 10) = 30.6, P < 0.001 \]. MTII increased body temperature only slightly for the first 2 h after injection. A post hoc comparison 60–90 min after injection revealed a marginally significant difference \( F(1, 1, 18) = 5.3, P = 0.034 \). There was also a tendency for MTII to increase locomotor activity during the first 3 h after injection.

As could be expected, fourth ventricular administration of SHU9119 (0.5 nmol) produced opposite effects, and, in contrast to the immediate effects of MTII, the effects of SHU9119 were delayed (Fig. 5). Both body temperature and heart rate were lower for several hours starting ~6 h after injection of SHU9119. A post hoc comparison 480–530 min after injection revealed a marginally significant relative hypothermia \( F(1, 1, 18) = 4.1, P = 0.058 \). The decrease in heart rate was not significant. The MC4R antagonist did not significantly affect locomotor activity at any time.

Retrograde tracing of medulla-projecting hypothalamic POMC neurons. The unilateral injections of CTB resulted in tracer diffusion to most of the NTS, dorsal motor nucleus, and...
area postrema caudal to the rostral end of the area postrema on the side of the injection, as well as limited diffusion to the medial portions of the NTS on the contralateral side (Fig. 6, the side of the injection, as well as limited diffusion to the area postrema caudal to the rostral end of the area postrema on the contralateral side (Fig. 6).

These injections resulted in dense retrograde labeling of neurons in the PVN and LH and sparser labeling in the basomedial hypothalamus, as reported earlier (11). Specifically, there were few retrogradely labeled neurons in the retrochiasmatic area and the more lateral aspects of the arcuate nucleus, the area of most POMC expression. The number of retrogradely labeled neurons was significantly smaller in the posterior half of the arcuate nucleus (Fig. 7).

Among the few retrogradely labeled neurons consistently found in each section, a small percentage exhibited α-MSH-ir or CART-ir (Fig. 6, C–E). Quantitative analyses of labeled neurons within an area, including the arcuate nucleus and its lateral extension that often also contains POMC positive neurons, showed that 6.4 ± 1.7% of all POMC neurons were retrogradely labeled with CTB and that 9.7 ± 2.5% of the retrogradely labeled neurons in the area expressed α-MSH. There was considerable variation in these percentages among the three rats, and some of it could be explained by the differences in the size of tracer diffusion in the DVC. In rat 1618, the injection site extended further laterally than in the two other animals (Fig. 6A), and this rat had the highest percentage of POMC neurons retrogradely labeled (8.3%) and the highest percentage of retrogradely labeled neurons expressing POMC (13.7%). Further analyses showed that a higher number of double-labeled neurons and a higher percentage of POMC neurons with projections to the DVC were located in the anterior half compared with the posterior half of the arcuate nucleus, whereas the percentage of retrogradely labeled neurons expressing POMC was similar (Fig. 7).

α-MSH-ir axons and terminals in the DVC. As reported earlier, α-MSH-ir fibers quite selectively innervate the DVC, with few fibers in surrounding areas of the dorsal medulla (Fig. 8). Varicose α-MSH-ir fibers were found in all NTS subnuclei and the dorsal motor nucleus, but only the rare fiber was present in the area postrema. The density of α-MSH-ir fibers was particularly high in the dorsal, commissural, and lateral NTS, including the interstitial and intermediate subnuclei around the solitary tract. In the dorsal motor nucleus, the highest density was found medially and in rostral sections.

Anatomical relationship between α-MSH-ir axons and identified NTS neurons. Neurons expressing tyrosine hydroxylase were often in close anatomical contact to α-MSH-ir varicose axons (Fig. 9). Similarly, we found frequent contacts between varicose α-MSH-ir axon profiles and NOS-ir neurons as well as GFP-expressing GABA neurons in the mouse (Fig. 9, C and D). In contrast, GLP-1-ir neurons in the caudal NTS were rarely contacted by α-MSH-ir axon profiles (Fig. 9B).

As previously reported, gastric infusion of a mixed meal stimulated the expression of c-Fos protein in neurons throughout the caudal NTS, particularly in the dorsomedial, medial, and commissural subnuclei (Fig. 9E). Mock gastric infusions resulted in very little c-Fos expression in the DVC (not shown). On double-labeled sections, many close anatomical appositions between varicose α-MSH-ir fibers and c-Fos-ir cell nuclei were present throughout the area of codistribution in the dorsal, commissural, and medial subnuclei of NTS.

In an attempt to identify NTS neurons involved in sympathetic outflow, we injected pseudorabies virus expressing red fluorescent protein into the interscapular BAT pads of rats. This resulted in trans-synaptic retrograde labeling of many neurons in the raphe nuclei and ventrolateral medulla, as well as a few neurons in the NTS, when the survival time was 4–5 days. Close appositions between α-MSH-ir varicose axon profiles and such BAT-projecting NTS neurons were present (Fig. 9D).

**DISCUSSION**

**Control of meal size.** The brain melanocortin system, with its transmission at the MC4R, has been demonstrated to mediate very potent effects on food intake and energy expenditure induced by leptin and other feedback signals (23, 38, 45).
Although the site of this action was originally thought to be in the hypothalamus, it was shown that injections of MC4R ligands into the caudal brain stem are sufficient to strongly modulate food intake (34, 77, 78). Here, we further show that the effects on spontaneous daily food intake by administration of MC4R ligands near the caudal brain stem or directly into the dorsal vagal complex (DVC) are due to changes in meal size. MTII suppresses food intake by selectively decreasing meal size but not frequency and thus increasing the satiating power of a given size meal. In contrast, SHU9119 increases food intake by selectively increasing meal size but not frequency and thus decreasing the satiating power of a given size meal. We also show that this

Fig. 6. Hypothalamic proopiomelanocortin (POMC)/cocaine- and amphetamine-related transcript (CART) neurons project to the rat dorsal vagal complex. A: verification of injection sites in the dorsal vagal complex, showing the extent of diffusion of the retrograde tracer cholera toxin B (CTB) at different rostrocaudal levels for the 3 rats (#1618, #1619, and #1620) quantitatively analyzed (see Fig. 7). B: fluorescent micrograph showing typical injection site of CTB tracer. C–C’: confocal images showing examples of neuron (arrows) in arcuate nucleus, retrogradely labeled with rhodamine (rhod) beads (C), expressing α-melanocyte-stimulating hormone (α-MSH)-immunoreactivity (C’), and merged images (C’’). D and E: examples of neurons in the arcuate nucleus retrogradely labeled from the dorsal vagal complex with Fluorogold (green) and either expressing α-MSH (D) or CART-immunoreactivity (E), shown in red. Double-labeled neurons appear in yellow (arrows). Scale bar = 600 μm (A), 400 μm (B), 20 μm (C), and 50 μm (D and E).

Fig. 7. Quantitative analysis of hypothalamic POMC neurons with projections to the dorsal vagal complex. Left: no. of POMC (α-MSH-immunoreactive) neurons and CTB retrogradely labeled neurons per section in the anterior (ant) and posterior (post) arcuate nucleus. Right: double-labeled neurons expressed as percentage of POMC neurons or as percentage of retrogradely labeled neurons. Analyses are based on a total of 793 POMC neurons, 502 retrogradely labeled neurons, and 50 double-labeled neurons counted. *P < 0.05 between anterior and posterior arcuate nucleus (t-test).
effect is not diet specific, as it was manifest with both regular chow and high-fat diet. The results with direct DVC injections suggest that this brain area is the site of action of the MC4R ligands. However, because we have not demonstrated that the same dose of SHU9119 used for direct parenchymal injections was without effect when injected into the fourth ventricle (78), we cannot rule out action at another site via backdiffusion into the fourth ventricle.

In light of earlier research, these findings are not surprising. Selective reduction of meal size has been demonstrated with MTII (4) or leptin (31) injected into the third ventricle and leptin injected peripherally (18). Furthermore, fourth ventricu-
ular MTII injection dose-dependently reduced scheduled 30-
min glucose intake (77). We are aware of only one demonstra-
tion of the opposite: intracerebroventricular ghrelin injection
increased mainly meal frequency but not meal size (30).

What is the mechanism of this selective effect on meal size?
Because the MC4R is highly expressed in the DVC (48, 52,
69), it seems most likely that fourth ventricular and locally
administered ligands directly bind to the MC4R on NTS and
dorsal motor nucleus neurons. The effect of MC4R ligands on
electrical activity of NTS neurons has not been studied. In
preliminary experiments, we found both excitatory and inhibi-
tory effects of MTII on identified NTS neurons (unpublished
observations). In cultured hypothalamic cell lines expressing
MC4R, α-MSH dose-dependently stimulated cAMP produc-
tion, and this effect was blocked by AgRP (47, 56). We have
recently demonstrated that fourth ventricular injection of MTII
and SHU9119 modulate spontaneous and CCK-induced phos-
phorylation of the extracellular signal-regulated kinases ERK1/2 (unpublished observations). Activation of this signal-
ing cascade, including phosphorylation of the transcription
factor CREB with MTII or α-MSH, has also been shown in
neurons of the PVN of the hypothalamus (17, 39, 64). To-
gether, these observations suggest that MC4R activation in
NTS neurons stimulates specific intracellular signaling cas-
cades leading to changes in cAMP, intracellular calcium,
membrane potential, firing rate, and gene transcription.

Smith (66) proposed a new classification of the factors
controlling meal size. He defined food stimuli contacting
preabsorptive receptors along the surface of the gut from the tip
of the tongue to the end of the small intestine as direct controls
and everything else as indirect controls. Indirect controls that
require the forebrain (metabolic, ecological, and rhythmic)
change meal size by modulating the potency of direct controls
(66). This distinction bears similarities to the concept of
short-term and long-term signals but in addition assigns an
anatomical site for the possible integration of these different
classes of satiety signals.

Most signals used by the direct controls, such as gastric
distension and CCK, reach the brain via vagal afferent neurons
(67) that terminate in the NTS, where glutamate is released as
principal transmitter, stimulating both NMDA and non-NMDA
glutamate receptors on second-order NTS neurons (10). It is
well known that ionotropic glutamate receptor stimulation
leads to rapid increases in intracellular calcium concentration
and neuronal firing, as well as activation of intracellular sig-
naling cascades, resulting in longer-lasting adaptive neuronal
responses as induced by induction of c-Fos expression. Stimu-
lation of c-Fos expression in NTS neurons by stimuli associ-
ated with the ingestion of food has been demonstrated in
numerous studies (10, 27, 28, 32, 73, 79). Any of these cellular
changes could be at the beginning of the neural process of
satiation. A meal is terminated when these changes in NTS
neurons are propagated to omotor control circuits in other
parts of the caudal brain stem.

The concept of integration of direct and indirect controls was
tested at both the behavioral and neural level. Leptin at doses
that did not reduce liquid diet intake, when administered to the
third ventricle alone, significantly increased the suppression of
intake induced by peripheral CCK administration or gastric
nutrient infusion; in parallel experiments, increased CCK and
gastric nutrient infusion induced c-Fos expression in the NTS
(25, 26). In another study, MTII did not augment feeding
inhibition induced by intraduodenal glucose infusion (4), and it
is thus possible that a more complete stimulation of sensors,
including gastric distension signals, might be necessary for the
synergism to occur. Finally, these authors showed that central
NPY injections had opposing effects on NTS neuronal firing
activity induced by gastric distension (65). However, in all
these studies, the “indirect” signal represented by leptin, MTII,
or NPY was delivered to the third ventricle, making it impos-
tible to pinpoint down the site(s) of action. Some of the effects
may have been due to activation of medulla-projecting hypo-
thalamic neurons and some to direct effects on NTS neurons.

Our demonstration that brain stem administration of MTII
and SHU9119 reciprocally affects food intake through exclu-
sive changes in meal size is consistent with the ideas of a brain
stem/NTS integrator of direct and indirect controls of satiation
and meal size. What we do not know is where the natural
ligands for the MC4R come from, the brain stem or the
hypothalamus.

Control of energy expenditure. Activation of brain stem
melanocortin receptors is known not only to suppress meal size
and food intake but also to stimulate uncoupling protein 1 in
BAT (76). In support of this notion, we show here that fourth
ventricular MTII and SHU9119 increase and decrease, respec-
tively, body temperature and that α-MSH-ir axon profiles are
in close anatomical contact with NTS neurons projecting to
BAT. By use of relatively long survival times, a considerable
number of NTS neurons are retrogradely infected with pseudo-
dorabies virus injected into the intercapsular BAT (6), suggest-
ing a polysynaptic projection pathway that likely relays in the
ventral medulla and spinal cord. The raphe pallidus nucleus in
the ventromedial medulla has been shown to be a major site of
sympathetic premotoneurons projecting to BAT (54). We have
also been able to demonstrate α-MSH-ir fibers in close prox-
imity to raphe pallidus neurons with BAT projections and in
the A1/C1 area of the ventrolateral medulla (unpublished
observations), suggesting multiple sites of endogenous mela-
nocortin action on sympathetic outflow. Thus fourth ventricu-
lar administration of MC4R ligands could have affected BAT
thermogenesis at any of these sites.

Innervation of the DVC by α-MSH-containing fibers. The
presence of α-MSH-ir and ACTH-ir fibers in the caudal med-
dulla of rat and monkey has been reported before (43, 44, 46,
55, 57), and our mapping study confirms the general pattern of
innervation, with axon profiles distributed throughout the ro-
stromedial extent of the NTS. To further characterize α-MSH
innervation of the NTS, we labeled α-MSH-ir axon profiles
together with specific NTS neuronal phenotypes that have been
implicated in the control of food intake. Although our double-
labeling study revealed close anatomical appositions between
α-MSH-ir axon profiles and catecholamine, GABA, and NOS
expressing neurons, there was no preferential innervation of
any of these neuron types. We observed many relatively thick
and straight, but highly varicose, as well as thin and profusely
branching axon profiles, but we did not observe axon
terminals wrapping around specific neurons. This might sug-
gest that α-MSH action does not strictly depend on close
synaptic transmission but possibly on volume release, affecting
any receptor in the vicinity of axons. It should also be kept in
mind that confocal microscopy does not reveal functional
synapses and that additional approaches will be necessary for proof of synaptic relationships.

Catecholaminergic neurons in the NTS are known to respond to visceral satiety signals, such as CCK and gastric distension (61, 62, 73, 79), and that their immunotoxich lesion abolishes CCK’s suppression of food intake (59). However, only a small fraction of neurons expressing c-Fos in response to visceral satiety signals are catecholaminergic. Our double-labeling study shows that many NTS neurons expressing c-Fos in response to gastric nutrient infusion are tightly surrounded by profusely branching α-MSH-ir fibers forming highly varicos terminals. These findings suggest that endogenously released α-MSH is in an excellent position to modulate both catecholaminergic and noncatecholaminergic NTS neurons involved in satiation.

GABA is an important inhibitory transmitter in the DVC, originating from both local neurons and distant projection neurons, some as far away as the forebrain (41). GABA-A receptors are located on central terminals of vagal afferents and on dendrites and somata of NTS neurons and vagal motoneurons (3). As summarized by Travaglì and Rogers (70), electrophysiological studies have clearly demonstrated a role for GABA and GABA-A receptors in the elaboration of vago-vagal reflexes, and functional studies have shown involvement in esophageal and gastrointestinal motor reflexes (74, 80) important for ingestive control.

Although we had difficulty demonstrating close anatomical contacts between α-MSH fibers and GLP-1-ir neurons, we cannot rule out such an interaction. GLP-1 neurons located in the mainly caudal NTS are thought to play a role in satiation, particularly satiation that is accompanied by signs of nausea and typically involving the oxytocin system, such as occurs after the ingestion of unusually large meals (60, 63, 71). Thus it is possible that the food intake-suppressing effects of α-MSH are partially mediated through brain stem GLP-1 neurons.

Origin of MC4R ligands. There are two populations of POMC neurons in the brain: one in the arcuate nucleus/retrochiasmatic area of the medial hypothalamus (44) and one in the NTS (29, 43, 57, 75). The brain stem population can be demonstrated with immunohistochemistry only by use of massive doses of intraventricular colchicine (14, 43, 57, 75) and with a riboprobe for in situ hybridization only after prolonged exposure (14), suggesting that the normal expression level is much lower than in the hypothalamus. Furthermore, RT-PCR analysis revealed the presence of the predicted 742-bp product in the rat and human medulla, but also in other parts of the rat brain (33). Thus the POMC gene is expressed in several areas of the rat brain, but the contribution of the low-level expression sites to the control of energy balance is not clear. Most recently, transgenic mice with enhanced green fluorescent protein (eGFP) expression under the POMC promoter were obtained to stain brain stem POMC neurons and to show that intraperitoneal CCK injection induced c-Fos expression in 30% of NTS POMC-eGFP neurons (29). Thus one potential source of α-MSH, the natural ligand for the MC4R, is the local POMC neurons in the NTS.

However, it is also likely that α-MSH is released from NTS-projecting hypothalamic POMC neurons. Early studies that used surgical lesions and transections at various levels between the hypothalamus and NTS clearly demonstrated a contribution of hypothalamic POMC neurons to the innervation of the rostral NTS by α-MSH-ir fibers (2, 57). Here we confirm with retrograde tracing that a small percentage of hypothalamic POMC neurons projects to the DVC. Compared with the PVN and LH, relatively few neurons in the mediodorsal hypothalamus project to the DVC, and even fewer express POMC. Our analysis shows a gradient along the anterior-posterior direction, with higher numbers of retrogradely labeled and double-labeled neurons, as well as a higher percentage of POMC neurons with DVC projections, in the anterior half of the arcuate nucleus. Given the average of approximately one double-labeled neuron per 30-μm section and taking into account that the POMC-containing portion of the arcuate nucleus is ~2 mm long, we estimate that there are only 50–100 POMC neurons with projections to the DVC. However, if these few neurons branch profusely in the DVC, they could still exert significant modulatory effects. It is also interesting to note that, among the retrogradely labeled neurons, only ~10% express α-MSH, with the remaining 90% expressing a neurochemical phenotype that remains to be determined.

In conclusion, the behavioral demonstration that melanocortin ligands administered near or within the caudal brain stem modulate food intake by selectively changing meal size strongly suggests that melanocortin signaling in the brain stem is necessary to alter food intake. The close appositions between α-MSH-ir axons and NTS neurons activated by gastric nutrient infusion provide a general anatomical basis for such modulation but do not distinguish between pre- and postsynaptic mechanisms. The presence of POMC neurons in the arcuate nucleus of the hypothalamus with projections to the DVC suggests that these neurons provide the melanocortin receptor ligand α-MSH; however, a role for the POMC neuron population in the NTS cannot be excluded.

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