Effects of Chronic Central Nervous System Administration of Agouti-Related Protein in Pair-Fed Animals

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The melanocortin receptor (MC3-R and MC4-R) antagonist, agouti-related protein (AGRP), is a potent stimulant of food intake. We examined the effect of chronic intracerebroventricular (ICV) AGRP treatment on energy metabolism and pituitary function in ad libitum fed rats and rats administered AGRP and then pair-fed to a saline control group. Chronic ICV AGRP (83-132) administration (1 nmol/day for 7 days) significantly increased food intake and body weight in ad libitum fed animals compared with saline-treated controls (body weight on day 7: 272 ± 6 [saline] vs. 319 ± 8 g [AGRP ad libitum fed]; *P* < 0.001). A significant increase in the epididymal fat pad weight, interscapular brown adipose tissue (BAT) weight, and plasma leptin was also observed in the ad libitum fed group. In the AGRP pair-fed group, a significant increase in the epididymal fat pad weight, BAT weight, and plasma leptin was again observed, suggesting that AGRP caused metabolic changes independent of increased food intake. BAT uncoupling protein 1 (UCP-1) content was significantly decreased compared with saline controls in both the AGRP ad libitum fed (21 ± 8% of saline control; *P* < 0.01) and AGRP pair-fed groups (24 ± 7% of saline control; *P* < 0.01). Plasma thyroid-stimulating hormone (TSH) was significantly suppressed compared with saline controls in both the AGRP ad libitum fed and AGRP pair-fed groups (3.5 ± 0.3 [saline] vs. 2.7 ± 0.4 [AGRP ad libitum fed] vs. 2.1 ± 0.2 ng/ml [AGRP pair-fed]; *P* < 0.01). This study demonstrates that independent of its orexigenic effects, chronic AGRP treatment decreased BAT UCP-1, suppressed plasma TSH, and increased fat mass and plasma leptin, suggesting that it may play a role in energy expenditure. ***Diabetes* 50:248–254, 2001**

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α-MSH, α-melanocyte–stimulating hormone; AGRP, agouti-related protein; Ang-II, angiotensin II; BAT, brown adipose tissue; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; FSH, follicle-stimulating hormone; HPA, hypothalamopituitary adrenal; ICV, intracerebroventricular; ir, immunoreactivity; LH, luteinizing hormone; LRHR, LH-releasing hormone; MC3-R, melanocortin 3 receptor; MC4-R, melanocortin 4 receptor; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus; RIA, radioimmunoassay; SHBG, sex hormone–binding globulin; TBS, Tris-buffered saline; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylene diamine; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; UCP-1, uncoupling protein 1; WAT, white adipose tissue.

The hypothalamic melanocortin system is a regulator of energy homeostasis. The agouti-related protein (AGRP), produced in the arcuate nucleus, is an antagonist at the melanocortin 3 and 4 receptors (MC3-R and MC4-R), which are distributed throughout the brain. The proopiomelanocortin (POMC) products, such as α-melanocyte–stimulating hormone (α-MSH), act as agonists at the melanocortin receptors (1–3). Hypothalamic POMC mRNA, AGRP mRNA, and the density of MC4-R have been shown to be altered with nutritional status (4,5). Defects of POMC processing and mutations of MC4-R in humans and mice are associated with gross obesity (6–11).

Administration of the synthetic MC3/4-R agonist MT II into the forebrain ventricles or directly into the paraventricular nucleus (PVN) of the hypothalamus of rats or mice produced a dosage-dependent reduction in food intake and body weight (12–14). A single injection of either the endogenous (AGRP [83-132]) or the synthetic (SHU9119 and HS014) MC3/4-R antagonists stimulated food intake in rodents (12,13,15,16). Chronic administration of the more selective MC4-R antagonist, HS028, for 7 days via an osmotic minipump significantly increased food intake and body weight. Tachyphylaxis to HS028 did not occur. However, neither energy expenditure nor pituitary function were examined in this study (17). Previous results from this department have demonstrated that AGRP (83-132), the endogenous hypothalamic melanocortin receptor antagonist, significantly increased 24-h food intake and antagonized the anorectic effects of α-MSH (16).

The melanocortin system also influences energy expenditure. Intracerebroventricular (ICV) administration of the MC3/4-R agonist, MT II, produced a dosage-dependent sympathoexcitation in brown adipose tissue (BAT) and renal and lumbar beds. These effects were antagonized by coadministration of SHU9119 (18). Recent investigations have suggested that the hypothalamic melanocortin system may mediate some of leptin's effects on food intake (14,15,19), body weight (14,15), the sympathetic nervous system (18,20), and gonadal function (21). A single ICV injection of leptin decreased food intake and body weight, and increased uncoupling protein 1 (UCP-1) mRNA expression in BAT.

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These effects were reversed by coadministration of the MC3/4-R antagonist SHU9119 (20). However, treatment with SHU9119 alone had no effect on these parameters (20). These observations suggest that agonism of hypothalamic melanocortin receptors increases sympathetic activity traffic to thermogenic and nonthermogenic tissues and may mediate some of the physiological actions of leptin.

AGRP and neuropeptide Y (NPY) are coproduced in cells of the medial arcuate nucleus of the hypothalamus, and considerable functional and anatomical overlap exists between them. Almost 95% of NPY mRNA-positive arcuate neurons coexpress AGRP mRNA, and NPY immunoreactivity (NPY-ir) is present in almost all AGRP-ir positive neurons (13,22,23). The NPY/AGRP fiber system projects to hypothalamic nuclei implicated in the regulation of food intake and neuroendocrine function (24–27). Both NPY and AGRP are potent orexigenic peptides when administered centrally (16,28). Although NPY is known to influence energy metabolism by decreasing brown fat thermogenesis and increasing white fat lipoprotein lipase enzymatic activity (29,30), the effects of AGRP are unknown. Acute ICV administration of either NPY or AGRP to intact male rats increased plasma leutinizing hormone (LH) (31,32). Both neuropeptides increased the release of LH-releasing hormone (LHRH) from medial basal hypothalamic explants (31,33). Chronic 7-day administration of NPY resulted in obesity and a dramatic suppression of plasma testosterone, LH, follicle-stimulating hormone (FSH), and seminal vesicle weight, and profoundly inhibited the somatotropic axis (34,35). However, the effects of chronic AGRP administration on energy expenditure and neuroendocrine function are unknown.

In recent studies, AGRP (83-132) significantly increased LHRH release in vitro from medial basal hypothalamic explants and increased plasma LH, FSH, and testosterone after ICV administration (31). Recent anatomical evidence suggests that α-MSH innervates pro–thyrotropin-releasing hormone (TRH) PVN neurones and that AGRP-ir axon varicosities are juxtaposed to all pro-TRH neurones receiving α-MSH innervations (27,36). ICV administration of α-MSH reversed the fasting-induced suppression of pro-TRH mRNA (36). In addition, recent studies from this department have shown that ICV administration of AGRP (83-132) significantly suppressed plasma TSH, whereas administration of α-MSH significantly increased plasma TSH (37). The aim of this study was to administer AGRP (83-132) chronically for 7 days and examine food intake, body weight, energy expenditure, and the hypothalamopituitary axis.

RESEARCH DESIGN AND METHODS

AGRP (83-132), an MC3/4-R antagonist (16,38), was purchased from Phoenix Pharmaceuticals (Mountain View, CA). Cannulation materials were supplied by Plastics One (Roanoke, VA).

Animals. Male Wistar rats (BSU, Imperial College School of Medicine, London, U.K.) weighing 250–300 g were maintained in individual cages under controlled temperature (21–23°C) and light conditions (12:12 h light:dark cycle, lights on 7:00 A.M.), with ad libitum access to food (RM1 diet; SDS, Witham, U.K.) and water, unless otherwise stated in the study protocol. Animal procedures undertaken were approved by the British Home Office Animals Scientific Procedures Act 1986.

ICV cannulation. Rats were anesthetized by intraperitoneal injection of a mixture of Ketalar (ketamine HCl 60 mg/kg; Parke-Davis, Pontypool, U.K.) and Rompun (xylazine 12 mg/kg; Bayer, Bury St. Edmunds, U.K.) and placed in a Kopf stereotaxic frame. To cannulate the third cerebral ventricle (ICV), permanent 22-gauge stainless steel guide cannulas were stereotactically placed 0.8 mm posterior to the bregma on the midline and implanted 6.5 mm below the outer surface of the skull. These coordinates were calculated using the rat brain atlas of Paxinos and Watson (39). The guide cannulas were held in place by dental cement glued to three stainless-steel screws driven into the skull. While anesthetized, all rats were given 1 ml amoxycillin sodium, i.p. (12.5 mg/ml; SmithKline Beecham, Welwyn Garden City, U.K.) dissolved in 0.9% saline to minimize the risk of postoperative infection. After surgery, a wire stylet was inserted into the guide cannulas to prevent blockage and infection. Rats were left for 1 week to recover from the surgical procedure. After this period, rats were given a further week of daily handling to habituate them to all experimental procedures and minimize stress. A positive dipsogenic response to ICV injection of angiotensin II (ANG II; 150 ng/rat), as previously described (16,40,41), verified cannula placement. If an animal ate <10 g of food in a 24-h period or lost >10 g body wt over a 48-h period, it was considered unwell and excluded from the study.

ICV injections. Substances were administered via a stainless steel injector placed in, and projecting 1 mm below, the tip of the guide cannulas. The required volumes were injected over 1 min and a further period of 30 s was allowed before removing the injector to ensure complete diffusion. A Hamilton gas-tight syringe was connected to an infusion pump to ensure an accurate and constant volume of delivery. The tubing was filled with saline solution, and a small air bubble (5:1) was drawn up at the distal end to separate it from the test solution. The entire injection process lasted ~2 min, after which the rats were returned to their cages. All compounds were dissolved in 0.9% saline and injected in a 10 µl volume.

Study design. Only rats that showed a prompt and sustained drinking response to ANG-II were used in the subsequent study. Rats were handled daily and their body weight and food intake monitored. Rats were randomized into one of three experimental groups (n = 16–18 per group): *1)* saline group, in which rats received saline injections daily for 7 days (days 0–6) and were allowed ad libitum access to food; *2)* AGRP ad libitum fed group, in which rats received daily injections of 1 nmol AGRP (83-132) for 7 days (days 0–6) and were allowed ad libitum access to food; and *3)* AGRP pair-fed group, in which rats received daily injections of 1 nmol AGRP (83-132) for 7 days (days 0–6) and were pair-fed to the saline group's mean daily food intake. The aim of this pair-fed group was to investigate which of the AGRP effects were dependent on increased body weight and food intake.

The mean starting body weight on day –1 was not significantly different among groups. All ICV injections were performed during the early light phase (8:00–11:00 A.M.). On day –1 all rats received a sham injection of saline to habituate them to the injection procedure. On day 0 the rats received their first injection of either saline or 1 nmol AGRP (83-132). The 1-nmol dosage of AGRP (83-132) was chosen from previous dosage-response curves (16) as significantly increasing 24-h food intake.

All rats were killed by decapitation during the early light phase (8:00–11:00 A.M.) on day 7. Trunk blood was collected into plastic tubes containing potassium EDTA (final concentration of 1.2–2 mg EDTA/ml blood) (Sarstedt, Leicester, U.K.). Whole brains were removed and the hypothalami were dissected, weighed, and assessed for gross anatomical changes. No alterations in hypothalamic weight (47.7 ± 2.8 [saline] vs. 47.0 ± 2.2 [AGRP pair-fed] vs. 47.3 ± 3.0 mg [AGRP ad libitum]) were seen and no gross anatomical changes were observed. Plasma was separated by centrifugation, frozen on dry ice, and stored at –70°C until assay. Epididymal fat pads (white adipose tissue [WAT], chosen as a representative fat pad), interscapular BAT, adrenals, testicles, and seminal vesicles were dissected and weighed. Interscapular BAT was snap frozen in liquid nitrogen for UCP-1 extraction and analysis by Western blotting.

Radioimmunoassay. Plasma pituitary hormone concentrations were estimated by radioimmunoassay (RIA) using reagents and methods provided by the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program (A. Parlow, Harbor University of CA, Los Angeles Medical Center) as previously described (31,42,43). Plasma total testosterone was measured using a solid-phase RIA (DPC, Los Angeles, CA); plasma corticosterone was measured by RIA (ICN Biomedicals, Costa Mesa, CA), as was plasma leptin (Linco Research, St. Charles, MO); and rat IGF-I was measured by RIA kit (DLS-2900; Diagnostic Systems Laboratory, Webster, TX).

Western blot analysis of UCP-1 content in BAT

Preparation of total cellular membranes. Each BAT pad was homogenized in 7 ml homogenization buffer (0.25 mol/l sucrose, 10 mmol/l Tris-HCl [pH 7.4], 1 mmol/l dithiothreitol, 1 mmol/l EDTA, and 1% bovine serum albumin [BSA]) at 4°C. Homogenates were centrifuged (Sorval RC centrifuge) at 1,000g for 15 min at 4°C. Supernatant was collected and recentrifuged at 100,000g for 60 min at 4°C. The resulting pellet was resuspended in 3 ml homogenization buffer without BSA and aliquotted. The protein concentration of each aliquot was assessed by the Biuret method (44). Aliquots were stored at –80°C until use.

SDS polyacrylamide gel electrophoresis of proteins. Electrophoresis was carried out in gel tanks using an adaptation of the method of Laemmli (45)

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and a discontinuous buffer system (Bio-Rad Unigel System, Bio-Rad, Richmond, CA). Acrylamide gels (12%) were made using acrylamide stock (30% acrylamide [37:5:1]) and resolving gel buffer (1.5 mol/l Tris-HCl, 0.4% [wt/vol] SDS; pH 8.8). Ammonium persulfate and *N,N,N′,N′*-tetramethylethylene diamine (TEMED) were added to polymerize the gel. A 4% stacking gel was added once the resolving gel had set, using the acrylamide stock and stacking buffer (0.5 mmol/l Tris-HCl, 0.4% [wt/vol] SDS; pH 6.8). Again ammonium persulfate and TEMED were added to catalyze polymerization. Samples of 1 μg total membrane protein for UCP-1 detection were solubilized in electrophoresis sample buffer (10% [wt/vol] SDS, 0.025% [wt/vol] bromophenol blue, 1% glycerol) to a final volume of 10 μl and loaded onto the gel. Gels were run at a constant 75 V in running buffer (0.025 mol/l Tris-HCl, 0.1% [wt/vol] SDS, 0.2 mol/l glycine; pH 8.3).

Western blotting. Western blotting was carried out according to the method of Burnette (46). Proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotechnologies, Buckinghamshire, U.K.) using the semidry transfer method on a Multiphore II Nova Blot Electrophoretic transfer unit (Amersham Pharmacia Biotechnologies) for 1.5 h at 25 V. After transfer, the nitrocellulose membrane was rinsed in distilled water and incubated at room temperature, with gentle agitation in 100 ml blocking buffer (Tris-buffered saline [TBS]/Tween [1% Tween 20, 10 mmol/l Tris, 0.9% NaCl; pH 7.4] with 5% [wt/vol] nonfat dried milk) to reduce nonspecific binding. Blots were then rinsed briefly in TBS/Tween and incubated with 50 ml primary antiserum 0.5 μg/μl (UCP-1 antibody; Research Diagnostics, Flanders, NJ) in TBS/Tween for 1.5 h at room temperature with gentle agitation. The blot was then washed six times for 5 min at room temperature and incubated for 1 h at room temperature under gentle agitation with 50 ml of a 1:4,000 dilution of anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS/Tween. Hybridized bands were then visualized using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotechnologies) and exposed to photographic film (Amersham Pharmacia Biotechnologies).

Quantification and statistical analysis. Results are presented as means ± SE. Western blot band intensities were quantified using the Bioimage Intelligent Quantifier program and expressed as percentage of control. Western blot results were analyzed by an unpaired Student's *t* test. For all other results, statistical significance between control and experimental group was determined by analysis of variance followed by a post hoc least significance differences test (Systat, Evanston, IL). In all cases, $P < 0.05$ was considered to be statistically significant.

RESULTS

Food intake. Before the onset of the study, there were no significant differences in food intake among the three experimental groups (24-h food intake on day −1: 25 ± 1 [saline] vs. 25 ± 0.7 [AGRP ad libitum fed] vs. 25 ± 0.9 g [AGRP pair-fed]). Treatment with 1 nmol/day AGRP (83-132) for 7 days significantly increased daily food intake compared with the saline control group (day +7 food intake: 21 ± 2 [saline] vs. 41 ± 2 g [AGRP ad libitum fed]; $P < 0.0001$ vs. saline) (Fig. 1). The AGRP pair-fed group received 1 nmol/day ICV-injected AGRP and was pair-fed to the saline group's mean daily food intake. Thus there was no difference in the daily food intake between the AGRP pair-fed and saline groups throughout the study.

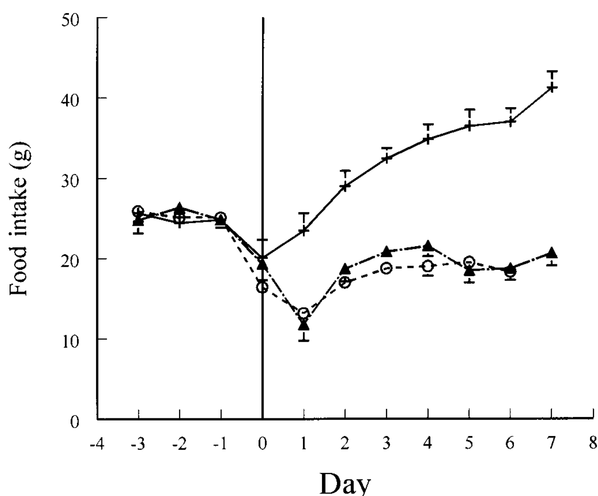

FIG. 1. Daily food intake after chronic administration of 1 nmol/day AGRP (83-132) for 7 days. +, AGRP ad libitum fed group; ○, AGRP pair-fed group; ▲, saline control group.

Body weight. Before the onset of the study, there was no significant difference in body weight between the three experimental groups (day −1 body weight: 296 ± 5 [saline] vs. 297 ± 6 [AGRP ad libitum fed] vs. 302 ± 4 g [AGRP pair-fed]). Treatment with 1 nmol/day AGRP (83-132) for 7 days significantly increased body weight in the AGRP ad libitum fed group (day +7 body weight: 272 ± 6 [saline] vs. 319 ± 8 g [AGRP ad libitum fed]; $P < 0.01$ vs. saline). The saline group body weight fell on day +1, and this weight was maintained during the entire study period. Other investigators (47,48), have previously observed a maintenance of body weight in a saline-injected control group. No significant difference in body weight was observed between the saline and AGRP pair-fed groups throughout the study (day +7 body weight: 271 ± 6 [saline] vs. 260 ± 4 g [AGRP pair-fed]; NS) (Fig. 2).

Fat pad weights and plasma leptin. Epididymal fat pad (WAT) weight was significantly greater in the AGRP ad libitum fed group than in the saline control group (1.8 ± 0.2 [saline] vs. 4.4 ± 0.3 g [AGRP ad libitum fed]; $P < 0.0001$ vs. saline). In addition, epididymal fat pad weight was significantly increased in the AGRP pair-fed group compared with the saline control group, despite there being no significant difference in food intake or body weight (1.8 ± 0.2 [saline] vs. 2.6 ± 0.07 g [AGRP pair-fed]; $P < 0.01$ vs. saline) (Table 1).

A significant increase in interscapular BAT weight was observed in both the AGRP ad libitum fed and AGRP pair-fed groups compared with the saline control group (0.31 ± 0.02 [saline] vs. 0.79 ± 0.07 [AGRP ad libitum fed; $P < 0.0001$ vs. saline] vs. 0.40 ± 0.02 g [AGRP pair-fed; $P < 0.01$ vs. saline]) (Table 1). Interestingly, despite similar daily food intake in the AGRP pair-fed and saline control groups, a significant increase in wet weight of both the BAT and WAT was observed in the AGRP pair-fed group.

Plasma leptin concentrations paralleled the changes in WAT weight. Plasma leptin increased significantly in the AGRP ad libitum fed group compared with the saline control group (0.86 ± 0.1 [saline] vs. 11.7 ± 1.9 ng/ml [AGRP ad libitum fed]; $P < 0.0001$ vs. saline). As observed with the WAT wet weight, plasma leptin was significantly increased in the AGRP pair-fed group compared with the saline control group (0.86 ± 0.1 [saline] vs. 1.7 ± 0.2 g [AGRP pair-fed]; $P < 0.05$ vs. saline), despite there being identical food intake and no significant difference in body weight (Table 1).

UCP-1 protein levels in BAT. The UCP-1 bands detected by Western blotting were of the expected size, 32 kDa (see Fig. 3 for representative Western blot). A significant suppression of UCP-1 was observed in the AGRP ad libitum fed group compared with the saline control group (100 ± 11.0 [saline] vs. 21.8 ± 8.3% [AGRP ad libitum fed]; $P < 0.001$ vs. saline) (Fig. 4). A similar magnitude of suppression of UCP-1 protein was observed in the AGRP pair-fed group despite no hyperphagia (100 ± 11.0 [saline] vs. 24.0 ± 6.8% [AGRP pair-fed]; $P < 0.001$ vs. saline) (Fig. 4).

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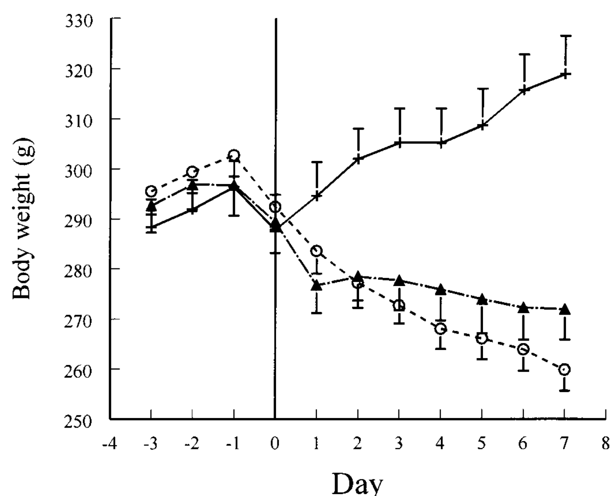

FIG. 2. Body weight changes after chronic administration of 1 nmol/day AGRP (83-132) for 7 days. +, AGRP ad libitum fed group; ○, AGRP pair-fed group; ▲, saline control group.

Organ weights. After 7 days of 1 nmol/day ICV-injected AGRP (83-132), no change was observed in adrenal (55.0 ± 1.8 [saline] vs. 58.8 ± 3.4 [AGRP ad libitum fed] vs. 53.5 ± 1.9 mg [AGRP pair-fed]; NS), testicular (3.4 ± 0.06 [saline] vs. 3.5 ± 0.08 [AGRP ad libitum fed] vs. 3.3 ± 0.06 g [AGRP pair-fed]; NS), or seminal vesicle weight (0.52 ± 0.01 [saline] vs. 0.52 ± 0.1 [AGRP ad libitum fed] vs. 0.51 ± 0.02 g [AGRP pair-fed]; NS).

Plasma hormones

Pituitary. A significant suppression of plasma TSH was observed in both the AGRP ad libitum fed and AGRP pair-fed groups when compared with the saline control group (2.7 ± 0.4 [AGRP ad libitum fed; $P < 0.05$ vs. saline] vs. 2.1 ± 0.2 [AGRP pair-fed; $P < 0.001$ vs. saline] vs. 3.5 ± 0.3 ng/ml [saline]) (Fig. 5). No significant change in plasma LH (0.23 ± 0.05 [AGRP ad libitum fed] vs. 0.24 ± 0.04 [AGRP pair-fed] vs. 0.21 ± 0.02 ng/ml [saline]) or prolactin (5.5 ± 1.2 [AGRP ad libitum fed] vs. 4.9 ± 1.7 [AGRP pair-fed] vs. 4.1 ± 0.8 ng/ml [saline]) was observed after chronic administration of AGRP (83-132).

Peripheral. A significant suppression in plasma IGF-I was observed in the AGRP ad libitum fed and AGRP pair-fed groups when compared with the saline control group (1486 ± 102 [AGRP ad libitum fed; $P < 0.05$ vs. saline] vs. 1320 ± 75 [AGRP pair-fed; $P < 0.001$ vs. saline] vs. 1737 ± 58 ng/ml [saline]) (Fig. 6). No change in corticosterone was observed in any of the experimental groups investigated (109 ± 29 [AGRP ad libitum fed] vs. 106 ± 15 [AGRP pair-fed] vs. 136 ± 32 ng/ml [saline]). A significant increase in plasma total testosterone was observed in the AGRP ad libitum fed group compared with the saline control group, but no significant difference was observed between the AGRP pair-fed group and the saline control group (1288 ± 276 [AGRP ad libitum fed; $P < 0.05$ vs. saline) vs. 670 ± 146 [AGRP pair-fed] vs. 719 ± 111 ng/dl [saline]). The observation that plasma total testosterone was significantly increased only in the AGRP ad libitum fed group may be secondary to changes in either sex hormone–binding globulin (SHBG) or body weight. Measurement of SHBG was beyond the scope of the current study.

DISCUSSION

Chronic 7-day ICV administration of the MC3/4-R antagonist AGRP (83-132) increased daily food intake and adipose tissue mass and caused an elevation of plasma leptin in ad libitum fed rats. No tachyphylaxis was observed.

Chronic AGRP treatment caused a dramatic fall in BAT UCP-1 and a suppression of plasma TSH. These observations add to our previous finding that acute ICV-administered AGRP decreased plasma TSH and that α-MSH stimulated plasma TSH (37), and agree with a previous report of MC4 agonism causing increased sympathetic activation of BAT (18). Thus hypothalamic MC3/4-R antagonism results not only in hyperphagia, but also in decreased sympathetic and hypothalamo-pituitary thyroid activity, presumably resulting in decreased energy expenditure, which contributes to the obesity.

Our study included an ICV-injected AGRP group that was pair-fed to the food intake of the saline controls to determine which effects of AGRP were due to an increased food intake. We observed a dramatic decrease in BAT UCP-1 levels and a suppression of plasma TSH in the pair-fed group. This suggests that AGRP caused decreased energy expenditure independent of daily food intake and body weight; this suggestion is also supported by the observation that agouti (A^{vy}) mice pair-fed to non-agouti controls still become obese (49).

Previous studies have suggested that the hypothalamic melanocortin system may mediate some of the central effects of leptin (14,15,20,50). Decreases in food intake and body weight and increases in UCP-1 mRNA seen after a single ICV injection of leptin are blocked by coadministration of SHU9119 (20). However, melanocortin agonists and antagonists acted neither alone nor in combination with leptin to influence the suppressed reproductive endocrine system of the *ob/ob* mouse (50), suggesting that melanocortins do not mediate the effects of leptin on the reproductive axis.

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TABLE 1
Comparison of epididymal fat pad weight, BAT, and plasma leptin in rats administered ICV AGRP (83-132) (1 nmol/day) chronically for 7 days

	Saline	AGRP pair-fed	AGRP ad libitum fed
Epididymal fat pad (g)	1.8 ± 0.2	2.6 ± 0.07†	4.4 ± 0.3‡
Interscapular BAT (g)	0.31 ± 0.02	0.40 ± 0.02†	0.79 ± 0.07‡
Plasma leptin (ng/ml)	0.86 ± 0.1	1.7 ± 0.2*	11.7 ± 1.9‡

Data are means ± SE. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ vs. saline.

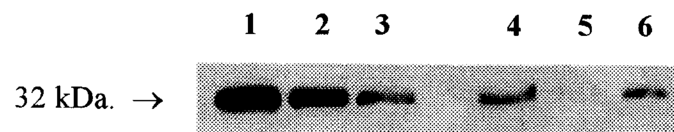

FIG. 3. Representative Western blot of UCP-1 protein in BAT. BAT homogenate proteins were separated on 12% SDS-PAGE. Proteins were transferred to nitrocellulose and the blot was probed with a UCP-1 antibody. Any hybridizing bands were visualized using an ECL system and photographic film. The picture above is a representative sample of the Western blots obtained in these studies. Each lane is from one animal and contains 1 µg of homogenate proteins. *Lanes 1* and *2*, saline-treated animals; *lanes 3* and *4*, AGRP pair-fed animals; *lanes 5* and *6*, AGRP ad libitum fed animals.

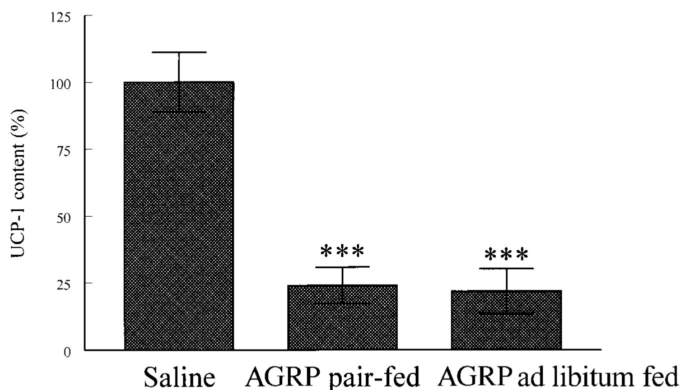

FIG. 4. Quantification (percentage) of UCP-1 in BAT after chronic administration of 1 nmol/day AGRP (83-132). ***P < 0.001 vs. saline.

After chronic AGRP (83-132) administration, no significant change was observed in testicular weight, seminal vesicle weight, plasma LH, or plasma prolactin. A significant increase in total plasma testosterone was observed in the AGRP ad libitum fed group; however, there was no difference between the AGRP pair-fed and saline control groups. These results suggest that the changes observed were dependent on an increased body weight. In humans (51) and rats (52), obesity is associated with decreased plasma testosterone, reduced testicular weight, and a decrease in SHBG. The current observed increase in plasma testosterone is a surprising and unexpected finding. Some investigators have suggested a role for the melanocortins in the acute control of the gonadal axis (21,31,53); however, the agouti mice (2), the POMC KO mice (6), and the MC4-R KO mice (11) are all fertile, suggesting that the melanocortin system is not essential for reproduction. Our results support these findings.

Uniquely among animal obesity models, those with altered melanocortin signaling exhibit altered somatic growth. Agouti (A^y) animals, for example, are 10–15% longer than their wild-type counterparts (11,21). MC4-R–deficient humans and mice also have an increased length (8,10,11). However, we found that chronic administration of AGRP decreased plasma IGF-I. This result was observed in both the AGRP pair-fed and AGRP ad libitum fed groups, suggesting that this suppression was independent of both food intake and body weight. This paradoxical result is worthy of further investigation, but may suggest a developmental role for the melanocortin system distinct from its role in the adult animal. The effect of AGRP on somatostatin or growth hormone releasing factor (GRF) release may be an important field of future study in this respect.

We observed no alteration in adrenal weight or plasma corticosterone in the present study. This observation is of interest as the MC4-R–deleted mouse and the agouti mouse (A^y, A^{vy}) are obese, but have normal corticosterone (11,54). In contrast, both chronic NPY-induced obesity (55,56) and leptin deficiency (*ob/ob* and *db/db* mice) (57,58) result in profound hypercorticosteronemia in rodents. Acute central administration of α-MSH increased plasma ACTH and corticosterone (59,60), but our results suggest that the melanocortin system may not play an important role in the long-term control of the hypothalamopituitary adrenal (HPA) axis.

Chronic AGRP treatment influenced body weight, energy expenditure, and pituitary function. It is interesting to contrast these observations with chronic NPY treatment, as both AGRP and NPY are coproduced in the arcuate nucleus of the hypothalamus. Both neuropeptides increased daily food intake and body adiposity and suppressed the somatotropic axis. The most striking differences were their influences over the reproductive and adrenal axes. NPY profoundly inhibited the gonadal axis, with reduction in seminal vesicle weight and a dramatic suppression of testosterone (34). Chronic NPY administration also activated the HPA axis. No such alterations were observed after chronic AGRP treatment. These differences suggest that the melanocortin system may be a better target for the treatment of obesity.

A reduction or absence of serum leptin results in complex adaptive neuroendocrine changes, including hypercorticosteronemia, infertility, and decreased metabolic rate (61). Current evidence suggests that leptin's inhibition of body weight and food intake could be mediated in part via the hypothalamic MC3/4-R (15,50). The current study suggests that the melanocortin system is an important regulator of food intake, the hypothalamopituitary thyroid axis, thermogenesis, and energy expenditure. However, the hypothalamic melanocortin system may not responsible for mediating other effects of leptin insufficiency, such as hypercorticosteronemia and infertility.

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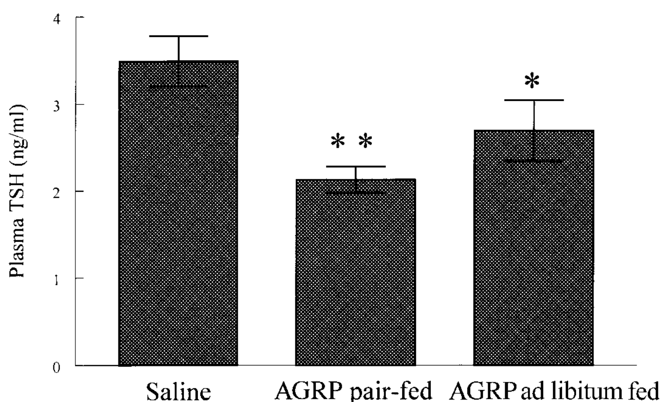

FIG. 5. Plasma TSH after chronic administration of 1 nmol/day AGRP (83-132). **P < 0.001; *P < 0.05 vs. saline.

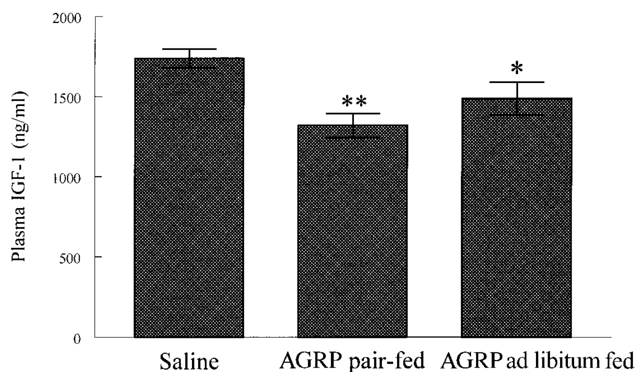

FIG. 6. Plasma IGF-I after chronic administration of 1 nmol/day AGRP (83-132). **P < 0.001; *P < 0.05 vs. saline.

In summary, we have demonstrated for the first time that chronic ICV administration of AGRP (83-132) increased food intake, body weight, and body adiposity. AGRP suppressed the somatotropic axis independently of food intake and did not alter the HPA axis. AGRP's influence on the gonadal axis was dependent on food intake. Chronic AGRP treatment resulted in a profound suppression of BAT UCP-1, decreased plasma TSH, and increased fat deposition. These metabolic effects were independent of food intake. These finding suggest that AGRP not only altered food intake but also decreased energy expenditure.

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