0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(11):4692–4697

doi: 10.1210/en.2003-0440

Brainstem Melanocortin 3/4 Receptor Stimulation Increases Uncoupling Protein Gene Expression in Brown Fat

D. L. WILLIAMS, R. R. BOWERS, T. J. BARTNESS, J. M. KAPLAN, AND H. J. GRILL

Department of Psychology (D.L.W., J.M.K., H.J.G.), University of Pennsylvania, Philadelphia, Pennsylvania 19104; Molecular and Cellular Biology (R.R.B.), Medical University of South Carolina, Charleston, South Carolina 29425; and Department of Biology (T.J.B.), Georgia State University, Atlanta, Georgia 30303

Central administration of melanocortin 3 and 4 receptor (MC3/4-R) agonists increases energy expenditure, with the hypothalamus commonly held as the primary site of action. It is also clear, however, that MC4-R are expressed in caudal brainstem structures of relevance to the control of energy expenditure. Three experiments investigated whether hindbrain MC-R contribute to the energy expenditure effects of central MC3/4-R agonist treatments; in each, we examined the effect of fourth intracerebroventricular (icv) administration of a MC3/4-R agonist, MTII (three injections, each separated by 12 h), on uncoupling protein 1 (UCP-1) gene expression in brown adipose tissue (BAT). First, we compared the effects of fourth and third icv administration of MTII and found that the hindbrain and forebrain treatments were equally effective at elevating UCP-1 mRNA expression in BAT compared with the respective vehicle-treated group results. A second experiment demonstrated that the fourth icv MTII-induced rise in UCP-1 expression was mediated by sympathetic outflow to BAT by showing that this response was abolished by surgical denervation of BAT. In the third experiment, we showed that chronic decerebrate rats, like their neurologically intact controls, elevated UCP-1 mRNA expression in response to fourth icv MTII administration. Taken together, the results indicate that: 1) there is an independent caudal brainstem MC3/4-R trigger for a sympathetically stimulated elevation in BAT UCP-1 gene expression, and 2) the MTII-induced rise in UCP-1 expression can be mediated by circuitry intrinsic to the caudal brainstem and spinal cord. (*Endocrinology* 144: 4692–4697, 2003)

A GROWING BODY of evidence identifies the central melanocortin system as an important contributor to the regulation of energy balance through coordinated actions on both food intake and energy expenditure. Ingestive behavioral effects of melanocortin 3 and 4 receptor (MC3/4-R) ligands have been well characterized, with robust and long-lasting hyperphagia after intracerebroventricular (icv) administration of antagonists, and pronounced hypophagia after agonist treatment (*e.g.* Refs. 1 and 2). In addition, mice lacking functional MC4-R are hyperphagic and obese (3, 4), as are mice that transgenically overexpress the endogenous antagonist for these receptors, agouti-related protein (5). MC3/4-R activity also has implications for energy expenditure. The MC3-R and MC4-R knockout mice both exhibit abnormalities in energy expenditure that contribute to their obese phenotypes (4, 6, 7). Increases in oxygen consumption follow acute (8) or chronic (9) icv injection of the MC3/4-R agonist, MTII (melanotan II; Phoenix Pharmaceuticals, Belmont, CA). In addition, third icv administration of MTII increases the firing rate of sympathetic nerves innervating interscapular brown adipose tissue (BAT) (10) and increases core body temperature (11). These findings are consistent with the suggestion that a salient portion of the energy expenditure response to MTII is mediated by sympathetic stimulation of BAT thermogenesis.

Current models of melanocortinergic control of energy balance focus on hypothalamic nuclei. The arcuate nucleus (ARC) contains sets of neurons that express agouti-related protein, and those that express proopiomelanocortin (POMC, the precursor of the endogenous MC3/4-R ligand α-MSH). These neurons project to other hypothalamic structures such as the paraventricular nucleus (PVN) and dorsomedial nucleus, sites where microinjection of a MC3/4-R agonist inhibits food intake (12, 13). Some of the same hypothalamic nuclei also are considered critical for the energy expenditure responses to MC3/4-R stimulation. For example, neuroendocrine responses to hypothalamic MC3/4-R stimulation, such as increased thyroid-releasing hormone release by the PVN, may contribute to the increase in energy expenditure (14–16). In addition, sympathetic nerve activity, acknowledged as a major stimulus for BAT thermogenic activity, is thought to be influenced by neurons that express MC3/4-R in the PVN and by POMC neurons in the ARC via their direct projections to sympathetic preganglionic neurons in the intermediolateral cell column (IML) of the spinal cord (17, 18).

The case for a hypothalamic melanocortin contribution to the control of energy balance is strong, but there also is evidence that focuses attention on other MC3/4-R populations in the central nervous system. Of particular interest are caudal brainstem regions of clear relevance to ingestive behavior control (19), within which MC4-R are expressed at high density, including the dorsal vagal complex (DVC), parabrachial nucleus (PBN), and nucleus raphe pallidus

Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate nucleus; BAT, brown adipose tissue; DVC, dorsal vagal complex; EWAT, epididymal white adipose tissue; IBAT, interscapular BAT; icv, intracerebroventricular; IML, intermediolateral cell column; MC3/4-R, melanocortin 3 and 4 receptor; MTII, melanotan II; NE, norepinephrine; NS, not significant; NTS, nucleus of the solitary tract; PBN, parabrachial nucleus; POMC, proopiomelanocortin; PVN, paraventricular nucleus; RNAP, RNA polymerase; RPaicv, nucleus raphe pallidus; RWAT, retroperitoneal white adipose tissue; UCP-1, uncoupling protein 1.

(RPa) (18, 20). It is already clear that hindbrain MC-R populations are relevant for the control of ingestive behavior. For example, the food intake responses to fourth icv delivery of MTII and SHU9119 (a MC3/4-R antagonist) are as strong as those obtained upon lateral icv delivery (1). Moreover, injections of MTII or SHU9119 into the DVC or PBN induce marked feeding responses with doses below threshold for fourth icv effects (21, 22). The energy expenditure effects of caudal brainstem MC3/4-R treatments have not been evaluated but are plausible in light of the direct and indirect projections from MC-R-rich hindbrain structures to IML neurons, and the correlation between neural activity in areas such as the RPa with BAT thermogenesis (23–26). Thus, there is an anatomical basis for the suggestion that like hypothalamic MC-R populations, receptors in the hindbrain influence both food intake and sympathetic activation of BAT.

In the present study, we address two hypotheses concerning caudal brainstem MC-R involvement in energy balance: 1) that hindbrain MC3/4-R agonist treatment stimulates uncoupling protein 1 (UCP-1) gene expression in BAT; and 2) that this effect is mediated by a mechanism(s) intrinsic to the caudal brainstem (*i.e.* independent of hypothalamic involvement). We examined the effect of central MTII treatment on UCP-1 mRNA expression in BAT because UCP-1 is the primary effector of thermogenesis in BAT and is stimulated by sympathetic nerve activation (reviewed in Refs. 27 and 28). Up-regulation of UCP-1 in response to activation of MC3/4-R has not been directly examined, although such an effect would be expected, at least for hypothalamic treatment, based on the previous demonstration of MTII-induced elevation in the activity of sympathetic nerves innervating interscapular BAT (IBAT; 10). We affirm this expectation in rats receiving third icv injections of MTII, and further show a comparable increase in UCP-1 expression after fourth icv treatment. That this caudal brainstem-elicited response is indeed mediated by sympathetic efferents is demonstrated by our finding that denervation of IBAT completely blocks the UCP-1 mRNA response to fourth icv MTII treatment. The question of whether there is a hypothalamic contribution to the hindbrain-triggered response is addressed by an experiment in which MTII is delivered to the fourth ventricle of chronic decerebrate rats. An increase in UCP-1 mRNA expression in response to MTII administration in decerebrate rats would add weight to the hypothesis that elevations in energy expenditure can be triggered by caudal brainstem MC3/4-R and would further demonstrate that the production of this response can be mediated entirely by neural circuits within the hindbrain and spinal cord.

Materials and Methods

Subjects

Naive male Sprague Dawley rats (Charles River, Wilmington, MA; 350–450 g) were housed in hanging stainless steel cages in a vivarium under a 12-h light, 12-h dark cycle. Pelleted food (Purina, St. Louis, MO) and water were available *ad libitum* except where otherwise noted. The experimental protocols used conform to institutional standards of animal care and the Guide for the Care and Use of Laboratory Animals (National Research Council 1996).

Intracerebroventricular cannulation

Each rat received a guide cannula (Plastics One, Roanoke, VA; 26-gauge), implanted 2.0 mm above either the third or fourth ventricle, under ketamine (90 mg/kg), xylazine (2.7 mg/kg) and acepromazine (0.64 mg/kg) anesthesia administered by im injection. Third-ventricle cannula placement was 2.0 mm posterior to bregma, −7.0 mm ventral to dura, and on the midline. Fourth-ventricle cannula placement was 2.5 mm anterior to occipital suture, −4.5 mm ventral to dura, and on the midline. The cannula was cemented to four jeweler's screws attached to the skull, and closed with an obturator. Rats recovered for at least 5 d, during which daily food intake and body weight were recorded. Intracerebroventricular cannula placement was evaluated after recovery from surgery through the measurement of a sympathetically mediated increase in plasma glucose 60 min after icv injection of 210 μg of 5-thio-D-glucose in 2 μl of artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA) (29). Only rats that showed at least a doubling of plasma glucose level in response to this treatment were used in experiments.

Ribonuclease protection assay

To generate a riboprobe for use in ribonuclease protection assays (RPAs), a full-length rat cDNA encoding UCP (GenBank accession no. NM_012682) (30) was digested with *Hin*dIII (cuts at nucleotide 683) and *Bam*HI (cuts at nuceotide 1014) to yield a 331-bp gene fragment that was subcloned into the Topo pCRII cloning vector (Invitrogen, Carlsbad, CA). Thereafter, the SP6 and T7 RNAP promoters flanking the multiple cloning site of Topo pCRII were used in *in vitro* transcription reactions to prepare run-off cRNA transcripts. The cRNA transcribed by one RNAP (RNA polymerase) is the sense strand, whereas the cRNA produced by the other RNAP was radiolabeled with [^{32}P]CTP and used as a riboprobe in RPAs, essentially as described previously (31). Briefly, 2×10^4 cpm of ^{32}P-labeled riboprobe was coprecipitated with 0.5 μg of adipose tissue RNA. An 18S rRNA riboprobe was included as an internal control to ensure that equal amounts of RNA from each sample were used. After ethanol precipitation and suspension in hybridization buffer, RNA was denatured at 80 C and hybridized at 55 C overnight. Unhybridized RNA was digested by adding 300 U of T1 ribonuclease to each sample and incubating at 37 C for 1 h, and the reaction was stopped with 5 mM EDTA. After precipitation, protected double-stranded RNA was resuspended in dye and electrophoretically resolved on 6% denaturing polyacrylamide gels containing 8 M urea. The gels were exposed to film, and the autoradiographs were scanned with a densitometer. Known amounts of sense strand standards were included in each assay to allow quantification of protected mRNA. Because the densitometric area of the autoradiographic bands is a linear function of the amount of sense strand added, we were able to generate a standard curve to quantify the message protected in each lane.

Experiment 1

Design and procedures. Twenty-five rats were implanted with third or fourth icv cannulae as described above. Our injection schedule was modeled after that used by Kotz and colleagues (32). All rats received three injections over the course of 24 h, separated by 12-h intervals. Injections took place in the middle of the light or dark phase. Each subject was assigned to one of three groups, as follows: 1) aCSF, *ad libitum* access to food (fourth icv, n = 4; third icv, n = 4); 2) MTII, *ad libitum* access to food (fourth icv, n = 5; third icv, n = 5); and 3) aCSF, pair-fed to MTII-treated rats' intake (fourth icv, n = 4; third icv, n = 3). For weight-related dependent measures, the pair-fed group was included to distinguish between the effect of drug and the effect of reduced intake. MTII was prepared by dissolving the peptide in aCSF. Injections were administered through 28-G stainless steel tubing that extended 2 mm below the guide cannula. A 2-μl volume of drug or aCSF was injected over 2 min using a Hamilton microsyringe. The injector was left in place for another minute before removal. The dose of 0.33 nmol MTII/injection was chosen because of its efficacy at decreasing food intake (1, 33). Immediately before the third injection, food was removed from all subjects. Two hours after the third injection, rats were euthanized with CO_2 gas. Retroperitoneal and epididymal white adipose tissue (RWAT, EWAT) and IBAT was dissected and weighed. IBAT was preserved in RNAlater (Ambion, Austin, TX) for UCP-1 mRNA analysis (as described above).

Statistical analysis. Two-way ANOVA [site of injection (third *vs.* fourth icv) X treatment group (aCSF, MTII)] was used to evaluate effects on UCP-1 mRNA level. For RWAT weight, EWAT weight, food intake, and body weight change during the 24 h of the experiment, three treatment groups were compared in the ANOVA: aCSF, MTII, and aCSF pair fed.

Tukey's honestly significant difference method was used for *post hoc* comparisons where appropriate.

Experiment 2

BAT denervation. Unliateral denervation of IBAT was performed as previously described (34) in anesthetized animals. Briefly, an incision was made anterior to the IBAT and the tissue was separated from the underlying muscle by blunt dissection. The intercostal nerve bundles entering each side of the pad were visualized, and a section of each nerve bundle was removed on one side of the pad. Half of the subjects were denervated on the left, and the other half were denervated on the right side. During the same surgery, rats were implanted with fourth icv cannulas as described above.

Surgical denervation of BAT was verified by measuring norepinephrine (NE) content in BAT using reverse-phase HPLC with electrochemical detection essentially after our modification (35) of the method of Mefford (36). Briefly, half of each hemisphere of BAT was thawed and homogenized in a solution containing 20 ng of dihydroxybenzylamine (internal standard) in 0.2 M perchloric acid and 1 mg /ml ascorbic acid. After centrifugation, catecholamines were extracted from the homogenate with alumina and eluted into 0.2 M perchloric acid and 1 mg /ml ascorbic acid. Catecholamines were assayed using an ESA (Bedford, MA) HPLC system with electrochemical detection (Cuolochem II). The mobile phase was Cat-A-Phase II and the column was a C-18 reverse phase column. Results were analyzed offline and expressed as ng of NE/g of BAT.

Design and procedures. Rats were given three injections of aCSF (2 μl) or MTII (0.33 nmol in 2 μl aCSF) into the fourth ventricle, on the same schedule as that described above for experiment 1. All rats were given *ad libitum* access to food in this experiment. Two hours after the third injection, the animals were euthanized with CO_2 gas, and IBAT was dissected and weighed. Half of each hemisphere of IBAT was preserved in RNAlater (Ambion, Austin, TX) for UCP-1 mRNA analysis (as described above), and the other half was rapidly frozen with methylbutane and dry ice for HPLC.

Statistical analysis. The effects of surgical denervation and MTII treatment on UCP-1 mRNA level in IBAT were assessed via two-way ANOVA [denervation (intact *vs.* denervated BAT hemisphere) X treatment group (vehicle *vs.* drug)]. *Post hoc* comparisons were made using Bonferroni-adjusted *t* tests where appropriate.

Experiment 3

Decerebration surgery. Supracollicular decerebration was performed in two stages separated by at least 1 wk, as previously described (37). Neurologically intact control rats were implanted with fourth icv cannulae, and decerebrate rats received fourth icv cannulae during the second hemisection surgery. Animals recovered for at least 1 wk before the experiment. The completeness of the intended transection was verified histologically after the experiment.

Subject maintenance. All nutrition was supplied via four daily gastric intubations of 9 ml of liquid diet (1:1 Borden's sweetened condensed milk/water, with 1 ml Polyvisol with iron added per 600 ml of diet). This maintenance diet provides 76 kcal/d. Feedings were separated by intervals of at least 2 h. All subjects gained weight on this diet during the period of recovery from surgery, but there were no group differences (chronic decerebrate mean, 346.4 g; control mean, 357.1 g).

Design and procedures. Half of the chronic decerebrate (n = 12) and neurologically intact control (n = 12) groups were given three fourth icv injections of aCSF (2 μl), and the other half were given MTII (0.33 nmol in 2 μl aCSF). As for experiments 1 and 2, there were three injections over a 24-h period, separated by 12-h intervals. Rats were tube-fed as usual during the first 12 h between injections, but were not fed thereafter. Two hours after the final injection, rats were euthanized with CO_2 gas. RWAT, EWAT, and IBAT was dissected and weighed. IBAT was preserved in RNAlater (Ambion) for UCP-1 mRNA analysis (as described above).

Statistical analysis. Two-way ANOVA (decerebration X drug treatment) was used to evaluate effects on UCP-1 mRNA level, RWAT weight, and EWAT weight. Tukey's honestly significant difference method was used for *post hoc* comparisons where appropriate.

Results

Experiment 1

As shown in Fig. 1, central MTII treatment significantly elevated IBAT UCP-1 mRNA (see Fig. 2 for a representative autoradiograph). Statistical support for these conclusions is derived from the significant main effect of drug treatment [$F_{(1, 14)} = 14.34$, $P < 0.01$] and the *post hoc* comparisons revealing that MTII-treated rats had significantly higher UCP-1 mRNA levels than aCSF-treated *ad libitum*-fed rats ($P < 0.01$). The observed effect of MTII on UCP-1 expression did not depend on site of administration, as demonstrated by the lack of a main effect [$F_{(1, 14)} = 1.20$, not significant (NS)] or interaction [$F_{(1, 14)} = 0.72$, NS]).

RWAT and EWAT weights were significantly reduced in subjects with reduced daily intake due to pair-feeding or MTII treatment (see Table 1). There were significant main effects of treatment group on WAT weight [RWAT $F_{(2, 18)} = 5.81$, $P < 0.05$; EWAT $F_{(2, 19)} = 7.5$, $P < 0.01$], but in each case,

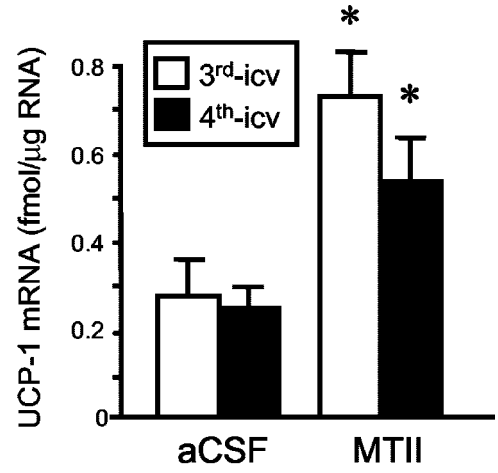

FIG. 1. UCP-1 mRNA expression (mean ± SE) in BAT after rats received third or fourth icv injections of either aCSF or MTII. *, Significant difference between aCSF and MTII treatment.

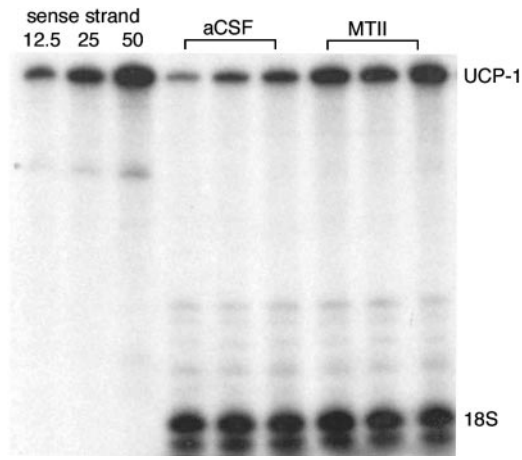

FIG. 2. Representative autoradiograph from experiment 1. UCP-1 mRNA is shown in the *upper bands* of the gel, and 18S rRNA is shown in the *lower bands*. Lanes 1–3 are UCP-1 mRNA sense strand, with 12.5 pg in lane 1, 25 pg in lane 2, and 50 pg in lane 3. Lanes 4–6 show RNA detected in BAT after third icv injections of aCSF. Lanes 7–9 show RNA detected after third icv MTII injections.

TABLE 1. Means (±SE) from experiment 1 for food intake, percentage of body weight change, and WAT depot weights for the three treatment groups, collapsed across fourth and third ventricle injection groups

	aCSF	MTII	Pair-fed
24-h Food intake (g)	24.03 (1.15)^a	12.37 (1.01)^b	12.33 (0.55)^b
% Body weight change	−0.73 (0.29)^a	−5.08 (0.60)^b	−5.84 (0.94)^b
RWAT weight (g)	4.30 (0.32)^a	2.70 (0.31)^b	2.96 (0.49)^b
EWAT weight (g)	4.54 (0.19)^a	3.53 (0.26)^b	3.31 (0.34)^b

Significant differences among groups are indicated by *differing superscript letters.*

the weights of the MTII-treated and vehicle-treated pair-fed groups were not distinguishable. Both MTII-treatment and pair-feeding significantly reduced WAT weight compared with vehicle treatment in *ad libitum*-fed rats (all $P < 0.05$). This finding should not be taken to indicate that central MTII treatment does not stimulate WAT lipolysis; more direct investigation of lipolysis in WAT would be required to make such a conclusion (see 38). The effects on WAT size did not depend on the ventricle injected [main effect of injection site: RWAT, $F_{(1, 18)} = 0.39$, NS; EWAT, $F_{(1, 19)} = 0.63$, NS].

In agreement with previous results (1), food intake was significantly reduced by MTII treatment [see Table 1; $F_{(1, 14)} = 62.83$, $P < 0.001$], with no effect of injection site [$F_{(1, 14)} = 1.22$, NS]. Rats with reduced intake due to MTII treatment or pair feeding lost a significant amount of body weight relative to aCSF-treated rats allowed to ingest *ad libitum* [$F_{(2, 19)} = 19.91$, $P < 0.001$, *post hoc* $P < 0.001$]. MTII injection did not reduce body weight to a greater degree than did pair feeding. Site of injection also had no influence on body weight.

Experiment 2

IBAT denervations were confirmed with HPLC as described above. Denervation of BAT reduced NE content by 83.4%, on average. Subjects were removed from the analysis if the NE content difference between intact and denervated BAT was less than 60% (only one such case was observed).

As shown in Fig. 3, MTII treatment increased UCP-1 mRNA expression only in the intact IBAT hemisphere [interaction between drug and denervation $F_{(1, 7)} = 8.60$, $P < 0.02$; *post hoc* comparisons: 1) intact IBAT, vehicle *vs.* MTII $P < 0.05$; 2) denervated IBAT, vehicle *vs.* MTII NS; 3) MTII-treated group, intact *vs.* denervated IBAT $P < 0.05$]. The results clearly indicate that the MTII-induced rise in UCP-1 mRNA expression is mediated by sympathetic nerve activity.

Experiment 3

Light microscopic analysis of cresyl violet-stained sagittal sections revealed that the transection was complete in all rats subject to two-stage decerebration. Figure 4 shows that UCP-1 mRNA was up-regulated by MTII treatment in both neurologically intact and decerebrate rats [$F_{(1, 20)} = 8.41$, $P < 0.01$]. Baseline UCP-1 mRNA expression was not altered by decerebration [$F_{(1, 20)} = 2.51$, NS]. Despite the apparent difference in the magnitude of the response between intact and decerebrate rats, there was no significant interaction between decerebration and drug treatment [$F_{(1, 20)} = 2.14$, NS]. Not surprisingly, in light of the continued gavage-feeding of all rats throughout the experiment, MTII treatment did not affect body weight or the size of either WAT depot examined (see Table 2).

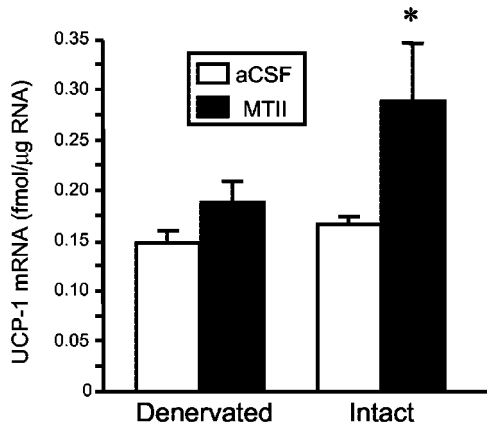

FIG. 3. UCP-1 mRNA expression in denervated or intact BAT after fourth icv treatment with aCSF or MTII. *, Significant difference between aCSF and MTII treatment.

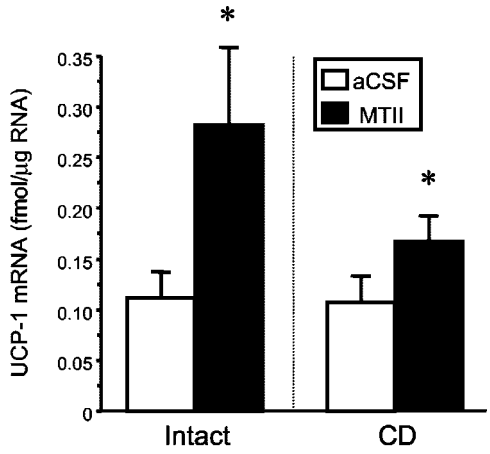

FIG. 4. UCP-1 mRNA expression in BAT after fourth icv treatment with aCSF or MTII. Results for neurologically intact rats are shown on the *left*, and those for chronic decerebrate (CD) rats are shown on the *right*. *, Significant difference between aCSF and MTII treatment.

Discussion

The present results establish that stimulation of central MC3/4-R increases UCP-1 expression in BAT. The sympathetic mediation of this effect is demonstrated by the abolition of the response to MTII in surgically denervated BAT. This result does not rule out involvement of endocrine factors (*e.g.* thyroid hormone) but does argue that sympathetic stimulation of BAT is necessary for the response. These findings are novel, although not unexpected.

The substantive contribution of the experiments reported here lies in expanding the range of MC-R that are potentially important for the control of energy expenditure beyond the

TABLE 2. Means (±SE) from experiment 3 for percentage of body weight change and WAT depot weights for the four treatment groups

	Controls		Decerebrates	
	aCSF	MTII	aCSF	MTII
% Body weight change	−0.49 (0.59)	−1.16 (0.52)	0.41 (0.34)	1.31 (0.61)
RWAT weight (g)	8.67 (0.57)	8.67 (0.63)	9.15 (0.65)	8.38 (0.57)
EWAT weight (g)	6.23 (0.46)	6.10 (0.58)	6.32 (0.30)	5.98 (0.44)

There were no significant differences between groups for these variables.

hypothalamus. The results are consistent with both stated hypotheses: that there is an independent trigger for the increase in UCP-1 mRNA after MC3/4-R stimulation within the caudal brainstem, and that this response can be mediated by circuitry contained entirely within the caudal brainstem and spinal cord.

The response to fourth icv administration of MTII seen in neurologically intact rats (experiments 1 and 2) supports the suggestion that MC-R in the hindbrain can activate sympathetic outflow to BAT. The fourth icv results in intact rats alone, however, do not address the extent of the central network involved in the mediation of the UCP-1 response. Indeed, one could hypothesize a hypothalamic contribution as part of a mechanism involving an ascending pathway driven by caudal brainstem MC3/4-R stimulation, hypothalamic integration, and a descending command to the sympathetic effectors. Such a long-loop arrangement is discounted in decerebrate rats with transections that eliminate all ascending projections to the hypothalamus, as well as any means for hypothalamic structures to directly engage sympathetic premotor neurons. MTII treatment did significantly elevate UCP-1 gene expression in decerebrate rats, and this effect was of appreciable magnitude (~50% increase) relative to those generally reported (*e.g.* Refs. 32 and 39). The UCP-1 response in decerebrates was not statistically distinguishable from that seen in intact controls (*i.e.* no group X treatment interaction), although the average effect size was smaller in decerebrates than in the controls. In any event, the demonstrable UCP-1 response in decerebrate rats, which was at least qualitatively similar to that observed in controls, affirms a caudal brainstem MC-R trigger for the response and, moreover, supports the hypothesis that there is mediating circuitry intrinsic to the brainstem and spinal cord through which changes in energy expenditure can be elicited.

The positive indications of a role for caudal brainstem MC-R in the control of energy expenditure do not discount potentially important hypothalamic contributions. Dual (or multiple) MC3/4-R triggers already have been demonstrated for feeding responses to MC3/4-R stimulation and antagonism, with ventricle-subthreshold doses of receptor ligands delivered to the hypothalamic (12) and caudal brainstem (21, 22) parenchyma, respectively, yielding significant and robust ingestive effects. We maintain a working hypothesis that energy expenditure responses are also subject to both hypothalamic and hindbrain melanocortinergic control. A multilevel control principle raises a number of issues for further investigation, including interactions between the relevant hypothalamic and brainstem structures, and sites of convergence for pathways linking the anatomically disparate MC-R triggers with the sympathetic effectors.

A better understanding of the brainstem contribution to melanocortinergic control of energy expenditure requires the localization of the MC-R that mediate the effects of fourth icv MTII administration. Although we think it unlikely, the possibility that the observed effects reflect diffusion of the agonist to receptors in the spinal cord has not been formally ruled out. The RPa is one candidate brainstem site of MTII action, given that pharmacological disinhibition of RPa neurons elicits increased firing of sympathetic nerves innervating BAT (20, 40, 41). MC4-R-bearing neurons in the nucleus of the solitary tract (NTS) may also give rise to thermogenic responses, either through direct projections to IML or indirectly, through projections to other nuclei within the hindbrain, such as the ventrolateral medulla (42). As is the case for the feeding response to MTII (21, 22), there may be more than one MC-R population in the caudal brainstem from which thermogenic responses can be initiated.

Although the present results reveal an effect of brainstem MC-R activation, they do not speak to the source of the endogenous ligand for the receptors stimulated by fourth icv infusion of MTII. There are only two known possibilities: POMC neurons in the ARC and those in the NTS (43–45). The ARC POMC neurons project to other areas of the hypothalamus and elsewhere in the forebrain, but they also project to the hindbrain and spinal cord (44). The POMC neurons of the NTS project primarily within the dorsal vagal complex and to other brainstem structures (46, 47). It is possible, then, that the MC-R stimulated by fourth icv treatments are normally supplied by POMC fibers originating from ARC, from NTS, or from both sites. A contribution of the NTS is an intriguing possibility, given that this region receives signals correlated with metabolic status that are known to influence energy expenditure (*e.g.* leptin) (48, 49). Very little, however, can be said about the POMC neurons in the NTS. Almost no information is available about colocalization of receptors and other neurotransmitters, or about the physiology of these neurons; and at this point, no functional role has been assigned to this potentially interesting POMC cell population. Considerably more information is available about the POMC neurons in ARC, which figure prominently in the contemporary model of central nervous system control of energy balance (*e.g.* Ref. 50). If ARC POMC cells can be implicated here, then there would be multiple pathways through which the ARC may regulate sympathetic outflow: a direct projection to IML; an indirect projection to IML via the PVN; and an indirect projection to IML via a caudal brainstem relay. The issue of ARC and NTS contributions to melanocortinergic control of energy expenditure, as well as multilevel interactions between receptor-containing structures, are important topics for future research.

Acknowledgments

Received April 9, 2003. Accepted July 23, 2003.

Address all correspondence and requests for reprints to: D. L. Williams, VA Puget Sound Health Care System, 1660 South Columbian Way, S-111-Endo, Seattle, Washington 98108. E-mail: dianalw@u.washington.edu.

This work was supported by National Science Foundation graduate research fellowship (to D.L.W.), NIH-DK35254 (to T.J.B.), NIH-DK-42284 (to J.M.K.), and NIH-DK-21397 (to H.J.G.).

References

1. **Grill HJ, Ginsberg AB, Seeley RJ, Kaplan JM** 1998 Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. J Neurosci 18:10128–10135
2. **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
3. **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
4. **Ste Marie L, Miura GI, Marsh DJ, Yagaloff K, Palmiter RD** 2000 A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors. Proc Natl Acad Sci USA 97:12339–12344
5. **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
6. **Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L, Metzger JM, Strack AM, Camacho RE, Mellin TN, Nunes CN, Min W, Fisher J, Gopal-Truter S, MacIntyre DE, Chen HY, Van Der Ploeg LH** 2000 Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat Genet 26:97–102
7. **Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, Baetscher M, Cone RD** 2000 A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. Endocrinology 141:3518–3521
8. **Hwa JJ, Ghibaudi L, Gao J, Parker EM** 2001 Central melanocortin system modulates energy intake and expenditure of obese and lean Zucker rats. Am J Physiol 281:R444–R451
9. **Jonsson L, Skarphedinsson JO, Skuladottir GV, Watanobe H, Schioth HB** 2002 Food conversion is transiently affected during 4-week chronic administration of melanocortin agonist and antagonist in rats. J Endocrinol 173:517–523
10. **Haynes WG, Morgan DA, Djalali A, Sivitz WI, Mark AL** 1999 Interactions between the melanocortin system and leptin in control of sympathetic nerve traffic. Hypertension 33:542–547
11. **Murphy B, Nunes CN, Ronan JJ, Hanaway M, Fairhurst AM, Mellin TN** 2000 Centrally administered MTII affects feeding, drinking, temperature, and activity in the Sprague-Dawley rat. J Appl Physiol 89:273–282
12. **Giraudo SQ, Billington CJ, Levine AS** 1998 Feeding effects of hypothalamic injection of melanocortin 4 receptor ligands. Brain Res 809:302–306
13. **Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DG, Small CJ, Edwards CM, Heath MM, Stanley SA, Seal LJ, Bhatti JR, Smith DM, Ghatei MA, Bloom SR** 2000 Hypothalamic localization of the feeding effect of agouti-related peptide and α-melanocyte-stimulating hormone. Diabetes 49:177–182
14. **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
15. **Kim MS, Small CJ, Russell SH, Morgan DG, Abbott CR, AlAhmed SH, Hay DL, Ghatei MA, Smith DM, 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
16. **Fekete C, Legradi G, Mihaly 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
17. **Elmquist JK** 2001 Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. Int J Obes Relat Metab Disord 25(Suppl 5):S78–S82
18. **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
19. **Grill HJ, Kaplan JM** 2002 The neuroanatomical axis for control of energy balance. Front Neuroendocrinol 23:2–40
20. **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
21. **Williams DL, Kaplan JM, Grill HJ** 2000 The role of the dorsal vagal complex and the vagus nerve in feeding effects of melanocortin-3/4 receptor stimulation. Endocrinology 141:1332–1337
22. **Grill HJ, Kaplan JM** 2001 Effects of urocortin, leptin and melanocortin receptor ligands delivered to the parabrachial nucleus. Appetite 37:140 (Abstract)
23. **Bamshad M, Song CK, Bartness TJ** 1999 CNS origins of the sympathetic nervous system outflow to brown adipose tissue. Am J Physiol 276:R1569–R1578
24. **Zhang YH, Lu J, Elmquist JK, Saper CB** 2000 Lipopolysaccharide activates specific populations of hypothalamic and brainstem neurons that project to the spinal cord. J Neurosci 20:6578–6586
25. **Morrison SF** 2001 Differential control of sympathetic outflow. Am J Physiol 281:R683–R698
26. **Morrison SF** 2001 Differential regulation of brown adipose and splanchnic sympathetic outflows in rat: roles of raphe and rostral ventrolateral medulla neurons. Clin Exp Pharmacol Physiol 28:138–143
27. **Lowell BB** 1998 Adaptive thermogenesis: turning on the heat. Curr Biol 8:R517–R520
28. **Lowell BB, Spiegelman BM** 2000 Towards a molecular understanding of adaptive thermogenesis. Nature 404:652–660
29. **Ritter RC, Slusser PG, Stone S** 1981 Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. Science 213:451–445
30. **Bouillaud F, Weissenbach J, Ricquier D** 1986 Complete cDNA-derived amino acid sequence of rat brown fat uncoupling protein. J Biol Chem 261:1487–1490
31. **Demas GE, Bowers RR, Bartness TJ, Gettys TW** 2000 Photoperiodic regulation of gene expression in brown and white adipose tissue of Siberian hamsters (*Phodopus sungorus*). Am J Physiol 282:R114–R121
32. **Kotz CM, Wang C, Levine AS, Billington CJ** 2002 Urocortin in the hypothalamic PVN increases leptin and affects uncoupling proteins-1 and -3 in rats. Am J Physiol 282:R546–R551
33. **Williams DL, Grill HJ, Weiss SM, Baird JP, Kaplan JM** 2002 Behavioral processes underlying the intake suppressive effects of melanocortin 3/4 receptor activation in the rat. Psychopharmacology 161:47–53
34. **Bartness TJ, Wade GN** 1984 Effects of interscapular brown adipose tissue denervation on body weight and energy metabolism in ovariectomized and estradiol-treated rats. Behav Neurosci 98:674–685
35. **Youngstrom TG, Bartness TJ** 1995 Catecholaminergic innervation of white adipose tissue in Siberian hamsters. Am J Physiol 268:R744–R751
36. **Mefford IN** 1981 Application of high performance liquid chromatography with electrochemical detection to neurochemical analysis: measurement of catecholamines, serotonin and metabolites in rat brain. J Neurosci Methods 3:207–224
37. **Grill HJ, Norgren R** 1978 Chronically decerebrate rats demonstrate satiation but not bait shyness. Science 201:267–269
38. **Obici S, Feng Z, Tan J, Liu L, Karkanias G, Rossetti L** 2001 Central melanocortin receptors regulate insulin action. J Clin Invest 108:1079–1085
39. **Commins S, Watson P, Levin N, Beiler R, Gettys T** 2000 Central leptin regulates the UCP1 and ob genes in brown and white adipose tissue via different betaadrenoceptor subtypes. J Biol Chem 275:33059–33067
40. **Morrison SF** 1999 RVLM and raphe differentially regulate sympathetic outflows to splanchnic and brown adipose tissue. Am J Physiol 276:R962–R973
41. **Morrison SF, Sved AF, Passerin AM** 1999 GABA-mediated inhibition of raphe pallidus neurons regulates sympathetic outflow to brown adipose tissue. Am J Physiol 276:R290–R297
42. **Aicher SA, Kurucz OS, Reis DJ, Milner TA** 1995 Nucleus tractus solitarius efferent terminals synapse on neurons in the caudal ventrolateral medulla that project to the rostral ventrolateral medulla. Brain Res 693:51–63
43. **Gee CE, Chen CL, Roberts JL, Thompson R, Watson SJ** 1983 Identification of proopiomelanocortin neurones in rat hypothalamus by in situ cDNA-mRNA hybridization. Nature 306:374–376
44. **Bagnol D, Lu XY, Kaelin CB, Day HE, Ollmann M, Gantz I, Akil H, Barsh GS, Watson SJ** 1999 Anatomy of an endogenous antagonist: relationship between agouti-related protein and proopiomelanocortin in brain. J Neurosci 19:RC26
45. **Bronstein DM, Schafer MK, Watson SJ, Akil H** 1992 Evidence that β-endorphin is synthesized in cells in the nucleus tractus solitarius: detection of POMC mRNA. Brain Res 587:269–275
46. **Palkovits M, Mezey E, Eskay RL** 1987 Pro-opiomelanocortin-derived peptides (ACTH/β-endorphin/α-MSH) in brainstem baroreceptor areas of the rat. Brain Res 436:323–338
47. **Palkovits M, Eskay RL** 1987 Distribution and possible origin of β-endorphin and ACTH in discrete brainstem nuclei of rats. Neuropeptides 9:123–137
48. **Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breininger J, Baskin DG** 2002 Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. Endocrinology 143:239–246
49. **Hosoi T, Kawagishi T, Okuma Y, Tanaka J, Nomura Y** 2002 Brain stem is a direct target for leptin's action in the central nervous system. Endocrinology 143:3498–3504
50. **Schwartz MW, Woods SC, Seeley RJ, Barsh GS, Baskin DG, Leibel RL** 2003 Is the energy homeostasis system inherently biased toward weight gain? Diabetes 52:232–238