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Differential Activation of the Sympathetic Innervation of Adipose Tissues by Melanocortin Receptor Stimulation

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Melanocortins are implicated in the control of energy intake/expenditure. Centrally administered melanotan II (MTII), a synthetic melanocortin 3/4-receptor agonist, decreases adiposity beyond that accountable by food intake decreases. Melanocortin-4 receptor (MC4-R) mRNA is expressed on sympathetic nervous system (SNS) outflow neurons to white adipose tissue (WAT) in Siberian hamsters, suggesting a role in lipid mobilization. Therefore, we tested whether third ventricular injections of MTII increased sympathetic drive to WAT and interscapular brown adipose tissue (IBAT) using norepinephrine turnover (NETO) as a measure of sympathetic drive. We also tested for MTII-induced changes in lipolysis-related WAT gene expression (β3-adrenoceptors, hormone sensitive lipase) and IBAT thermogenesis (β3-adrenoceptor, uncoupling protein-1). Finally, we tested whether third ventricularly injected MTII, a highly selective MC4-R agonist (cyclo[β-Ala-His-D-Phe-Arg-Trp-Glu]NH2) increased or agouti-related protein decreased IBAT temperature in hamsters implanted with sc IBAT temperature transponders. Centrally administered MTII provoked differential sympathetic drives to WAT and IBAT (increased inguinal WAT, dorsosubcutaneous WAT and IBAT NETO, but not epididymal WAT and retroperitoneal WAT NETO). MTII also increased circulating concentrations of the lipolytic products free fatty acids and glycerol but not plasma catecholamines, suggesting lipid mobilization via WAT SNS innervation and not via adrenal medullary catecholamines. WAT or IBAT gene expression was largely unaffected by acute MTII treatment, but IBAT temperature was increased by MTII and the MC4-R agonist and decreased by agouti-related protein. Collectively, this is the first demonstration of central melanocortin agonist stimulation of WAT lipolysis through the SNS and confirms melanocortin-induced changes in BAT thermogenesis. (*Endocrinology* 148: 5339–5347, 2007)

OBESITY IS LITERALLY and figuratively a growing problem internationally, reaching even nonheavily industrialized nations (1, 2). Obesity is associated with several secondary health risks including increased incidence of type 2 diabetes, some cancers, and stroke (for review see Refs. 3–5). The severity of these secondary health consequences of obesity are substantially reduced through decreases in lipid stores, especially viscerally located white adipose tissue (WAT) (*e.g.* Refs. 6, 7). In rodents and humans, the mobilization of lipid from the major WAT depots is not uniform in response to energetically demanding conditions (for review see Refs. 8–11). The exact mechanism underlying differential lipolysis across WAT depots is not precisely known but likely involves divergent sympathetic nervous system (SNS) outflow circuits from brain to fat (12, 13).

It is clear that the sympathetic nervous system is the primary initiator of lipolysis in rodents (for review see Refs. 9, 10) as well as humans (for review see Ref. 14). The evidence for the involvement of the SNS in WAT lipid mobilization through its principal postganglionic neurotransmitter, norepinephrine (NE) includes neuroanatomical tract tracing of the postganglionic sympathetic innervation of WAT (12) as well as the central origins of the SNS outflow to WAT, as identified using a viral transneuronal retrograde tract tracer, the pseudorabies virus (PRV) (13, 15–18). Neurochemically, conditions promoting lipid mobilization such as short photoperiod exposure in Siberian hamsters (19, 20) or fasting or cold exposure in laboratory rats (21–24) all increase WAT NE turnover (NETO) (12, 25, 26), a measure of sympathetic drive (27). Finally, surgical or selective chemical destruction of the SNS innervation of WAT at the level of the fat pad blocks lipid mobilization under a variety of lipid-promoting stimuli [*e.g.* fasting (12, 28, 29), estradiol treatment of ovariectomized animals (30), short-day exposure of Siberian hamsters (31, 32)].

NETO measures in WAT are not uniform across the fat pads and typically correspond to proportional decreases in WAT mass, an integrative measure of lipid mobilization [(12); *cf.*, Ref. 33]. Thus, the notion of Cannon (34) that at times of emergency, a general SNS discharge is triggered preparing animals for fight or flight and provoking an all-or-nothing sympathetic response is not true for WAT nor does it hold across other tissues (for review see Ref. 35). Therefore, the trafficking of sympathetic outflow between tissues or among the same tissue type in different locations, such as with WAT, is one of the great mysteries of regulatory biology. A number

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Abbreviations: AgRP, Agouti-related protein; AMPT, α-methyl-*p*-tyrosine; β3-AR, β3-adrenoceptor; BAT, brown adipose tissue; DHBA, dihydroxybenzylamine; DWAT, dorsosubcutaneous WAT; EWAT, epididymal WAT; HSL, hormone sensitive lipase; IBAT, interscapular BAT; icv, intraventricular; IWAT, inguinal WAT; MCR, melanocortin receptor; MTII, melanotan II; NE, norepinephrine; NETO, norepinephrine turnover; PRV, pseudorabies virus; RWAT, retroperitoneal WAT; SNS, sympathetic nervous system; UCP, uncoupling protein; WAT, white adipose tissue.

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of possible neuroanatomical answers to this mystery exists, however. We previously have shown that the postganglionic sympathetic innervation of different WAT pads is relatively separate (12) and could easily account for fat pad-specific differences in NETO and thereby differential rates of lipid mobilization. Alternatively, or in addition to this peripheral viscerotopic separation of sympathetic nerves, viscerotopy could occur centrally at rostral aspects of the neuroaxis as suggested recently using two viral tract tracers (13). Finally, the balance between the number and/or affinity of β- and α_2-adrenoceptors possessed by white adipocytes that, when activated, trigger lipolysis and antilipolysis, respectively (for review see Refs. 36, 37), could also be an important factor in the differential mobilization of lipid from WAT. Regardless, these neuroanatomical scenarios are complicated by the fact that the neurochemical phenotypes of the neurons comprising the sympathetic circuits to WAT are largely unknown. We have, however, previously shown the involvement of several neuropeptides, neurotransmitters, or their synthetic enzymes as part of the SNS outflow to WAT (38). In addition, and perhaps more importantly, we recently identified high colocalizations of melanocortin-4 receptor (MC4-R) mRNA on SNS outflow neurons to WAT, the latter labeled via the PRV (18).

The melanocortin system has been implicated in the control of lipid mobilization. Specifically, intraventricular (icv) administration of melanotan II (MTII) a synthetic version of the natural agonist of the MC3/4-Rs, αMSH, triggers decreases in body fat of laboratory rats (39) that cannot be accounted for by the well-established MTII-induced inhibition of food intake (*e.g.* Refs. 40, 41). That is, rats receiving MTII that are pair fed to MTII-treated *ad libitum*-fed control rats have an exaggerated body fat loss, implicating both lipid mobilization and increases in thermogenesis that could be stimulated by the melanocortins (39). Although there is evidence for the involvement of both MC4-R and MC3-R in the effects of melanocortins on energy balance (42, 43), the vast preponderance of the data suggest MC4-R is considered to be the primary receptor involved in these responses. The study by Raposinho *et al.* (39), demonstrating the food intake-independent decreases in body fat by MTII, did not address the underlying mechanism responsible for these decreases; however, it seems likely that, given our finding of MC4-Rs on sympathetic outflow neurons to WAT (18) and brown adipose tissue (BAT) (Song, C. K., C. H. Vaughan, E. Keen-Rhinehart, D. Richard, R. B. Harris, and T. J. Bartness, manuscript in preparation, and Refs. 44, 45), it might be via the SNS innervation of these tissues. Thus, MC4-R activation may increase the sympathetic drive to WAT to trigger lipolysis via NE stimulation of β_3-adrenoceptors thought to be involved principally in lipolysis in rodents (37, 46). In addition, MC4-R activation may increase the sympathetic drive to BAT to trigger thermogenesis via NE stimulation of β_3-adrenoceptors thought to be principally involved in this response (for review see Ref. 46). This, in turn, may result in increases in the activity of uncoupling protein (UCP)-1 (47–49), the mitochondrial membrane protein responsible for heat production in this tissue (for review see Ref. 50).

Therefore, the purpose of the present study was to test whether third ventricular injections of MTII increased sympathetic drive to the major WAT pads and interscapular BAT (IBAT), the predominant BAT pad (for review see Ref. 51), using NETO as a neurochemical measure of sympathetic drive. In addition, we measured quantitative changes in gene expression for several factors involved in lipolysis [β_3-adrenoceptor (β3-ARs), hormone-sensitive lipase (HSL)] and BAT thermogenesis (β3-AR, UCP-1). Finally, we tested whether melanocortin receptor agonism via third ventricularly injected MTII, a MC3/4-R agonist, or a cyclic analog of α-MSH, cyclo(β-Ala-His-D-Phe-Arg-Trp-Glu)-NH2, which is a highly selective agonist for the MC4-R (52), would increase IBAT temperature *in vivo* in awake hamsters with temperature transponders implanted under the IBAT pad as well as whether agouti-related protein (AgRP), a MC3/4-R inverse agonist, would conversely decrease IBAT temperature.

Materials and Methods

Animals

One hundred thirty-six male Siberian hamsters (*Phodopus sungorus*), 3–4 mo old, were obtained from our breeding colony. The hamsters were single housed in plastic cages (23 × 26 × 30 cm) and maintained under a long-day photoperiod (16-h light, 8-h dark, lights on at 0300 h) at 22 ± 1 C. Food (Purina Rodent Chow no. 5001, St. Louis, MO) and tap water were available *ad libitum* throughout the experiment. Body mass was monitored for 2 wk, at which time the hamsters were submitted to icv cannula implantation. Body mass was monitored for 2 wk after cannula implantation and at which time they were divided into three groups matched for body mass and percent body mass change. Housing and all procedures were approved by the Georgia State University Institutional Animals Care and Use Committee and were in accordance with the Public Health Service and U.S. Department of Agriculture guidelines.

Intraventricular cannula implantation

Cannulae were stereotaxically implanted into the third ventricle as described previously (53). Briefly, the animals were anesthetized with isoflurane, and the fur at the top of the head was removed to exposure the area to be incised. After exposure of the skull, a hole was trephined at the intersection of bregma and the midsaggital sinus and the guide cannula (26-gauge stainless steel; Plastics One, Roanoke, VA) was positioned using the following stereotaxic coordinates: level skull, anterior-lateral from bregma, 0 mm; medial-lateral from midsaggital sinus, 0 mm; and dorsal-ventral, −5.5 mm from the top of the skull, which targeted placement just above the third ventricle. The guide cannula was secured to the skull with 3/16-mm jeweler's screws, cyanoacrylate glue, and dental acrylic. A removable obturator (Plastics One) sealed the opening in the guide cannula throughout the experiment, except when it was removed for the injections and adaptation to simulate the experimental injection procedures.

Intraventricular injection protocol

All hamsters were adapted to the microinjection procedure for 3 d before the actual experimental procedure. Each animal was lightly restrained by hand, and the cannula obturator was removed and put back, simulating the handling associated with the injection procedure.

On the day of the experiments, the hamsters were transferred from the vivarium to a testing room at 0600 h, and food was removed from the hamster's pouches and their cages. After removing the obturator, an inner cannula (33-gauge stainless steel; Plastics One) that was custom fit to extend 6.0 mm below the top of the skull (0.5 mm beyond the tip of the guide cannula) was inserted and connected at the other end to a 1-μl microsyringe via polyethylene tubing. All injections were equivolemic (0.4 μl) and given between 0800 and 1000 h. Sterile saline (vehicle) or 0.5 or 5 nmol MTII [doses determined from its inhibitory effect on food intake in this species when given icv (54, 55)] was injected while the animals were lightly restrained by hand during the 30-sec injection period. The injection needle remained in place approximately 30 sec

before withdrawal to minimize efflux up the cannula tract. Hamsters were then placed back into their respective cages.

Cannula verification

At the end of the experiment, 0.4 μl of India ink was injected to confirm placement of the cannula in the third ventricle. The brains were removed and postfixed in 10% paraformaldehyde for a minimum of 1 wk. Each brain was sliced at 20 μm on a freezing stage sliding microtome for cannula site verification. Cannulae were considered to be located in the third ventricle if the dye was visible in any part of this ventricle. Only the data from animals with confirmed third ventricle cannula placements were included in the analysis.

NETO and tissue preparation

NETO was measured using the α-methyl-*p*-tyrosine (AMPT) method (56). AMPT is a competitive inhibitor of tyrosine hydroxylase, the rate-limiting enzyme in catecholamines biosynthesis. After AMPT administration, the endogenous tissue levels of NE decline at a rate proportional to the initial NE concentrations (56). AMPT methyl ester hydrochloride (Sigma Aldrich, St. Louis, MO) was prepared by first adding an aliquot of glacial acetic acid (1 μl/mg AMPT) and then diluting to the final concentration with 0.15 M NaCl. At the beginning of the experiment, half of these animals were untreated and killed at 0 h to obtain baseline tissue NE content, whereas the other half was injected ip with AMPT (250 mg AMPT per kilogram; 25 mg/ml) between 0800 and 1000 h. A supplemental dose of AMPT (125 mg/kg body mass, at a concentration of 12.5 mg/ml) was administered to these animals 2 h after the initial injection to assure the maintenance of tyrosine hydroxylase inhibition. Thirty minutes after the first injection of AMPT, each animal received an icv injection of saline or MTII (0.5 or 5 nmol). The doses of MTII was chosen based on third ventricular injections of this melanocortin receptor agonist that inhibit food intake in this species (54, 55) and are higher than the usual doses of MTII used in laboratory rats and mice with some exception (*e.g.* Ref. 57). The animals were then killed 4 h after the initial AMPT injection by decapitation (*i.e.* 3.5 h after icv MTII or saline). Inguinal WAT (IWAT), retroperitoneal WAT (RWAT), epididymal WAT (EWAT), dorsosubcutaneous WAT (DWAT), and IBAT were rapidly removed, weighed, frozen in liquid nitrogen, and stored at −80 C until assayed for catecholamine content to determine NETO.

The NE tissue content was measured using reverse-phase HPLC with electrochemical detection, following our modification (12) of the method of Mefford (58). Briefly, tissue was thawed and homogenized in a solution containing dihydroxybenzylamine (DHBA; internal standard) in 0.2 M perchloric acid with 1 mg/ml ascorbic acid. The amount of tissue processed and DHBA added were varied to obtain NE values within the range of the standards (~250 mg of WAT was used with 50 ng of DHBA added; ~50 mg of IBAT was used with 100 ng of DHBA added). After centrifugation for 15 min (7500 × *g* at 5 C), catecholamines were extracted from the homogenate with alumina and were eluted into the perchloric acid/ascorbic acid. The catecholamines were assayed using an ESA Biosciences (Chelmsford, MA) HPLC system with electrochemical detection (Coulochem II). The mobile phase was Cat-A-Phase II, and the column was a HR-80 reverse phase column. NETO was calculated in IWAT, EWAT, RWAT, DWAT and IBAT by subtracting the NE content (ng NE per tissue) from the 0-h group from the 4-h group according to the method of Brodie *et al.* (10). The log of NE content was plotted *vs.* time and the least-square straight line provided the fractional turnover rate, *k* (Fig. 1). Specifically and briefly, calculations were made according to the following formula: $k = (\lg[NE]_0 - \lg[NE]_4/(0.434 \times 4)$ and $K = k[NE]_0$, where k is the constant rate of NE efflux (also known as fractional turnover rate), $[NE]_0$ is the initial NE concentration, $[NE]_4$ is the final NE concentration, and K = NETO. Representative values from a separate set of animals (n = 6 per time point) killed at 0, 2, and 4 h to demonstrate linearity of NE disappearance and associated turnover measures is exemplified for RWAT (Fig. 1). Similar linearity was found for all WAT pads (data not shown), except EWAT, which was nonlinear, as we have seen previously (12, 59), likely due to its very low baseline sympathetic drive.

Plasma hormones and metabolites

Because of the likelihood that AMPT administration would alter several physiological systems that might impact concentrations of hormones and circulating metabolic fuels, we conducted a parallel experiment to the NETO experiment in which everything was done exactly the same except there was no AMPT administration. Instead, trunk blood was collected after decapitation from hamsters injected at time 0 with the saline vehicle or 0.5 or 5 nmol MTII at 1 or 3.5 h after injection. The blood was centrifuged for 20 min (3000 × *g* at 5 C). Plasma was removed and stored at −80 C until assayed. We measured plasma glucose (Ascesion Elite blood glucose strips; Bayer Corp., Mishawaka, IN), free fatty acids (NEFA C kit; Wako Chemicals, Richmond, VA), and glycerol, the latter based on the Wieland method (48) adapted to fluorometric analysis in microplates according to the method of Laurell and Tibbling (60). Plasma leptin was measured with a commercial kit (mouse leptin ELISA kit; Linco Research Inc., St. Charles, MO). Plasma epinephrine and NE were measured using a commercial kit (plasma catecholamine analysis kit; ESA Biosciences, Inc., Chelmsford, MA) by HPLC (see above).

IBAT temperature

Another set of hamsters (n = 27) was used for IBAT temperature tests. Hamsters were anesthetized with isoflurane, and a temperature transponder [Implantable Programable Temperature Transponder 300 (IPTT-300); BioMedic Data Systems, Seaford, DE] was implanted under the IBAT pads such that temperature from both pads could be measured. The transponder was secured to the surrounding muscle. Hamsters also received a third ventricular cannula as above. After a 2-wk postsurgical recovery period, the animals were adapted to the handling procedure for the icv injections once each day for 3 d as above. In addition, the temperature sensing wand also was used (see below) to adapt the animals to several low beeping sounds produced by the recording apparatus (DAS 5002 Notebook System; BioMedic Data Systems) when acquiring the temperature.

On the test day, food was removed from the pouches of the hamsters and from their cages at 0600 h, but water was present. Two hours later, the temperature of IBAT was measured to determine the beginning baseline. The hamsters were then immediately injected with the saline vehicle or 0.5 or 5.0 nmol MTII (0.4 μl) into the third ventricle as above. Injections were made using a counterbalanced schedule to control for order effects of the injection, such that each animal received all three

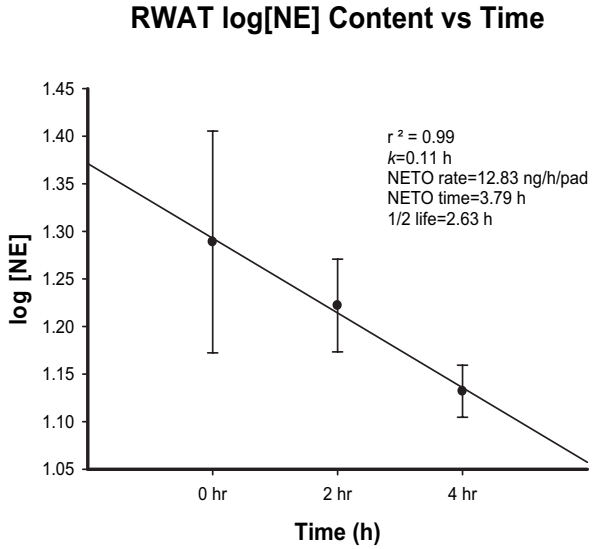

FIG. 1. Log[NE content] (log[NE]) *vs.* time for the RWAT pad. The slope (b) was determined by the least squares method and the constant rate of NE efflux, *i.e.* fractional turnover is k = b/0.434. NETO rate is estimated by multiplying the constant rate (*k*) by NE concentration at time 0. NETO time is the reciprocal of *k*, and half-life is NETO time multiplied by 0.693 (91).

TABLE 1. Primers for real-time RT-PCR for UCP-1, HSL, β3-AR, and 18s rRNA

	Forward primer (5′-3′)	Reverse primer (5′-3′)
β3-AR	GAGCCAGTGGTGGCGTGTAGG	ACAGCAGCGATTGGAGT
UCP-1	GATCCAAGGTGAAGGCCAGG	GGTGGTGTCTTTCGAACAGTTG
HSL	CAACATGGCATCAACCACTG	GCCTGGGATCAGAGGTGATG
18S rRNA	ACGGAAGGGCACCACCAGGA	CACCACCACCCACGGATTCG

injections; 10 d occurred between tests to minimize carryover effects. IBAT temperature was assessed at 30, 60, 90, 120, and 180 min by passing the temperature sensing wand 10–20 mm above the back of the animal in its cage. At the end of that series of injections, an additional 10-d washout period occurred before injection of saline or the specific MC4-R agonist cyclo(β-Ala-His-D-Phe-Arg-Trp-Glu)-NH2 (Phoenix Pharmaceuticals, Burlingame, CA), referred hereafter as the MC4-agonist, at 0.5, 2.5, or 5.0 nmol in a counterbalanced order (doses chosen as mole equivalents to MTII doses for ease of comparison). IBAT temperature was measured as above. Finally, after a 10-d washout period, the hamsters were injected with saline or AgRP [AgRP (83–132); Phoenix Pharmaceuticals] at 0.5, 2.5, or 5.0 nmol AgRP and IBAT temperature measured as above to test for decreases in IBAT temperature, given its opposing effect on melanocortin receptors (doses chosen on the ability of AgRP to stimulate food intake and hoarding in Siberian hamsters) (53). At the conclusion of the experiment, each hamster was injected peripherally with 0.8 mg/kg of the pan β-adrenoceptor agonist, isoproterenol, and IBAT temperature recorded for 60 min as a positive control to assess transponder function.

Real-time RT-PCR

IWAT, RWAT, EWAT, and IBAT pads (from animals treated with saline or 0.5 or 5.0 nmol MTII) were dissected, flash frozen in liquid nitrogen, and stored in −80 C until further processed to measure HSL, β3-AR and UCP-1 gene expression. Total RNA was extracted using RNEasy lipid tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The concentration and quality of the total RNA were determined by UV spectrophotometry and electrophoretically on a denaturing gel. Five micrograms of the extracted RNA were then used for first-strand cDNA synthesis using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed on ABI PRISM 7500 (Applied Biosystems, Foster City, CA) with the following amplification conditions: 95 C for 10 min; 40 cycles of 95 C for 15 sec, 60 C for 1 min with SYBR green (SYBR Green ER qPCR SuperMix Universal; Invitrogen) as the detector fluorophore. Immediately after real-time PCR amplification, a melt curve analysis was performed to ensure the specificity of the amplification. MTII-induced changes in gene expression were analyzed by relative quantitation standardized to 18S rRNA and calibrated against that of saline treated control animals. Change in ΔCt value values [*i.e.* differences in threshold cycles or for the mRNA of interest and 18S rRNA] were determined for each sample and relative mRNA expression compared as 1/difference in threshold cycles.

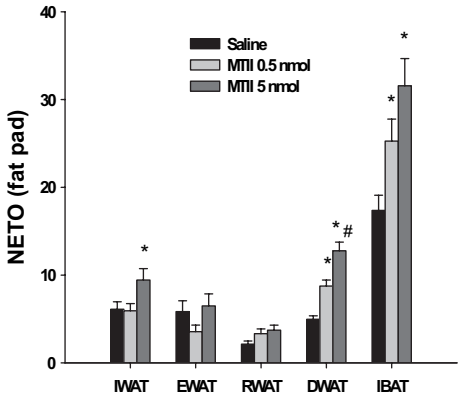

FIG. 2. Mean ± SEM NETO expressed as NE (nanogram per tissue per hour) after third icv MTII or saline in IWAT, EWAT, RWAT, DWAT, and IBAT. *, $P < 0.05$ *vs.* saline; #, $P < 0.05$ *vs.* 0.5 nmol MTII.

To obtain hamster specific primer sequences for each of the genes (Table 1), a portion of each hamster gene was cloned using degenerate primers and hamster cDNA (61). The clones were sequenced and showed between 70 and 98% amino acid identity with their murine orthologs, depending on the gene. The hamster sequence was then used to design primers for real-time PCR.

Statistical analysis

The relative gene expression for β3-AR, UCP-1, and HSL mRNA was compared between groups using one-way ANOVA. Similarly, NETO and circulating concentrations of glucose, glycerol, free fatty acids, leptin, epinephrine, and NE were statistically analyzed using a one-way ANOVA (saline control *vs.* MTII). IBAT temperature was statistically analyzed using a two-way repeated-measures ANOVA (drug × time; 2 × 6). Duncan's new multiple-range tests (SigmaStat version 2.0; Systat Software, San Jose, CA) were used as *post hoc* tests when appropriate. Differences between the means for all tests were considered statistically significant if $P < 0.05$. Exact probabilities and test values were omitted for simplicity and clarity of the presentation of the results.

Results

NETO

NETO is presented on a whole-organ basis to reflect the overall sympathetic drive and physiological impact for each tissue. Third ventricular injection of MTII increased NETO differentially across the adipose tissue depots assayed here. Specifically, only the highest MTII dose (5 nmol) significantly increased NETO in the sc IWAT pad ($P < 0.05$; Fig. 2), whereas MTII produced significant dose-dependent increases in NETO in another sc depot, DWAT ($P < 0.05$; Fig. 2). NETO in RWAT and EWAT, both internally located WAT pads (Siberian hamsters have no scrotum, so EWAT is located in the peritoneal cavity) were not significantly increased by MTII, although the increase in RWAT NETO almost reached statistical significance ($P < 0.06$; Fig. 2). Both doses of MTII significantly increased IBAT NETO ($P < 0.05$; Fig. 2), approaching but not reaching a statistically significant dose-dependent increase ($P < 0.06$).

Circulating factors

Plasma glucose concentration only was significantly increased 1 h after MTII injection at the highest dose (5 nmol; $P < 0.05$; Table 2). Both plasma glycerol and free fatty acid concentrations were significantly increased at both MTII doses and at both times (1 and 3.5 h after injection; Tables 2 and 3; $P < 0.05$) but not dose dependently. Plasma epinephrine and NE concentrations were not significantly affected by MTII at either dose or either time point (Tables 2 and 3). Plasma leptin concentrations were not affected by MTII at either dose or either time point as well (Tables 2 and 3).

IBAT temperature

Third ventricular MTII significantly increased IBAT temperature at 60 min after injection through the end of the

TABLE 2. Effect of third icv MTII on plasma glucose, glycerol, free fatty acids, epinephrine, NE, and leptin 1 h after MTII administration

	Saline	0.5 nmol MTII	5 nmol MTII
Glucose (mg/dl)	145.67 ± 9.79	147.88 ± 8.12	201.38 ± 21.70[a,b]
Glycerol (μM)	215.08 ± 48.77	386.78 ± 26.31[a]	429.31 ± 42.30[a]
Free fatty acids (mEq/liter)	0.92 ± 0.15	1.57 ± 0.12[a]	1.77 ± 0.06[a]
Leptin (ng/ml)	6.90 ± 0.89	9.19 ± 1.21	5.54 ± 0.65
NE (ng/ml)	15.30 ± 2.26	20.37 ± 2.65	17.97 ± 2.35
Epinephrine (ng/ml)	10.19 ± 2.11	10.18 ± 2.36	9.28 ± 1.59

[a] $P < 0.05$ *vs.* saline.
[b] $P < 0.05$ *vs.* 0.5 nmol MTII.

experiment ($P < 0.05$; Fig. 3) for both MTII doses. The MTII-induced peak increase in IBAT temperature (~2 C, compared with saline control; Fig. 3) occurred at 5 nmol MTII, with a smaller increase in IBAT temperature (~0.6 C) at the lower MTII dose (0.5 nmol), and this occurred between 60 and 220 min after injection.

The MC4-R-specific agonist also significantly increased IBAT temperature at 60, 120, and 220 min after injection for all doses and all but the 2.5 nmol dose at 180 min ($P < 0.05$; Fig. 3). By contrast, AgRP significantly decreased IBAT temperature at the two lowest doses (0.5 and 2.5 nmol) 60, 120, and 180 min after icv injection ($P < 0.05$; Fig. 3), whereas the highest dose (5 nmol) did not alter IBAT temperature (Fig. 3).

Real-time RT-PCR

There were very few changes in gene expression with MTII treatment, regardless of the dose of this agonist (Table 4). This is not surprising because the treatment was acute (single icv injection of MTII) and the animals were killed shortly thereafter (3.5 h). There were, however, some statistically significant changes. Specifically, EWAT β3-AR gene expression was significantly decreased by both doses of MTII, compared with their saline controls ($P < 0.05$; Table 4). In addition, IBAT β3-AR gene expression was significantly decreased but only by the high dose of MTII (5.0 nmol; $P < 0.05$; Table 4). There were no significant changes in HSL gene expression in any WAT pad or UCP-1 gene expression in IBAT (Table 4).

Discussion

The results of the present study show for the first time that central melanocortin receptor agonism with MTII provokes differential sympathetic drive of WAT and BAT, as measured by NETO as well as confirming previous involvement of the melanocortins in IBAT temperature. Because plasma free fatty acids and glycerol concentrations were increased by third ventricular injection of MTII *vs.* saline, indicating increased lipolysis, and because circulating catecholamines were not affected, indicating a lack of adrenal medulla stimulation, these results suggest that central melanocortin receptor agonism triggers WAT lipolysis, perhaps via the stimulation of MC4-R mRNAs located on sympathetic outflow neurons ultimately innervating WAT (18), but because we used MTII, a MC3- and MC4-R agonist, an effect based on MC3-R agonism cannot be ruled out. In addition, the dose of MTII, although generally high by laboratory rat and mouse standards, is not for Siberian hamsters [at least in terms of its ability to inhibit food intake; *e.g.* Refs. 54, 55, and 62)]. Because of the relative size of the MTII dose, there is the possibility, as there often is for centrally administered substances, of leakage into the periphery. Such leakage would not explain the differential increases in WAT NETO across the fat depots but could be consistent with the ability of peripherally injected MTII to cause decreases in WAT mass (63), although no measures or corollaries of lipolysis were measured in that study. Moreover, peripherally administered MTII likely has both peripheral and centrally mediated effects, the latter possibly having its mechanism of action through the melanocortin/SNS circuits to WAT (18).

Gene expression for factors involved in lipolysis (HSL, β3-AR) and BAT thermogenesis (UCP-1, β3-AR) were generally not changed, except for small but significant decreases in EWAT β3-AR and IBAT β3-AR gene expression. The lack of substantial changes in the expression of any of these genes likely is due to the acute nature (single injection, 3.5 h test) of the MTII treatment.

We did not find a decrease in WAT mass after MTII treatment, an effect that can indicate lipid mobilization (data not shown), which is not surprising giving the short duration of MTII treatment. As noted above, the combination of increased plasma glycerol and free fatty acid concentrations with no change in epinephrine or NE plasma concentrations indicates lipolysis via the SNS innervation of WAT. The possibility of central MTII triggered increases in lipolytic hormones, such as

TABLE 3. Effect of third icv MTII on plasma glucose, glycerol, free fatty acids, epinephrine, NE, and leptin 3.5 h after MTII administration

	Saline	0.5 nmol MTII	5 nmol MTII
Glucose (mg/dl)	158.00 ± 10.28	130.70 ± 5.52	131.00 ± 5.58
Glycerol (μM)	149.06 ± 16.88	281.29 ± 27.13*	280.48 ± 19.45*
Free fatty acids (mEq/liter)	0.66 ± 0.09	1.70 ± 0.08*	1.82 ± 0.12*
Leptin (ng/ml)	6.34 ± 1.07	5.42 ± 0.79	4.79 ± 0.69
NE (ng/ml)	14.87 ± 2.23	12.28 ± 1.66	16.59 ± 2.89
Epinephrine (ng/ml)	7.07 ± 0.86	8.48 ± 1.19	9.27 ± 1.61

*, $P < 0.05$ *vs.* saline.

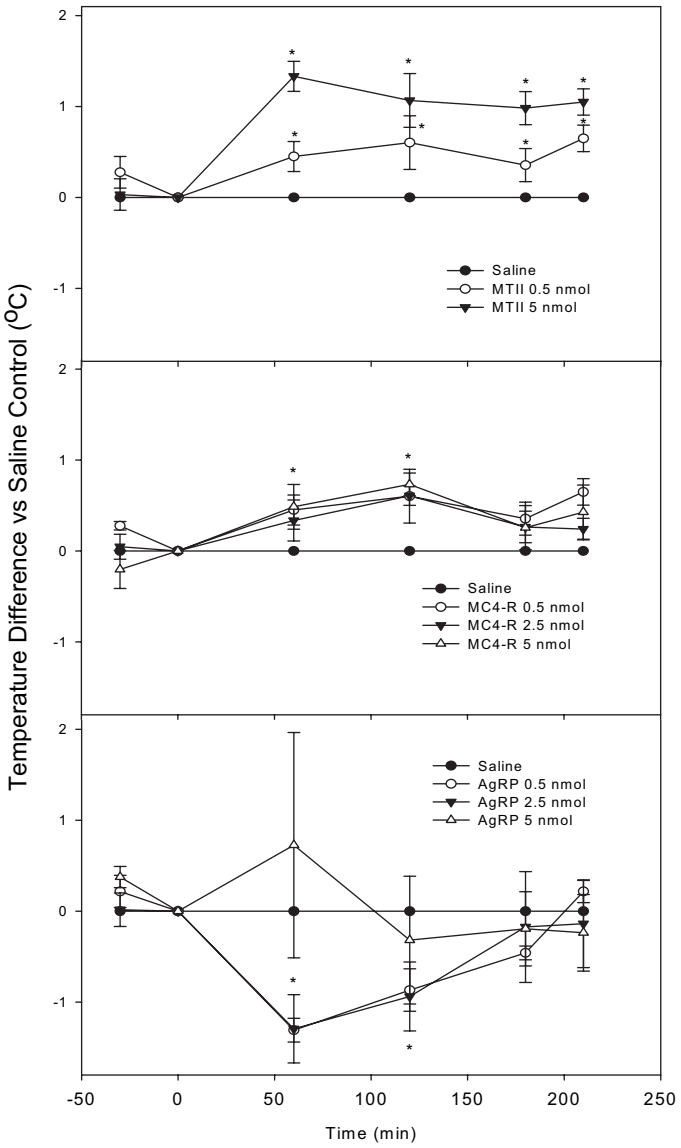

FIG. 3. Mean + SEM difference in IBAT temperature (C) from saline controls for third icv MTII (*top*), the MC4-R agonist, cyclo[β-Ala-His-D-Phe-Arg-Trp-Glu]NH2 (*middle*), and AgRP (83–132) (*bottom*) on IBAT temperature. *, $P < 0.05$ *vs.* saline.

glucagon (64–66), cannot be completely ruled out. There is also the possibility that the central MTII reached the periphery because MTII can directly stimulate white adipocyte lipolysis *in vitro* (67). This possiblity of leakage of MTII into the periphery seems unlikely because it does not explain the increase in NETO to some but not all WAT pads.

The most notable, although not surprising, finding of the present study was the fat pad-specific nature of the increases in NETO. MTII triggered increases NETO in IWAT and DWAT as well as IBAT but not EWAT or RWAT, although there was a suggestive increase in NETO for the latter. Fat pad-specific effects of stimuli on lipolysis are typical, rather than the exception, as are fat pad-specific increases in lipid accretion (for review see Ref. 11). That is, fat pad-specific responses are almost invariably found if more than one fat pad is assayed. For example, the *in vitro* lipolytic response to adrenoceptor stimulation varies among adipocytes harvested from various fat pads (*e.g.* Refs. 68 and 69), and disparate decreases in WAT pad mass and/or fat cell size, indicative of increased lipid mobilization, occur in response to several stimuli including fasting (*e.g.* Refs. 22 and 70) and short winter-like photoperiods in Siberian hamsters (20, 71–73). The differential decreases in WAT mass typically are associated with differential sympathetic drives, as indicated by NETO (*cf.* Ref. 33). Thus, the greater the decreases in WAT mass from short photoperiod-exposed Siberian hamsters, the greater the NETO (12). Evidence for nearly separate populations of sympathetic outflow neurons to different WAT pads has been demonstrated for postganglionic sympathetic neurons in the sympathetic chain of Siberian hamsters (12) as well as for preganglionic sympathetic neurons in the intermediolateral horn of the spinal cord and forebrain of laboratory rats (13). Moreover, in terms of the latter, using two strains of the transneuronal viral tract tracer PRV, separate sympathetic innervations of sc WAT *vs.* visceral WAT have been shown (13). These data fit nicely with the current results in which icv MTII strongly stimulated sc (IWAT and DWAT) NETO but either weakly stimulated (RWAT) or did not stimulate (EWAT) internal fat. The likely site(s) of action for the icv-delivered MTII is unknown and could be anywhere periventricularly from the hypothalamus to the spinal cord in which MC4-Rs are located on sympathetic outflow neurons from brain to WAT (18).

The lack of a MTII-induced increase in EWAT NETO is similar to our findings in which other lipid-promoting stimuli such as glucoprivation [using 2-deoxy-D-glucose; (10)] and cold exposure and/or fasting also do not increase EWAT NETO, but all increase NETO to DWAT, IWAT, and RWAT (our unpublished observations). We have hypothesized that lipid or growth factors associated with the EWAT pad are protected from the effects of most lipid-promoting stimuli to preserve spermatogenesis because removal of EWAT severely inhibits spermatogenesis in laboratory rats (74). Moreover, EWAT removal in Syrian hamsters also blocks spermatogenesis, but removal of other fat pads, some producing even larger lipid deficits than EWAT removal, does not affect spermatogenesis (Chu, Y., G. G. Huddleston, R. R. Bowers, A. N. Clancy, R. B. Harris, and T. J. Bartness, manuscript in preparation).

Central MTII administration did not affect circulating concentrations of the largely adipocyte-derived cytokine, leptin. The SNS innervation of WAT is one of the regulators of leptin secretion; with conditions that increase WAT sympathetic drive [*e.g.* cold exposure (25), fasting (26)], decrease WAT leptin synthesis/release (*e.g.* Refs. 75–77), as does β3-AR stimulation (*e.g.* Refs. 78–82). MTII treatment that increased sympathetic drive (NETO) to IWAT and DWAT did not inhibit circulating leptin concentrations at either time point after injection, a somewhat surprising result, given the increased synthesis/release of leptin by sc *vs.* visceral WAT (83, 84). The likely explanation for the lack of inhibition was that MTII was given acutely, and chronic treatment may be required for significant inhibition of leptin synthesis/secretion.

MTII treatment increased plasma glucose concentrations only at the 1-h postinjection time for the highest dose of the melanocortin receptor agonist. Typically, increases in sympathetic drive to WAT and/or BAT are associated with decreases in circulating glucose concentrations, an effect usually due to increases in glucose uptake by these tissues (*e.g.* Ref. 85). Because the liver is sympathetically innervated (*e.g.* Refs. 13 and

TABLE 4. Effects of icv MTII on real-time RT-PCR quantitative gene expression for factors affecting lipolysis (HSL, β3-AR) and thermogenesis (β3-R, UCP-1)

	IWAT	EWAT	RWAT	IBAT
HSL				
Saline	0.050 ± 0.002	0.042 ± 0.002	0.042 ± 0.001	
0.5 nM	0.053 ± 0.001	0.039 ± 0.001	0.040 ± 0.001	
5.0 nM	0.052 ± 0.001	0.040 ± 0.001	0.041 ± 0.001	
β3-AR				
Saline	0.049 ± 0.001	0.052 ± 0.003	0.052 ± 0.001	0.064 ± 0.001
0.5 nM	0.047 ± 0.001	0.046 ± 0.002[a]	0.049 ± 0.001	0.062 ± 0.001
5.0 nM	0.046 ± 0.001	0.045 ± 0.001[a]	0.048 ± 0.002	0.060 ± 0.001[a]
UCP-1				
Saline				0.041 ± 0.001
0.5 nM				0.040 ± 0.001
5.0 nM				0.040 ± 0.001

[a] Compared with saline control ($P < 0.05$).

86) and because increases in sympathetic drive to the liver increase glycogenolysis, thereby increasing liver glucose output (*e.g.* Refs. 87 and 88), our central MTII injection likely stimulated SNS outflow to the liver, although no one has demonstrated MC4-Rs on these sympathetic circuit neurons to the liver. This possible sympathetic nerve-mediated mechanism for MTII-triggered increased plasma glucose concentrations is buttressed by our finding that plasma concentrations of adrenal medullary catecholamines were not increased.

Finally, the melanocortins, in addition to affecting energy intake, also affect energy output [oxygen consumption (89, 90)]. Some of these changes likely are due to the stimulatory effects of MC4-R stimulation on BAT thermogenesis. For example, central α-MSH or MTII administration increases sympathetic nerve activity to IBAT (47, 49), whereas central AgRP administration decreases sympathetic nerve activity to IBAT (49). In parallel to these effects, central MTII increases IBAT temperature and AgRP decreases it (49). In the present experiment, we found complementary results; third ventricular MTII, a MC3/4-R agonist, significantly increased IBAT NETO and increased IBAT temperature, whereas AgRP, the inverse agonist, decreased IBAT temperature. Moreover, this effect likely was mediated via stimulation of brain MC4-Rs because a similar albeit a smaller effect was seen after icv injection of a highly specific MC4-R agonist (cyclo[β-Ala-His-D-Phe-Arg-Trp-Glu]NH2). The central sites reached by our icv injections are not known; however, we determined some likely possible periventricular sites in a recent study in which there was a high (average ~60% or greater) coexpression of MC4-R mRNA with neurons that are part of the sympathetic outflow from brain to IBAT as labeled by PRV (Song, C. K., C. H. Vaughan, E. Keen-Rhinehart, D. Richard, R. B. Harris, and T. J. Bartness, manuscript in preparation and Ref. 18) such as the hypothalamic paraventricular nucleus, dorsomedial nucleus, lateral hypothalamus in the forebrain, periaqueductal gray in the midbrain, various brain stem raphe (*e.g.* raphe pallidus) and reticular regions (*e.g.* lateral reticular), the nucleus of the solitary tract, and the intermediolateral horn of the spinal cord. While our double-labeling study was in preparation, similar high levels of coexpression of the MC4-R with sympathetic outflow to IBAT was found at comparable sites in murine brain (45). The ability of agonism of MC4-Rs in these sites to stimulate IBAT thermogenesis remains to be tested completely, but we have preliminary data that suggest MTII injected in the hypothalamic paraventricular nucleus and nucleus of the solitary tract of brainstem in Siberian hamsters both significantly increase IBAT temperature (Song, C. K., C. H. Vaughan, E. Keen-Rhinehart, D. Richard, R. B. Harris, and T. J. Bartness, manuscript in preparation).

Collectively, the results of these experiments show for the first time that central MTII stimulates the sympathetic drive to WAT (as indicated by significant increases in WAT NETO and the lack of increases in circulating catecholamines) to increase lipid mobilization (as indicated by increased circulating concentrations of free fatty acids and glycerol). Moreover, the MTII-stimulated increased sympathetic drive to WAT was not uniform with increases in sc WAT NETO and lesser or no change in internal WAT NETO. This latter finding adds to the evidence of differential sympathetic drives to peripheral tissues and more specifically among WAT depots (*e.g.* Refs. 10 and 12). Finally, the present data add to the accumulating evidence for the role of the melanocortin receptors in BAT thermogenesis.

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