0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(11):4816–4821
Copyright © 2004 by The Endocrine Society
doi: 10.1210/en.2004-0476

Effect of Agouti-Related Protein in Regulation of the Hypothalamic-Pituitary-Thyroid Axis in the Melanocortin 4 Receptor Knockout Mouse

CSABA FEKETE, DANIEL L. MARKS, SUMIT SARKAR, CHARLES H. EMERSON, WILLIAM M. RAND, ROGER D. CONE, AND RONALD M. LECHAN

Tupper Research Institute and Department of Medicine (C.F., S.S., R.M.L.), Division of Endocrinology, Diabetes, Metabolism and Molecular Medicine, Tufts-New England Medical Center, and Departments of Community Health (W.M.R.) and Neuroscience (R.M.L.), Tufts University School of Medicine, Boston, Massachusetts 02111; Division of Endocrine Neurobiology (C.F.), Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest 1083, Hungary; Department of Medicine, Division of Endocrinology (C.H.E.), University of Massachusetts Medical School, Worcester, Massachusetts 01655; and Vollum Institute, Oregon Health and Science University (R.D.C), Department of Pediatrics (D.L.M.), Center for the Study of Weight Regulation (D.L.M., R.D.C.), Portland Oregon 97239

Agouti-related protein (AGRP) is thought to be one of the neuropeptides mediating the effects of leptin on appetite and satiety. The central administration of AGRP not only stimulates food intake, but also inhibits the hypothalamic-pituitary-thyroid axis (HPT) axis, closely replicating the central hypothyroid state induced by fasting. AGRP binds as an endogenous antagonist or inverse agonist of the central melanocortin receptors but has also been hypothesized to have melanocortin receptor-independent effects. Thus, we determined whether the central effects of AGRP on the HPT axis are altered in mice with selective deletion of the melanocortin 4 receptor (MC4-R). AGRP or artificial cerebrospinal fluid was administered daily into the lateral ventricle of adult, male MC4-R knockout and wild-type (WT) mice for 3 d. AGRP significantly increased the cumulative food intake and weight of white and brown adipose tissue, suppressed circulating levels of T_4 [control *vs.* AGRP in WT (μg/dl): 4.54 ± 0.16 *vs.* 3.87 ± 21], and inhibited proTRH mRNA content in the hypothalamic paraventricular nucleus of WT mice (control *vs.* AGRP in WT (density units ± SEM): 4.65 ± 0.50 *vs.* 2.47 ± 0.17). In contrast, no significant effects of AGRP were observed in any of these parameters in the MC4-R knockout mice. These data suggest that AGRP signaling to TRH hypophysiotropic neurons in the paraventricular nucleus is primarily mediated by the MC4-R and therefore, binding to the MC3-R or other putative AGRP receptors may have only a minor role. (*Endocrinology* 145: 4816–4821, 2004)

THE HYPOTHALAMIC-PITUITARY-THYROID (HPT) axis plays important role in the regulation of energy homeostasis (1, 2) via the effects of thyroid hormone to increase oxygen consumption and heat generation (1, 2). Thus, inhibition of the HPT axis during fasting (3–5) would appear to be an important adaptive mechanism to conserve energy stores. The state of central hypothyroidism induced by fasting is orchestrated by changes of circulating levels of a protein, leptin, which declines with fasting and is restored to normal levels by refeeding (4). Thus, if leptin is administered exogenously to fasting animals, the reduction in circulating levels of thyroid hormones, TSH, and hypophysiotropic proTRH mRNA in the hypothalamic paraventricular nucleus (PVN) can be prevented (3). The primary action of leptin on the HPT axis is mediated by the hypothalamic arcuate nucleus through direct axonal projections to the PVN. If the arcuate nucleus is ablated, not only is the response of the thyroid axis to fasting abolished, but its response to the exogenous administration of leptin is lost as well (6).

One of the most important arcuate nucleus-derived neuropeptides mediating the effects of leptin on energy homeostasis is agouti-related protein (AGRP) (7, 8). Central administration of AGRP not only markedly stimulates food intake, but also simultaneously inhibits the hypothalamic-pituitary-thyroid axis (HPT) axis, closely replicating the central hypothyroid state associated with fasting (9). Because AGRP is an endogenous antagonist at melanocortin receptors (8), it is presumed that its primary role on the HPT axis may be to prevent the stimulatory effects of α-MSH on the TRH gene (9, 10). Although practically all hypophysiotropic TRH neurons receive contacts by axons containing AGRP (10, 11), only approximately 30% of TRH neurons in the medial parvocellular subdivision are contacted by axons containing α-MSH (10), and only approximately 48% have been shown to express melanocortin 4 receptor (MC4-R) mRNA (12). Furthermore, the MC4-R knockout (KO) mouse retains significant responses to centrally administered AGRP (13). Thus, the possibility that AGRP might exert its actions on the HPT axis by a receptor other than the MC4-R must be considered.

To determine the importance of the MC4-R in mediating the inhibitory effects of AGRP on the HPT axis, we compared

Abbreviations: aCSF, Artificial cerebrospinal fluid; AGRP, agouti-related protein; GABA, γ-aminobutyric acid; HPT, hypothalamic-pituitary-thyroid; KO, knockout; MC4-R, melanocortin 4 receptor; PVN, paraventricular nucleus; WT, wild-type.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from endo.endojournals.org on February 23, 2005

the effects of central AGRP administration on circulating levels of T_4, as well as the relative expression of TRH mRNA in hypophysiotropic neurons in the PVN of wild-type (WT) and MC4-R KO mice.

Materials and Methods

Animals

The experiments were carried out on adult, male, WT (C57BL/6J) and MC4-R KO mice (C57BL/6J background) (14). The animals were housed individually in cages under standard environmental conditions (light between 0600 and 1800 h, temperature 22 ± 1 C, mouse chow and water *ad libitum*). All experimental protocols were reviewed and approved by the Animal Welfare Committee at Tufts-New England Medical Center and Oregon Health and Science University.

AGRP infusion

The WT and MC4-R KO mice were implanted with 25-gauge stainless steel guide cannula (Small Parts Inc., Miami Lakes, FL) into the lateral cerebral ventricle under stereotaxic control using a Cartesian stereotaxic apparatus (coordinates from Bregma anteroventral −0.2; lateral 1.0; dorsoventral 2.0) through a burr hole in the skull. The cannula was secured to the skull with Crazy Glue (Electron Microscopy Sciences, Fort Washington, PA) and dental cement, and temporarily occluded with a dummy cannula. Bacitracin ointment was applied to the interface of the cement and the skin. Animals were weighed daily and any animal showing signs of illness or weight loss was removed from the study and euthanized. One week after icv cannulation, both WT and MC4-R KO mice were divided into two groups. In the first group, WT (n = 8) and MC4-R KO (n = 9) mice were injected icv with 5 μl artificial cerebrospinal fluid (aCSF) [140 mM NaCl, 3.35 mM KCl, 1.15 mM $MgCl_2$, 1.26 mM Ca Cl_2, 1.2 mM Na_2HPO_4, 0.3 mM NaH_2PO_4 (pH 7.4)] containing 0.05% BSA every day between 1400 and 1600 h for 3 d. In the second group, WT (n = 8) and MC4-R KO (n = 10) groups were injected icv with 2.5 μg AGRP (Phoenix Pharmaceuticals Inc., Belmont, CA) in 5 μl aCSF every day between 1400 and 1600 h for 3 d. All icv injections were made through a 31-gauge needle that extended 0.5 mm below the guide cannula, connected by durametervinyl tubing (Scientific Commodities Inc., Lake Havasu City, AZ) to a 50-μl Hamilton syringe and infused over 2 min. Animals were weighed and food intake was assessed daily. On the fourth day of the experiment between 0900 and 1200 h, the animals were anesthetized with sodium pentobarbital, blood was taken from inferior vena cava for measurement of serum T_4, and the animals immediately perfused with fixative as described below. Blood was collected into polypropylene tubes, centrifuged for 15 min at 4000 rpm, and the plasma stored at −80 C until assayed. Brown adipose tissue was carefully dissected from the interscapular area and weighed.

Tissue preparation for in situ hybridization histochemistry

Under sodium pentobarbital anesthesia, the animals were perfused transcardially with 10 ml 0.01 M PBS (pH 7.4), containing 15,000 U/liter heparin sulfate followed by 40 ml 4% paraformaldehyde in PBS. The brains were removed and postfixed by immersion in the same fixative for 2 h at room temperature. Tissue blocks containing the hypothalamus were cryoprotected in 20% sucrose in PBS at 4 C overnight, then snap frozen on dry ice. Serial 18-μm-thick coronal sections through the rostrocaudal extent of the PVN were cut on a cryostat (Leica CM3050 S, Leica Microsystems, Nussloch GmbH, Germany) and adhered to Superfrost/Plus glass slides (Fisher Scientific Co., Pittsburgh, PA) to obtain three sets of slides, each set containing every third section through the PVN. Cannula placement was confirmed by light microscopic examination. The tissue sections were desiccated overnight at 42 C and stored at −80 C until prepared for *in situ* hybridization histochemistry.

In situ hybridization histochemistry

Every third section of the PVN was hybridized with a 265-base single-stranded [^{35}S]UTP-labeled cRNA probe for mouse proTRH (gift from Dr. Satoh, Gunma University School of Medicine, Maebashi, Japan) following methods as previously described (15, 16). The hybridization was performed under plastic coverslips in a buffer containing 50% formamide, a 2-fold concentration of standard sodium citrate (2× SSC), 10% dextran sulfate, 0.5% sodium dodecyl sulfate, 250 μg/ml denatured salmon sperm DNA, and 6×10^5 cpm of radiolabeled probe for 16 h at 56 C. Slides were dipped into Kodak NTB2 autoradiography emulsion (Eastman Kodak, Rochester, NY), and the autoradiograms were developed after 4 d of exposure at 4 C. The specificity of hybridization was confirmed using sense probes which resulted in the absence of specific hybridization in the PVN.

Image analysis

Autoradiograms were visualized under dark-field illumination using a COHU 4910 video camera (COHU, Inc., San Diego, CA). The images were captured with a color PCI frame grabber board (Scion Corp., Frederick, MD) and analyzed with a Macintosh G4 computer using Scion *Image*. Background density points were removed by thresholding the image and integrated density values (density × area) of hybridized neurons in the same region of each side of the PVN were measured in four consecutive sections on each side of the PVN for each animal. Nonlinearity of radioactivity in the emulsion was evaluated by comparing density values with a calibration curve created from autoradiograms of known dilutions of the radiolabeled probes immobilized on glass slides in 2% gelatin fixed with 4% formaldehyde and exposed and developed simultaneously with the *in situ* hybridization autoradiograms.

Hormone measurements

Plasma T_4 and TSH concentrations were measured by RIA. Plasma T_4 levels were measured with specific RIA using antiserum from Ventrex (Portland, ME) and [^{125}I]-labeled T_4 obtained from NEN Life Science Products (Boston, MA). The details of the assay have been reported previously (17). The Cobra 500 program was used for data reduction and calculation of the RIA results. The mouse TSH assay was performed using the rat TSH assay kit RPA 554 obtained from GE Healthcare (formerly Amersham Bioscience, Piscataway NJ). Sera from hypothyroid, methimazole-treated mice contained 14.86 ng/ml TSH. Observed TSH concentrations for the hypothyroid sera and its next three 1:1 serial dilutions (1/2, 1/4, and 1/8) were linear. The value for TSH was within 4% or less of the value predicted by the degree of dilution of the hypothyroid sera using the equation Value = 1 − [(0.743 − Observed Value)*(0.743)]. This equation, therefore, was used to obtain the values for the mouse serum samples in the RPA 554 rat TSH assay.

Statistical analysis

Results are presented as means and SES of the mean (SEM). The data were analyzed using one-way ANOVA with linear contrasts for the individual comparisons. SPSS version 11.5 (SPSS Inc., Chicago, IL) was used for the analysis with P values less than 0.05 considered statistically significant.

Results

Effects of central AGRP administration on food intake, body weight, and fat pad weight of WT and MC4-R KO mice

AGRP-treated WT mice consumed significantly more food and gained more weight than aCSF-treated WT animals over the duration of the study (Table 1). In WT mice, AGRP administration also increased the weight of epididymal white fat and interscapular brown adipose tissue. Control MC4-R KO mice were heavier, and had a significantly larger epididymal fat pad, but had similar size brown adipose tissue as the aCSF-treated WT mice. A tendency for increased food consumption was observed in the MC4-R KO mice relative to control WT mice during the experiment, but the increase in food intake was not significant. In contrast to the WT animals, AGRP treatment had no significant effect on food intake, weight gain, and the weight of epididymal white

Downloaded from endo.endojournals.org on February 23, 2005

TABLE 1. Effects of central AGRP administration on food intake, body weight, brown adipose tissue and epididymal fat pad weight, and plasma thyroid hormone levels of WT and MC4-R KO mice

	WT control (n = 8)	WT AGRP (n = 8)	MC4-R KO Control (n = 9)	MC4-R KO AGRP (n = 9)	WT control *vs.* WT AGRP *P* value	MC4-R KO control *vs.* MC4-R KO AGRP *P* value
Cumulative food intake	7.95 ± 0.71	10.15 ± 0.18	9.47 ± 0.65	10.67 ± 0.65	0.017	0.16
Body weight gain	−0.3 ± 0.26	1.49 ± 0.17	−0.79 ± 0.34	−0.34 ± 0.30	<0.001	0.26
Epididymal white fat normalized to weight	9.70 ± 0.53	11.97 ± 0.72	24.61 ± 4.12	18.86 ± 1.56	0.02	0.22
Brown adipose tissue normalized to weight	3.55 ± 0.31	6.24 ± 0.31	3.19 ± 0.28	4.10 ± 0.39	<0.001	0.051
T_4 (μg/dl)	4.55 ± 0.16	3.87 ± 0.21	4.84 ± 0.27	4.31 ± 0.09	0.024	0.057
TSH (ng/ml)	2.31 ± 0.13	2.56 ± 0.15	2.40 ± 0.15	2.62 ± 0.14	0.46	0.54
TRH mRNA (integrated density units)	4.65 ± 0.50	2.46 ± 0.17	4.21 ± 0.41	3.45 ± 0.35	<0.001	0.15

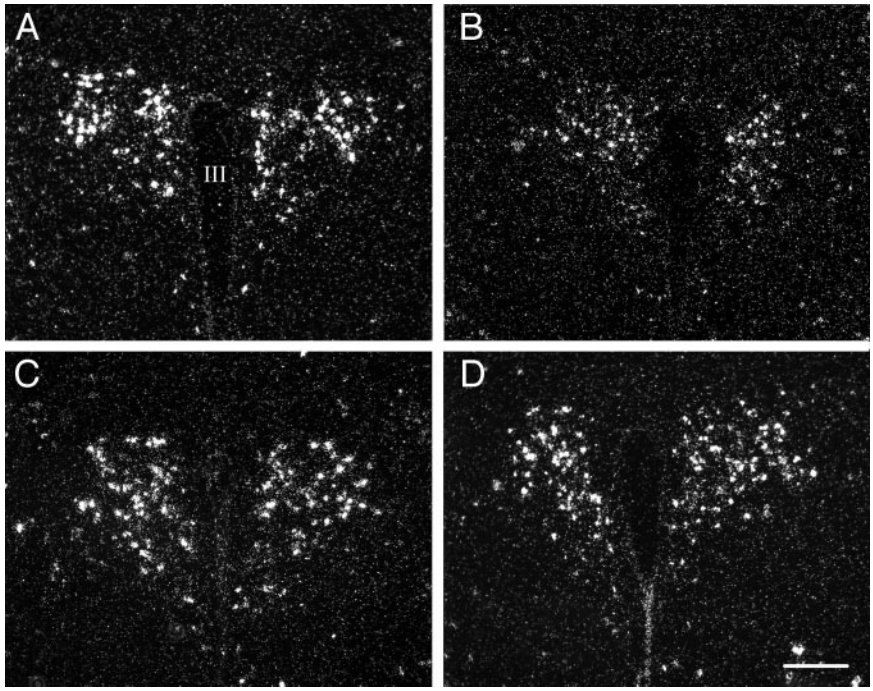

FIG. 1. Dark-field illumination photomicrographs of proTRH mRNA in the periventricular and medial parvocellular subdivisions of the hypothalamic PVN in aCSF-treated WT (A), AGRP-treated WT (B), aCSF-treated MC4-R KO (C), and AGRP-treated MC4-R KO (D) mice. Note the marked reduction in silver grains over neurons of the PVN in AGRP-treated WT mice, whereas the density of silver grains is unchanged in the AGRP-treated MC4-R KO mice. III, Third ventricle. *Scale bar*, 200 μm.

fat in MC4-R KO mice. However, a tendency for increased weight of brown adipose tissue was noticed in these animals.

Effect of central AGRP treatment on the hypothalamic-pituitary-thyroid axis of WT and MC4-R KO mice

In aCSF-treated WT mice, neurons containing proTRH mRNA were readily visualized by *in situ* hybridization histochemistry, symmetrically distributed in the medial and periventricular parvocellular subdivisions of the PVN on either side of the third ventricle (Fig. 1A). AGRP administration reduced hybridization signal over these neurons (Fig. 1B), which when analyzed semiquantitatively was approximately 50% of control values (Fig. 2). Administration of AGRP to WT animals resulted in a significant reduction in circulating levels of T_4 but did not change TSH levels (Table 1).

TRH mRNA in MC4-R KO animals was also readily visualized in the PVN (Fig. 1C). Semiquantitative analyses demonstrated the TRH mRNA levels of aCSF-treated WT and MC4-R KO animals were not significantly different (Fig. 2). Circulating T_4 and TSH levels were also similar in WT controls and MC4-R KO animals receiving aCSF (Table 1). In contrast with WT animals, however, the central administration of AGRP did not reduce proTRH mRNA in the PVN of

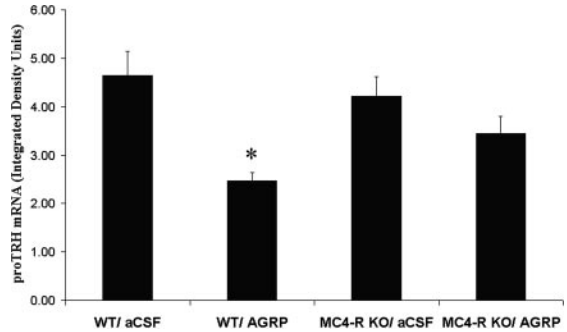

FIG. 2. Computerized image analysis of proTRH mRNA content in the PVN of aCSF-treated WT, AGRP-treated WT, aCSF-treated MC4-R KO and AGRP-treated MC4-R KO mice. *, Statistically different from aCSF-treated animals ($P < 0.05$, Newman-Keuls test).

Downloaded from endo.endojournals.org on February 23, 2005

MC4-R KO mice (Figs. 1D and 2). AGRP also had no significant effect on TSH and thyroid hormone levels in MC4-R KO mice, although a tendency for a decrease in T_4 level was observed (Table 1).

Discussion

Previous studies from our laboratories have demonstrated that AGRP-immunoreactive axons densely innervates hypophysiotropic TRH neurons in the PVN (10, 11), and that the exogenous administration of AGRP markedly inhibits the HPT axis in rats (9). Because fasting up-regulates the synthesis of AGRP in the arcuate nucleus neurons (18), the only source for AGRP-containing axons in the brain (19), we have proposed that AGRP is an important mediator of the inhibitory effects of fasting on the HPT axis (9).

AGRP is known to exert its central effects as an endogenous antagonist or inverse agonist at melanocortin receptors, including MC-4 and MC-3 receptors. Both receptor subtypes are believed to be involved in the regulation of energy homeostasis (14, 20). Targeted disruption of MC4-R in mice results in an obesity syndrome associated with hyperphagia, hyperinsulinemia, and increased linear growth (14), resembling the obesity syndrome of agouti mice (21), and a similar syndrome has also been observed in humans with mutations of the MC4-R (22).

In accordance with previous reports (14, 23), the MC4-R KO mice were heavier and had increased fat deposition compared with WT animals. Interestingly, however, although a tendency for hyperphagia was observed in the MC4-R KO mice, the increase of food intake was not significantly different in these animals from WT controls. Similar findings were published by Butler *et al.* (23), who observed significantly increased food intake in MC4-R KO mice only on high-fat diet. Food intake of MC4-R KO mice maintained on a standard low-fat diet did not differ from that of WT animals (23).

Whereas the MC4-R is abundantly expressed in the PVN (24, 25), only approximately 48% of TRH neurons in the PVN express this receptor (12). Nevertheless, by double-labeling anatomical studies (10, 11), all TRH neurons in the PVN are contacted by axon terminals containing AGRP, raising the possibility that AGRP might exert its effect on the HPT axis via more than one receptor.

Another melanocortin receptor, the MC3-R, is also present in the PVN (26). Because AGRP acts as an antagonist at both MC4- and MC3-receptors (8) and the MC3 receptor is involved in the regulation of energy homeostasis (20), AGRP may also influence the HPT axis by binding to the MC3-R. In addition, the observation that long-term effects of AGRP on food intake do not chronically block α-MSH action (27), further raises the possibility that AGRP may act on receptors other than the melanocortin receptors. Structural similarities between the C-terminal, biologically active region of the AGRP molecule with ω-agatoxin IVB, a spider venom toxin with P-type Ca^{2+}-channel blocking properties (28), has raised the additional possibility that AGRP may inhibit Ca^{2+}-currents analogous to the effects of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), acting on the $GABA_B$ receptor (28).

To determine the importance of the MC4-R in mediating the actions of AGRP on the HPT axis, we determined the effect of the central administration of synthetic AGRP on metabolic parameters and thyroid function in mice with targeted deletion of the MC4-R. Daily administration of AGRP into the lateral cerebral ventricle of WT mice significantly increased food intake, weight gain, accumulation of white and brown adipose, lowered circulating levels of T_4 but had no significant effects on TSH, and suppressed proTRH mRNA levels in neurons in the hypothalamic PVN. These responses are similar to that previously observed in the rat (28), suggesting the importance of this response across animal species. Observations by Mihály *et al.* (29) that AGRP is expressed in the hypothalamic infundibular nucleus in man, the homolog of the arcuate nucleus, and that TRH neurons in the PVN are densely innervated by AGRP, suggest that this response may also be important in man.

In contrast with observations in WT mice, AGRP administration to MC4-R KO mice had little effect on most of the parameters studied. Similar observations were observed by other groups when they used a comparable dose of AGRP (13). In particular, proTRH mRNA levels in the PVN in AGRP-treated MC4-R KO mice were not significantly different than aCSF-treated MC4-R KO mice. These data suggest that the MC4-R is of critical importance in mediating the effects of AGRP on hypophysiotropic TRH gene expression. Thus, we presume that either a greater percentage of proTRH neurons in the PVN express MC4-R than reported (12), or the effect of AGRP on TRH neurons via MC4-R are mediated both directly and indirectly. Indirect effects may be mediated through ultrashort loops among TRH neurons in the PVN (30), or possibly through monosynaptic projections from neurons in other regions of the brain. The hypothalamic dorsomedial nucleus, for example, is heavily innervated by axon terminals containing α-MSH and AGRP (19, 31), expresses MC4-R (25), and projects heavily to TRH neurons in the PVN (32). An indirect effect of AGRP may also be mediated by neurons of the arcuate nucleus, lateral hypothalamus and PVN that express c-*fos* after AGRP administration (33).

Whereas circulating T_4 levels were not significantly different in MC4-R KO animals treated with AGRP, compared with MC4-R KO animals treated with aCSF, the mean T_4 level was lower after AGRP treatment and closely approached statistical significance. Therefore, we cannot exclude with certainty the possibility that with a larger sample size, AGRP might be at least partially effective in lowering thyroid hormone levels. Given that proTRH mRNA was not affected by AGRP in MC4-R KO animals, any inhibitory effect of AGRP would have to be exerted downstream of hypophysiotropic TRH gene expression. One possibility to consider is that melanocortin signaling at the MC3-R exerts posttranslational effects on hypophysiotropic TRH neurons that are sufficient to sustain normal levels of TRH in these animals. Kim *et al.* (34), however, have demonstrated that γ_2-MSH, a selective MC3-R agonist, is inhibitory to TRH release *in vitro*. Thus, were the MC3-R to have an important role in the regulation of hypophysiotropic TRH, one might have expected activation of the HPT axis after AGRP as a result of antagonism of this receptor. Of interest, anterior pituitary cells have been

Downloaded from endo.endojournals.org on February 23, 2005

reported to express the MC3-R (35), raising the possibility that AGRP may exert inhibitory effect on the thyrotrophs via the MC3-R. Alternatively, because AGRP stimulates the hypothalamic-pituitary-adrenal axis through a mechanism that is presumed other than action on MC4-R (36), and corticosterone can inhibit TSH secretion (37), AGRP may have yet other mechanisms of action to inhibit thyroid hormone levels. No significant reduction in TSH levels were observed in either the WT or MC4-R KO mice after AGRP infusion, however, although the absence of a rise in TSH in WT AGRP-infused mice when circulating T_4 levels were low could be interpreted as inappropriately normal.

Because AGRP would appear to have an important effect in modulating hypophysiotropic TRH primarily through the MC4-R, and the MC4-R agonist, α-MSH, exerts potent stimulatory effects on the HPT axis, one might have predicted that the MC4-R KO mice would have abnormalities in thyroid function. Because AGRP inhibits the HPT axis of *ad libitum*-fed WT animals, it is reasonable to hypothesize that stimulation of melanocortin receptors either by α-MSH (10) or by constitutive activation of melanocortin receptors (38–40) when AGRP secretion is inhibited, may be important to establish normal circulating levels of thyroid hormone. Thus, lack of MC4-R might be expected to result in inhibition of the HPT axis. Nevertheless, proTRH mRNA levels in the PVN of MC4-R KO mice and circulating levels of T_4 were comparable with that of the control mice. One must assume, therefore, that to maintain normal thyroid function, MC4-R KO mice might evoke one or more compensatory mechanisms that allow activation of the HPT axis. Because the administration of CART to fasted animals prevents fasting-induced inhibition of the HPT axis (41), up-regulation of CART may be involved in this compensatory mechanism (42). Other possibilities to consider include GABA, galanin, and catecholamines, because these neurotransmitter systems are involved in the regulation of energy metabolism and directly innervate the hypophysiotropic TRH neurons (43–48). Elevated circulating levels of leptin in the MC4-R KO mice (14) might also contribute to this compensatory response. Whereas most of the effects of leptin to regulate the HPT axis may be mediated through its effects on α-MSH and AGRP-synthesizing neurons in the hypothalamic arcuate nucleus (6), recent evidence by Huo *et al.* (49) and Guo *et al.* (50), have provided data to suggest a direct action of leptin on at least a subpopulation of TRH neurons in the PVN.

In summary, these data indicate that AGRP has a similar inhibitory effect in mice as in rats. Furthermore, this effect is primarily mediated through MC4-R. Consequently, MC3-R or other putative AGRP receptors may play only a minor role in regulation of the HPT axis by AGRP.

Acknowledgments

We thank Dr. Antonio C. Bianco of the Thyroid Unit, Brigham and Women's Hospital, Boston, Massachusetts, for the gift of sera from hypothyroid mice and assistance regarding the assay of mouse TSH using the RPA 554 rat TSH assay.

Received April 14, 2004. Accepted July 8, 2004.

Address all correspondence and requests for reprints to: Ronald M. Lechan, M.D., Ph.D, Professor of Medicine, Division of Endocrinology, Box No. 268, Tufts-New England Medical Center, 750 Washington Street, Boston, Massachusetts 02111. E-mail: rlechan@tufts-nemc.org.

This work was supported by National Institutes of Health Grants DK-37021, DK062207, DK51730, and Sixth EU Program (LSHM-CT-2003-503041).

References

1. **de Jesus LA, Carvalho SD, Ribeiro MO, Schneider M, Kim SW, Harney JW, Larsen PR, Bianco** 2001 The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. J Clin Invest 108:1379–1385
2. **Lanni A, Moreno M, Lombardi A, de Lange P, Goglia F** 2001 Control of energy metabolism by iodothyronines. J Endocrinol Invest 24:897–913
3. **Légrádi G, Emerson CH, Ahima RS, Flier JS, Lechan RM** 1997 Leptin prevents fasting-induced suppression of prothyrotropin-releasing hormone messenger ribonucleic acid in neurons of the hypothalamic paraventricular nucleus. Endocrinology 138:2569–2576
4. **Rondeel JM, Heide R, de Greef WJ, van Toor H, van Haasteren GA, Klootwijk W, Visser TJ** 1992 Effect of starvation and subsequent refeeding on thyroid function and release of hypothalamic thyrotropin-releasing hormone. Neuroendocrinology 56:348–353
5. **van Haasteren GA, Linkels E, Klootwijk W, van Toor H, Rondeel JM, Themmen AP, de Jong FH, Valentijn K, Vaudry H, Bauer K, Visser TJ, de Greef WJ** 1995 Starvation-induced changes in the hypothalamic content of prothyrotrophin-releasing hormone (proTRH) mRNA and the hypothalamic release of proTRH-derived peptides: role of the adrenal gland. J Endocrinol 145:143–153
6. **Légrádi G, Emerson CH, Ahima RS, Rand WM, Flier JS, Lechan RM** 1998 Arcuate nucleus ablation prevents fasting-induced suppression of ProTRH mRNA in the hypothalamic paraventricular nucleus. Neuroendocrinology 68:89–97
7. **Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG** 2000 Central nervous system control of food intake. Nature 404:661–671
8. **Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS** 1997 Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 278:135–138
9. **Fekete C, Sarkar S, Rand WM, Harney JW, Emerson CH, Bianco AC, Lechan RM** 2002 Agouti-related protein (AGRP) has a central inhibitory action on the hypothalamic-pituitary-thyroid (HPT) axis; comparisons between the effect of AGRP and neuropeptide Y on energy homeostasis and the HPT axis. Endocrinology 143:3846–3853
10. **Fekete C, Légrádi G, Mihály E, Huang QH, Tatro JB, Rand WM, Emerson CH, Lechan RM** 2000 α-Melanocyte-stimulating hormone is contained in nerve terminals innervating thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and prevents fasting-induced suppression of prothyrotropin-releasing hormone gene expression. J Neurosci 20:1550–1558
11. **Légrádi G, Lechan RM** 1999 Agouti-related protein containing nerve terminals innervate thyrotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. Endocrinology 140:3643–3652
12. **Harris M, Aschkenasi C, Elias CF, Chandrankunnel A, Nillni EA, Bjoorbaek C, Elmquist JK, Flier JS, Hollenberg AN** 2001 Transcriptional regulation of the thyrotropin-releasing hormone gene by leptin and melanocortin signaling. J Clin Invest 107:111–120
13. **Marsh DJ, Miura GI, Yagaloff KA, Schwartz MW, Barsh GS, Palmiter RD** 1999 Effects of neuropeptide Y deficiency on hypothalamic agouti-related protein expression and responsiveness to melanocortin analogues. Brain Res 848:66–77
14. **Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F** 1997 Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88:131–141
15. **Dyess EM, Segerson TP, Liposits Z, Paull WK, Kaplan MM, Wu P, Jackson IM, Lechan RM** 1988 Triiodothyronine exerts direct cell-specific regulation of thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus. Endocrinology 123:2291–2297
16. **Kakucska I, Rand W, Lechan RM** 1992 Thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus is dependent upon feedback regulation by both triiodothyronine and thyroxine. Endocrinology 130:2845–2850
17. **Castro MI, Alex S, Young RA, Braverman LE, Emerson CH** 1986 Total and free serum thyroid hormone concentrations in fetal and adult pregnant and nonpregnant guinea pigs. Endocrinology 118:533–537
18. **Hahn TM, Breininger JF, Baskin DG, Schwartz MW** 1998 Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat Neurosci 1:271–272
19. **Broberger C, Johansen J, Johansson C, Schalling M, Hokfelt T** 1998 The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proc Natl Acad Sci USA 95:15043–15048
20. **Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA,**

Downloaded from endo.endojournals.org on February 23, 2005

Dekoning J, Baetscher M, Cone RD 2000 A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. Endocrinology 141: 3518–3521

21. **Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD** 1997 Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 385:165–168
22. **Hinney A, Schmidt A, Nottebom K, Heibult O, Becker I, Ziegler A, Gerber G, Sina M, Gorg T, Mayer H, Siegfried W, Fichter M, Remschmidt H, Hebebrand J** 1999 Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. J Clin Endocrinol Metab 84:1483–1486
23. **Butler AA, Cone RD** 2003 Knockout studies defining different roles for melanocortin receptors in energy homeostasis. Ann NY Acad Sci 994:240–245
24. **Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD** 1994 Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol 8:1298–1308
25. **Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB, Elmquist JK** 2003 Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. J Comp Neurol 457:213–235
26. **Iqbal J, Pompolo S, Dumont LM, Wu CS, Mountjoy KG, Henry BA, Clarke IJ** 2001 Long-term alterations in body weight do not affect the expression of melanocortin receptor-3 and -4 mRNA in the ovine hypothalamus. Neuroscience 105:931–940
27. **Hagan MM, Rushing PA, Pritchard LM, Schwartz MW, Strack AM, Van Der Ploeg LH, Woods SC, Seeley RJ** 2000 Long-term orexigenic effects of AgRP-(83–132) involve mechanisms other than melanocortin receptor blockade. Am J Physiol Regul Integr Comp Physiol 279:R47–R52
28. **Bures EJ, Hui JO, Young Y, Chow DT, Katta V, Rohde MF, Zeni L, Rosenfeld RD, Stark KL, Haniu M** 1998 Determination of disulfide structure in agouti-related protein (AGRP) by stepwise reduction and alkylation. Biochemistry 37:12172–12177
29. **Mihály E, Fekete C, Tatro JB, Liposits Z, Stopa EG, Lechan RM** 2000 Hypophysiotropic thyrotropin-releasing hormone-synthesizing neurons in the human hypothalamus are innervated by neuropeptide Y, agouti-related protein, and α-melanocyte-stimulating hormone. J Clin Endocrinol Metab 85: 2596–2603
30. **Toni R, Jackson IM, Lechan RM** 1990 Thyrotropin-releasing-hormone-immunoreactive innervation of thyrotropin-releasing-hormone-tuberoinfundibular neurons in rat hypothalamus: anatomical basis to suggest ultrashort feedback regulation. Neuroendocrinology 52:422–428
31. **Jacobowitz DM, TL OD** 1978 α-Melanocyte stimulating hormone: immunohistochemical identification and mapping in neurons of rat brain. Proc Natl Acad Sci USA 75:6300–6304
32. **Mihály E, Fekete C, Légrádi G, Lechan RM** 2001 Hypothalamic dorsomedial nucleus neurons innervate thyrotropin-releasing hormone-synthesizing neurons in the paraventricular nucleus. Brain Res 891:20–31
33. **Zheng H, Corkern MM, Crousillac SM, Patterson LM, Phifer CB, Berthoud HR** 2002 Neurochemical phenotype of hypothalamic neurons showing Fos expression 23 h after intracranial AgRP. Am J Physiol Regul Integr Comp Physiol 282:R1773–R17781
34. **Kim MS, Small CJ, Russell SH, Morgan DG, Abbott CR, alAhmed SH, Hay DL, Ghati MA, Smith SM, Bloom SR** 2002 Effects of melanocortin receptor ligands on thyrotropin-releasing hormone release: evidence for the differential roles of melanocortin 3 and 4 receptors. J Neuroendocrinol 14:276–282
35. **Matsumura R, Takagi C, Kakeya T, Okuda K, Takeuchi S, Takahashi S** 2003 α-Melanocyte-stimulating hormone stimulates prolactin secretion through melanocortin-3 receptors expressed in mammotropes in the mouse pituitary. Neuroendocrinology 78:96–104
36. **Dhillo WS, Small CJ, Seal LJ, Kim MS, Stanley SA, Murphy KG, Ghatei MA, Bloom SR** 2002 The hypothalamic melanocortin system stimulates the hypothalamo-pituitary-adrenal axis in vitro and in vivo in male rats. Neuroendocrinology 75:209–216
37. **Samuels MH, McDaniel PA** 1997 Thyrotropin levels during hydrocortisone infusions that mimic fasting-induced cortisol elevations: a clinical research center study. J Clin Endocrinol Metab 82:3700–3704
38. **Holst B, Schwartz TW, Adan RA, Kas MJ, Nijenhuis WA, Oosterom J** 2003 Molecular mechanism of agonism and inverse agonism in the melanocortin receptors: Zn^{2+} as a structural and functional probe. Ann NY Acad Sci 994:1–11
39. **Adan RA, Kas MJ, Nijenhuis WA, Oosterom J** 2003 Inverse agonism gains weight. AgRP(83–132) acts as an inverse agonist on the human-melanocortin-4 receptor. Trends Pharmacol Sci 24:315–321
40. **Nijenhuis WA, Oosterom J, Adan RA** 2001 AgRP(83–132) acts as an inverse agonist on the human-melanocortin-4 receptor. Mol Endocrinol 15:164–171
41. **Fekete C, Mihály M, Luo LG, Kelly J, Clausen JT, Mao Q, Rand WM, Moss LG, Kuhar M, Emerson CH, Jackson IMD, Lechan RM** 2000 Association of CART-immunoreactive elements with thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and its role in the regulation of the hypothalamic-pituitary-thyroid axis during fasting. J Neurosci 20:9224–9234
42. **Arens J, Moar KM, Eiden S, Weide K, Schmidt I, Mercer JG, Simon E, Korf HW** 2003 Age-dependent hypothalamic expression of neuropeptides in wild-type and melanocortin-4 receptor-deficient mice. Physiol Genom 16:38–46
43. **Fekete C, Wittmann G, Liposits Z, Lechan RM** 2002 GABA-ergic innervation of thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus of the rat. Brain Res 957:251–258
44. **Liposits Z, Paull WK, Wu P, Jackson IM, Lechan RM** 1987 Hypophysiotrophic thyrotropin releasing hormone (TRH) synthesizing neurons. Ultrastructure, adrenergic innervation and putative transmitter action. Histochemistry 88: 1–10
45. **Wittmann G, Sarkar S, Hrabovszky E, Liposits Z, Lechan RM, Fekete C** 2004 Galanin- but not galanin-like peptide-containing axon terminals innervate hypophysiotropic TRH-synthesizing neurons in the hypothalamic paraventricular nucleus. Brain Res 1002:43–50
46. **Kyrkouli SE, Stanley BG, Leibowitz SF** 1986 Galanin: stimulation of feeding induced by medial hypothalamic injection of this novel peptide. Eur J Pharmacol 122:159–160
47. **Kelly J, Alheid GF, Newberg A, Grossman SP** 1977 GABA stimulation and blockade in the hypothalamus and midbrain: effects on feeding and locomotor activity. Pharmacol Biochem Behav 7:537–541
48. **Shor-Posner G, Azar AP, Jhanwar-Uniyal M, Filart R, Leibowitz SF** 1986 Destruction of noradrenergic innervation to the paraventricular nucleus: deficits in food intake, macronutrient selection, and compensatory eating after food deprivation. Pharmacol Biochem Behav 25:381–392
49. **Huo L, Munzberg H, Nillni EA, Bjorbaek C** 2004 Role of STAT3 in regulation of hypothalamic *trh* gene expression by leptin. Endocrinology 145:2516–2523
50. **Guo F, Bakal K, Minokoshi Y, Hollenberg AN, Huo L, Munzberg H, Nillni EA, Bjorbaek C** 2004 Leptin signaling targets the thyrotropin-releasing hormone gene promoter *in vivo.* Endocrinology 145:2221–2227

Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from endo.endojournals.org on February 23, 2005