The medial preoptic nucleus as a site of the thermogenic and metabolic actions of Melanotan II in male rats.

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The present study was designed to investigate the role of the medial preoptic nucleus (MPO) as a site of the thermogenic and metabolic effects of the α-MSH analog Melanotan II (MTII). We also assessed the involvement of the dorsomedial hypothalamic nucleus (DMH) by investigating the effects of the MPO infusion of MTII in rats with DMH lesions produced by kainic acid. Infusion of MTII in the MPO led to increases in interscapular BAT (iBAT) temperature and iBAT uptake of $^{14}$C-bromopalmitate. Both increases were blocked by DMH lesions. iBAT temperature increase (area under curve) and $^{14}$C-bromopalmitate uptake emerged as two correlated variables ($r = 0.63$, $P < 0.001$). DMH lesions also blocked MTII-induced expression of mRNAs coding for proteins involved in (i) thermogenesis [type II iodothyronine deiodinase ($Dio2$) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha ($Pgc1α$)], (ii) lipolysis [hormone sensitive lipase ($Hsl$)] and (iii) lipogenesis [diacylglycerol O-acyltransferase 2 ($Dgat2$), fatty acid synthase ($Fas$)], in iBAT of rats killed one hour after MPO infusion of MTII. MTII also stimulated expression of genes in iWAT but only in rats with DMH lesions. These genes included glucose transporter member 4 ($Glut4$), glycerol-3-phosphate acyltransferase 3 ($Gpat3$), $Dgat1$, $Dgat2$, triglyceride lipase ($Atgl$), $Hsl$ and carnitine palmitoyltransferase 1 β ($Cpt1β$). Altogether, the present results reveal the MPO as a site of the thermogenic and metabolic actions of MTII. They also contribute to establish the MPO-DMH duet as a significant target for melanocortins to modulate energy homeostasis.
INTRODUCTION

The role of the brain melanocortin system in energy balance regulation is acknowledged (7, 13, 18, 33, 44). This system comprises the proopiocortin (POMC) neurons of the hypothalamic arcuate nucleus (ARC) and the widely distributed melanocortin-4 receptors (MC4R), which are important partners in controlling food intake and energy expenditure. POMC and Mc4r knockout or loss-of-function mutations are associated with severe obesity in laboratory rodents (21) as well as in humans (16). Consistent with this, the POMC product α-melanocyte-stimulating hormone (αMSH) and the αMSH synthetic analog Melanotan II, which represent MC4R/MC3R agonists, suppress food intake and stimulate energy expenditure (14, 22).

In addition to reducing food intake, MC4R agonism has been reported to increase energy expenditure through stimulating brown adipose tissue (BAT) thermogenesis (6, 15, 39, 41, 42). Increases in BAT temperature (39, 41), BAT sympathetic nervous system (SNS) activity (15) and BAT expression of uncoupling protein 1 (UCP1) and accessory thermogenic genes (43) have all been observed after brain ventricular and parenchymal injections of MC4R agonists. BAT involvement in MC4R-mediated thermogenic effects is also supported by observations obtained in Mc4r knockout or SHU9119 (MC4R antagonist)-treated animals, which exhibit diminished BAT thermogenic capacity/activity (22, 42). Additionally, the role of the MC4R in controlling BAT thermogenesis is anatomically supported by retrograde viral transneuronal tracing studies, which have demonstrated the strong connection between MC4R mRNA-expressing neurons and the SNS outflow to iBAT (41).

There is good evidence that the paraventricular hypothalamic (PVH) (41), ARC (12), and brainstem (39) are sites of the thermogenic action of MC4R agonists. However, the brain circuits
involved in the MC4R-mediated control of BAT thermogenesis remain to be fully unraveled. Several brain regions expressing the MC4R have not been systematically investigated as sites of the MC4R-mediated action on BAT thermogenesis. Among those regions, the preoptic area (POA) is certainly a worthy candidate. The POA represents the second hypothalamic region, next to the PVH, with the highest proportion of neurons connected to BAT (via the SNS) that express the MC4R (41). Within the POA, MC4R mRNA is widely expressed in the medial preoptic nucleus (MPO) (24), which is involved in thermoregulatory thermogenesis (30, 31). The MPO hosts inhibitory neurons that modulate the activity of the excitatory neurons of the dorsomedial hypothalamic nucleus (DMH) that control interscapular BAT (iBAT) activity via the raphe pallidus (RPa) (27, 34). The observation that inhibition of neurons found in the rostral RPa blocks the thermogenic effect of ventricular injection of MTII (15) supports the involvement of a MPO-DMH-RPa-SNS circuit in the MC4R-mediated effect on iBAT activity.

The objective of the present study was to verify whether the MPO could be a site where MTII could stimulate brown fat thermogenesis and metabolism with the complicity of the DMH. The effects of the MPO infusion of MTII on iBAT temperature as well as on iBAT $^{14}$C-bromopalmitate and $^3$H-deoxyglucose uptake were assessed in male Wistar rats with or without DMH lesions. The uptake of the radiolabelled energy substrate was also measured in inguinal white adipose tissue (iWAT), liver and heart. Furthermore, iBAT and iWAT expression of thermogenic and metabolic genes was measured in animals killed one hour following MTII with or without DMH lesions. iWAT was studied since it represents a WAT depot where brown adipocytes can develop under appropriate adrenergic stimulation (3) and since it is, similar to iBAT, connected to the MPO-MC4R neurons via the SNS (40).

MATERIALS AND METHODS
Animals

Male Wistar rats (Charles River Laboratories, St. Constant, QC, Canada), initially weighting ~300 g, were individually housed at 23 ± 1°C under a 12:12-h light-dark cycle (light on at 06:00). The rats had ad libitum access to water and pelleted standard chow (Charles River Rodent Diet no. 5075). All animal treatments and procedures were approved by the Université Laval Animal Ethics Committee and were in agreement with the Canadian Guide for the Care and Use of Laboratory Animals.

Drugs

The MC4R/MC3R agonist MTII (Phoenix Pharmaceutical Inc, Burlingame CA, USA) was dissolved in aCSF and administered at dose of 200 ng in a volume 200 nl (100 nl in each side of the MPO). Kainic acid (1 mg/ml, Sigma) was dissolved in sterile saline and adjusted to pH 7.2–7.4 by using 0.1 M NaOH. The dose of MTII (22) and kainic acid (26) were selected from previous studies (26).

Brain preparation

At time of killing, rats were anesthetized with 1.5 ml mixture containing 20 mg/ml ketamine and 2.5 mg/ml xylazine and intracardially perfused with 50 ml of ice-cold isotonic saline followed by 500 ml of paraformaldehyde (4%) solution. Brains were removed and kept in paraformaldehyde for an additional period of 7 days. They were then transferred overnight to a solution containing paraformaldehyde (4%) and sucrose (10%) and sliced from the olfactory bulb to the brainstem using a sliding microtome (Histoslide 2000, Reichert-Jung, Heidelberg, Germany). Thirty-µm thick sections of the PVH were collected and stored at −30°C in a cold cryoprotecting solution containing sodium phosphate buffer (50-mM), ethylene glycol (30%),
and glycerol (20%). The sections were examined for the cannula placement or underwent a Nissl staining to verify the lesion.

**MPO MTII infusions**

Rats were chronically implanted with bilateral (1.0 mm between tubes) guide-cannulas (26-gauge — Plastics One, Roanoke, VA, USA), aimed at the MPO, using the following coordinates relative to bregma: 0.5 mm on each side of the midline, 0.6 mm posterior and 6.5 mm ventral to the brain surface (37). The cannula was fixed in place using dental cement (Lang Dental Manufacturing co., Inc, Wheeling, IL, USA) and anchoring screws. Sterile obturators were inserted into the guide-cannulas to avoid blockage. The placement of the cannulas was further confirmed under microscope the end of the study and the injection sited were estimated. (Figure 1, A). Each surgery was done 1 week before the experiment to allow complete recovery at the time of infusion. Brain MTII infusions were carried out using $\mu$l Hamilton syringe connected to a syringe pump (Harvard Apparatus 55-2226). In each protocol, the solutions were carefully infused in the brain during 30 sec (200 nl/min).

**DMH lesions**

DMH lesions were performed prior to the MPO guide-cannula implantation. Briefly, a 26-gauge bilateral (1.0 mm between tubes) injection cannula (Plastics One, Roanoke, VA, USA) was stereotaxically aimed at the DMH. The coordinates were as follows: 0.5 mm on each side of the midline, 3.2 mm posterior to bregma and 8.5 mm ventral to the brain surface (37). Bilateral infusions of 200 nl of kainic acid or sterile saline (control animals) were performed over a 10-minute period. The cannula was left in place for another 10 minutes to allow the complete diffusion of the kainic acid. Kainic acid damages perikarya and dendrites of neurons harboring kainate receptors while preserving the fibers of passage of other neurons having their bodies
outside the targeted nuclei (4). DMH lesions are illustrated in figure 1, B. Rats with DMH lesions were subjected to the MTII infusions 10 days after the injections of kainic acid.

**iBAT thermogenesis and iBAT and iWAT energy substrate uptakes**

Four (4) groups of rats with or without DMH lesions were used to assess the effects of aCSF or MPO-infused MTII on iBAT thermogenesis. iBAT temperature was assessed under isoflurane anesthesia, using a copper-constantan thermocouple (Physitemp Instruments, Inc. Clifton, NJ, USA) inserted into the right iBAT pad. During the measurement, core temperature was monitored with a second thermocouple inserted 6 cm into the rectum (20). Skin temperature was also assessed with a third copper-constantan thermocouple placed on the left-rear leg. Core and skin measurements were performed before the MPO infusions in order to avoid body temperature variations. Measurement of CO₂ exhaled was assessed using a CO₂ analyser (model S-3A, Applied Electrochemistry Incorporated, Sunnyvale, CA, USA). CO₂ exhaled was used to gauge whole-body thermogenesis. VO₂ could not be measured for technical reasons inherent to the use of the isoflurane mask during the MPO infusions of MTII.

In addition to the iBAT temperature and CO₂ exhaled measurements, we also measured iBAT, iWAT, liver and heart ¹⁴C-bromopalmitate ([¹⁴C]-2-bromopalmitic acid) and ³H-deoxyglucose ([¹,²-³H(N)]-Deoxy-D-glucose) uptakes. ¹⁴C-bromopalmitate was used to assess non-esterified fatty acid (NEFA) uptake (28, 29) and to estimate lipid oxidation (10, 29, 36) and esterification (29), whereas ³H-deoxyglucose was used to trace glucose uptake (17). Both radioactive tracers (Moravek Biochemicals, Inc. Brea, CA, USA) were dissolved in normal saline supplemented with 4% bovine serum albumin (BSA). All experiments were performed after a 12-hour fasting period under anesthesia with 2.0% (vol/vol) isoflurane (Abbott Laboratories, Montreal, Canada) delivered through a nose cone. Catheters (PE-50) were placed into a carotid
artery for blood sampling. Thirty minutes after the MPO infusion of MTII, an intravenous bolus of 20 µCi of each tracer were injected into the tail vein, in a total volume of 350 µl. Arterial blood sampling was done from carotid prior to the MTII infusion at time -30, -15, 0 minutes for the measurement of plasma levels of insulin, NEFA and triglycerides (TG) as previously described (28). Additionally, blood was sampled at time 31, 32, 33, 35, 40, 50 and 60 minutes post MTII infusion to determine the radioactivity clearance. Sixty minutes after MTII infusion, animals were euthanized with an overdose of anesthetic and tissues were collected on ice, weighed and stored at -80°C pending analyses. iBAT, iWAT, liver and heart-specific uptakes of 14C-bromopalmitate and 3H-deoxyglucose were determined on samples of tissues (~250 mg), which were homogenized (Polytron PT10-35, Kinematica, inc. Bohemia, NY, USA) in ice-cold Chappell-Perry buffer (0.075 M sucrose, 0.225 M sorbitol, 1 mM EGTA, 0.1% fatty-acid-free BSA, and 10 mM Tris–HCl, pH 7.4). An aliquot was kept to determine total [14C and 3H] activity in the tissue as previously described (29). Fractional uptakes of 14C-bromopalmitate and 3H-deoxyglucose were determined by measuring [14C] and [3H] activity in tissue extracts using a scintillation counter (liquid scintillation analyzer Tri-Carb 2900TR, PerkinElmer, Montreal, QC, Canada). Uptakes data were expressed as the percentage of injected dose (% of ID) of [14C] and [3H] recovered per gram of tissues.

**iBAT and iWAT expression of metabolic genes**

We determined the expression of genes involved in (i) energy substrate uptake [fatty acid binding protein 1 (Fabp1) and glucose transporter member 4 (Glut4)], (ii) lipid synthesis [diacylglycerol O-acyltransferase 1 and 2 (Dgat1 and Dgat2), fatty acid synthase (Fas), glycerol-3-phosphate acyltransferase 3 (Gpat3)] (iii) lipolysis [triglyceride lipase (Atgl), hormone sensitive lipase (Hsl)] and (iv) thermogenesis-related genes [carnitine palmitoyltransferase 1 β (Cpt1 β),
Type II iodothyronine deiodinase (Dio2), peroxisome proliferator-activated receptor-γ coactivator 1-alpha (Pgc1α), Ucp1. Briefly, RNA was isolated from iBAT and iWAT depots with QIAzol and the RNeasy Lipid Tissue Kit (QIAGEN, Mississauga, ON, Canada). Expand reverse transcriptase (Roche, Laval, QC, Canada) was used for cDNA synthesis, according to manufacturer's instructions. cDNAs were diluted in DNase-free water (1:25) before quantification by real-time PCR. The primers used for the PCR reactions are shown in Table 1. The mRNA transcript levels were measured in duplicates through chemical detection of the PCR products with SYBR® Green JumpStart™ Taq ReadyMix™ (#S5193, Sigma Aldrich, USA) using a Rotor Gene 3000 system (Montreal Biotech, Montreal, QC, Canada). At the end of each run, melt curve analyses were performed. A few samples, representative of each experimental group, were run on agarose gel and sequenced to ensure the specificity of the amplification. Slope of standard curve was used to evaluate PCR efficiency; an efficiency between 90 and 100% was considered acceptable. Results were expressed as the ratio of the expression of the target gene to that of the housekeeping gene acidic ribosomal phosphoprotein (Arbp), which was selected as no significant variation in its expression was observed after our treatment.

Statistical analyses

Values obtained from rats with improper placement of cannula were excluded from the statistical analyses. Results are expressed as means ± SEMs. Comparisons were done on normally distributed data using two-way ANOVAs followed by Bonferroni post hoc tests to assess the differences between various conditions with Graph pad Prism Software version 6.0 for Mac (San Diego, CA, USA). We considered the following comparisons: Differences were considered statistically significant when P values were less than 0.05.
RESULTS

MTII infusion in the MPO led to an increase (above aCSF) in iBAT temperature, which was abolished by DMH lesions (Figure 2, A-B). The infusion of MTII also produced an elevation in exhaled CO₂ (Figure 2, C-D), which did not reach statistical significance in rats with DMH lesions. The correlation between iBAT temperature elevation and whole body exhaled CO₂ (Figure 2, E) proved to be highly significant (r = 0.79, P < 0.001).

Together with measuring iBAT thermogenesis and exhaled CO₂, we also measured ¹⁴C-bromopalmitate and ³H-deoxyglucose uptakes in iBAT, iWAT, liver and heart (Figure 3). MPO infusion of MTII led to an increase (above aCSF) in ¹⁴C-bromopalmitate uptake in iBAT, which was prevented by DMH lesions (Figure 3, A). However, we did not observe any increase in iBAT ³H-deoxyglucose uptake following MTII (Figure 3, B), revealing NEFA uptake as a better predictor of iBAT thermogenesis than glucose uptake in the context of the present study. Correlation analyses, which showed a significant Pearson's r (0.63, P < 0.001) solely between iBAT temperature elevation and ¹⁴C-bromopalmitate uptake (Figure 3, C-D), support this deduction. MTII infusion in MPO increased (above aCSF) ¹⁴C-bromopalmitate and ³H-deoxyglucose uptakes in iWAT (Figure 3, E-F). The effect was however statistically significant (P < 0.05) only for the ¹⁴C-bromopalmitate uptake. MPO infusion of MTII also stimulated liver ¹⁴C-bromopalmitate uptake, which was prevented by DMH lesions (Figure 3, G). MTII and DMH lesions did not influence liver ³H-deoxyglucose uptake (Figure 3, H). Finally, MTII did not increase heart ¹⁴C-bromopalmitate uptake (Figure 3, I), whereas DMH lesions significantly (P < 0.05) reduced heart ³H-deoxyglucose uptake, regardless of whether aCSF or MTII was injected in the MPO (Figure 3, H).
The effects of MTII and DMH lesions on the expression of genes involved in iBAT thermogenesis and metabolism are illustrated in figure 4A. We observed that MTII increased expression of genes involved in lipogenesis (Fas and Dgat2), lipolysis (Hsl) and thermogenesis (Pgc1a and Dio2) in rats with sham lesions. The effects of MTII were however abolished following DMH lesions. As revealed by the correlations presented in Figure 4, B-C, 14C-bromopalmitate uptake in iBAT correlated with the expression of Hsl (r = 0.62, P < 0.001), which is implicated in lipolysis, and Dgat2 (r = 0.51, P < 0.01), which is implicated in the synthesis of TG. Figure 4, D illustrates the effects of the various experimental treatments on the iWAT expression of the genes reported for iBAT in Figure 4, A. In animals with sham lesions, MTII did not lead to any increase in gene expression. In fact, it reduced Glut4 and Gpat3 expressions. However, in rats with DMH lesions, MTII induced Glut4, Gpat3, Dgat1, Dgat2, Hsl, Atgl and Cpt1β. As illustrated in Figure 4, E-F, the mRNA expression of Fas, which is implicated in the synthesis of TG, correlated with 14C-bromopalmitate (r = 0.46, P = 0.02) and 3H-deoxyglucose (r = 0.59, P < 0.01) uptakes in iWAT.

Table 2 presents the differences (Δ) between post- and pre-infusion levels of circulating NEFA, TG, and insulin. Rats with DMH lesions showed a Δ insulin that was significantly lower than that of aCSF animals with sham lesions. Finally, the averaged weight of rats with DMH lesions at the time of MTII infusion was significantly lower (P < 0.05) than that of animals with sham lesions (331.9 ± 3.9 vs 347.8 ± 4.2). Such an effect had been reporter before (4).
DISCUSSION

The present results point to the MPO as a site of the thermogenic and metabolic actions of MTII, the synthetic analog of α-MSH and a MC4R/MC3R agonist. The MPO injection of MTII led to an increase in iBAT temperature, which was accompanied by increases in iBAT $^{14}$C-bromopalmitate uptake and iBAT expression of genes involved in thermogenesis and energy metabolism. This study also demonstrated the role of DMH in the effects of the MPO infusion of MTII. DMH lesions caused by a neurotoxic dose of kainic acid blunted the MTII-induced increases in iBAT temperature, $^{14}$C-bromopalmitate uptake and iBAT expression of metabolic/thermogenic genes. Finally, the present results also suggest that the MPO melanocortin receptors could, through the MPO-DMH duet, be involved in energy substrate metabolism in other tissues such as iWAT and liver.

The injection of MTII into the MPO led to an increase in iBAT temperature, which was abolished by DMH lesions. Together with revealing a new site of the thermogenic action of MTII, these findings further support the role of the POA (which comprises the MPO) and DMH in the control of iBAT thermogenesis. These two structures have indeed been flagged to for their participation in cold-induced thermogenesis (30). The POA has also been reported to be the site of the thermogenic action of corticotropin-releasing factor (8, 11) and prostaglandin E2 (2, 27), which seemingly act by releasing the inhibitory effect of POA GABA neurons on the DMH to stimulate the RPa neurons governing the SNS outflow to iBAT (8, 30). The DMH neurons are likely to be glutamatergic excitatory neurons (32). Within the POA, both the MC4R and MC3R are expressed in relative abundance in the MPO (33). However, the MC4R is most probably the melanocortin receptor mediating the thermogenic effect of MTII since it is expressed in neurons that are polysynaptically connected to iBAT (41) and iWAT (40) via the SNS. Further supporting
this assumption are recent results from our group demonstrating that the iBAT-stimulating effect of a single icv (3rd ventricle) administration of MTII can be blocked by HS024 (a MC4R antagonist) also administered into the 3rd ventricle, 15 minutes prior to MTII. (Monge-Roferallo, B; Labbé, S and Richard, D, unpublished results). Finally, the observation that the MTII infusion in the MPO caused an increase in BAT thermogenesis further supports the integrative role of the POA in the effects of the melanocortins in energy balance regulation. This role was suggested in a previous investigation pointing to the MPO as one of the major sites of the hypophagic action of α-MSH (23).

The DMH-dependent stimulating effect of the MPO infusion of MTII on iBAT temperature was accompanied by an increase in iBAT 14C-bromopalmitate uptake. This tracer is taken up by cells as native NEFA and is trapped in mitochondria (29). 14C-bromopalmitate uptake correlated well with the change in iBAT temperature. This was not the case with 3H-deoxyglucose uptake. We cannot provide a clear explanation for the observation that MTII increased BAT uptake of 14C-bromopalmitate but not that of 3H-deoxyglucose, which appeared as a poor predictor of the MPO MTII effects on BAT thermogenesis. That 3H-deoxyglucose uptake would have been more over a measurement period longer than 60 minutes remains a possibility. 14C-bromopalmitate uptake also significantly correlated with iBAT Hsl and Dgat2 mRNA expression further indicating that 14C-bromopalmitate uptake can be used to predict lipid oxidation (10, 29, 36) and esterification (29). Noteworthily, Hsl (lipolysis) and Dgat2 (lipogenesis) were among those genes, which also included Fatp1 (fatty acid uptake), Fas (de novo lipogenesis) and Pgc1a and Dio2 (thermogenesis), whose expression underwent a DMH-dependent stimulating effect of MTII. We did not observe any induction of Ucp1 following
MTII, likely due to the short time period (one hour) elapsing between the MTII treatment and the killing of the rats.

We also observed DMH-dependent metabolic effects of the MPO infusion of MTII in tissues other than iBAT. Indeed, MPO infusion of MTII increased $^{14}$C-bromopalmitate uptake in the liver and iWAT and DMH lesions blunted those effects. Such findings further support the extensive role for the MPO melanocortin receptors and POA/DMH duet in energy metabolism (30, 38). Given the recognition of iWAT as a WAT depot capable of developing brown adipocytes (beige depot) (3) and the neuroanatomical link between iWAT and the POA via the SNS (40), we choose to examine the same panel of genes in this fat depot as the one we scrutinized in iBAT. The stimulating effects of MTII were however only seen in iWAT of rats with DMH lesions. Indeed, in those rats, MTII induced genes involved in the glucose transport ($\text{Glut4}$), lipogenesis ($\text{Gpat3, Dgat1 and Dgat2}$), lipolysis ($\text{Hsl, Atgl}$) and thermogenesis/metabolism ($\text{Cpt1\beta}$). The different effects seen in iBAT and iWAT cannot be easily explained. Nonetheless, these findings demonstrate the importance of the DMH in modulating and potentially fine-tuning energy homeostasis. DMH comprises excitatory glutamatergic neurons that were reported to be key players in the control of SNS-mediated iBAT thermogenesis (31, 32, 35). It seems reasonable to assume that those glutamatergic neurons were affected by kainic acid (1, 4), which prevented the temperature rise in iBAT induced by the MPO infusion of MTII. On the other hand, the increased expression of genes such as $\text{Hsl, Atgl}$ and $\text{Cpt1\beta}$, which we saw in iWAT after MTII only in rats with damaged DMH, suggests an enhanced iWAT metabolic activity, reminiscent of the effects seen after DMH NPY knockdown (5). Knockdown of DMH NPY has indeed been reported to stimulate metabolism and the browning of iWAT (due to the development of brown adipocytes) (5). The DMH neurons can
produce NPY in neurons that also expressed CART (25) and enhanced expression of NPY in the 
DMH has been associated with excess fat deposition (45), high fat feeding (25) and lactation (9). 
The possibility that the NPY-expressing neurons could have been damaged by kainic acid cannot 
be excluded. Further studies are however needed to verify this possibility and further clarify the 
complex interplay between the POA and DMH in iWAT metabolism.

In conclusion, the present results revealed the MPO as a site of the thermogenic and 
metabolic actions of MTII. They also established the MPO-DMH duet as a target for the 
melanocortin effects on energy homeostasis
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Perspectives and significance

BAT is a thermogenic organ richly innervated by the SNS. BAT is involved in thermoregulatory thermogenesis and in energy balance regulation. Accordingly, the brain regions connected to BAT via the SNS outflow are known to play a role in thermoregulation as well as energy balance regulation. One of the brain regions recognized to be a major site of control of the SNS-mediated BAT thermogenesis is the preoptic area (30). The preoptic area comprises neurons expressing the MC4R mRNA (19), which are concentrated in the MPO. Moreover, we know from previous investigations (41) that the MPO MC4R neurons are connected to iBAT. In this study, we demonstrated that the infusion of MTII into the MPO could stimulate iBAT thermogenesis, which strongly supports the view that the MPO MC4R neurons are thermogenic neurons. We additionally demonstrate the involvement of the DMH in the thermogenic response led to by the MPO infusion of MTII. Altogether the present study further emphasizes the role of the MC4R and hence the melanocortin system in the control of BAT thermogenesis. It also supports the role of the MPO-DMH duet in the control of BAT thermogenesis. Moreover, the present results lay the foundations for future studies aimed at identifying the signatures of the MPO and DMH neurons involved in the MTII effects on iBAT thermogenesis to ultimately further delineate the neuronal circuits responsible for SNS-mediated BAT thermogenesis. Finally, the DMH, with its NPY-expressing neurons, would seem of particular importance not only for the control of the SNS-mediated BAT thermogenesis but also for the modulation of WAT metabolism and possibly the brown adipocyte development in the inguinal depot of fat.
REFERENCES


LEGENDS

Figure 1. A) Schematic representation of the MPO infusions of MTII in the MPO. The different groups are presented as follows: white circles, aCSF-Sham lesions; black circles, MTII-Sham lesions; white squares, aCSF-DMH lesions; black squares, MTII-DMH lesions). B) Pictures of brain coronal sections taken at the level of DMH in rats with sham and DMH lesions.

Figure 2. Effects of the MPO infusion of MTII in rats with sham and DMH lesions on iBAT and whole body thermogenesis. Four distinct groups of 6-7 rats were used to test the effect of MTII (0 or 100 ng). The area under the curve (AUC — from baseline) for Δ-iBAT temperature (A, B), CO₂ exhaled (indirect assessment of whole body thermogenesis) (C, D) were measured over 60 minutes following the MTII infusion. (E) Correlation between the AUC of Δ-iBAT temperature and CO₂ exhaled. * P < 0.05, MTII vs. aCSF within the same level of lesions (Sham or DMH); # P < 0.05, Sham vs DMH lesions within the same level of MPO infusion (aCSF or MTII).

Figure 3. Effects of the MPO infusion of MTII on iBAT, iWAT, liver and heart metabolic activity in rats with sham and DMH lesions. Four distinct groups of 6-7 rats were used to test the effect of MTII (0 or 100 ng) on ¹⁴C-bromopalmitate (A) and ³H-deoxyglucose (B) uptakes in iBAT, on ¹⁴C-bromopalmitate (E) and ³H-deoxyglucose (F) uptakes in iWAT, on ¹⁴C-bromopalmitate (G) and ³H-deoxyglucose (H) uptakes in liver and on ¹⁴C-bromopalmitate (I) and ³H-deoxyglucose (J) uptakes in heart. (C) Correlation between ¹⁴C-bromopalmitate uptake and AUC of Δ-iBAT temperature. (D) Correlation between ³H-deoxyglucose uptake and AUC of Δ-iBAT temperature. * P < 0.05, MTII vs. aCSF within the same level of lesions (Sham or DMH); # P < 0.05, Sham vs DMH lesions within the same level of MPO infusion (aCSF or MTII).
**Figure 4.** Effects of the MPO infusion of MTII on iBAT and iWAT gene expression in rats with sham and DMH lesions. Four distinct groups of 6-7 rats were used to test the effects of MTII (0 or 100 ng) on iBAT (A) and iWAT (D) mRNA expression, which were measured in rats sacrificed 1 hour after MTII infusion. (C) Correlation between $^{14}$C-bromopalmitate uptake and Hsl mRNA expression in iBAT. (D) Correlation between $^{14}$C-bromopalmitate uptake and Dgat2 mRNA expression in iBAT. (E) Correlation between $^{14}$C-bromopalmitate uptake and Fas mRNA expression in iWAT. (F) Correlation between between $^{3}$H-deoxyglucose uptake and Fas mRNA expression in iWAT. * $P < 0.05$, MTII vs. aCSF within the same level of lesions (Sham or DMH); # $P < 0.05$, Sham vs DMH lesions within the same level of MPO infusion (aCSF or MTII).
Bregma - 0.48mm

Bregma - 0.60mm

Bregma - 0.72mm

Bregma - 0.84mm

Δ-iBAT temperature 30 minutes after MTII infusion

| 0.3°C | 1.3°C | 2.3°C |

A

B

Sham-lesions

DMH-lesions
Brown adipose tissue

% Injected dose of $[^{14}C]$-bromopalmitate / gram

Sham-lesions  |  DMH-lesions
---|---
aCSF | MTII

$P < 0.001$  
$r = 0.632$

AUC Δ-iBAT temperature

-50  |  0  |  50  |  100  |  150

Inguinal white adipose tissue

% Injected dose of $[^{3}H]$-deoxyglucose / gram

Sham-lesions  |  DMH-lesions
---|---
aCSF | MTII

$P = 0.11$  
$r = 0.31$

Liver

% Injected dose of $[^{3}H]$-deoxyglucose / gram

Sham-lesions  |  DMH-lesions
---|---
aCSF | MTII

Heart

% Injected dose of $[^{14}C]$-bromopalmitate / gram

Sham-lesions  |  DMH-lesions
---|---
aCSF | MTII
**iBAT gene expression / ARBP mRNA**

- aCSF-Sham-lesions
- MTII-Sham-lesions
- aCSF-DMH-lesions
- MTII-DMH-lesions

**iWAT gene expression / ARBP mRNA**

- aCSF-Sham-lesions
- MTII-Sham-lesions
- aCSF-DMH-lesions
- MTII-DMH-lesions

**Brown adipose tissue**

- Hsl mRNA expression
  - $r = 0.62$ P < 0.001

- Dgat2 mRNA expression
  - $r = 0.51$ P < 0.01

**Inguinal white adipose tissue**

- Fas mRNA expression
  - $r = 0.46$ P = 0.02

- Fas mRNA expression
  - $r = 0.59$ P < 0.01
Table 1. qPCR gene information.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>5’ Primer (5’-3’)</th>
<th>3’ Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbp</td>
<td>NM_022402.2</td>
<td>TAAAGACTGGAGACAAGGTG</td>
<td>GTGTAGTCAGTCTCCACAGA</td>
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<tr>
<td>Atgl</td>
<td>NM_001108509.2</td>
<td>CACTTTAGCTCCAAGGATGA</td>
<td>TGGTTCAGTAGGCCATTCCT</td>
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<tr>
<td>Cpt1b</td>
<td>NM_013200</td>
<td>CGGAAGCACAACAGGAAGTA</td>
<td>GCAGCTTCAGGGGTTGTCGGAATA</td>
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<tr>
<td>Dgat1</td>
<td>NM_053437</td>
<td>TATTACTTCTCTTTGCTCC</td>
<td>AAAGTAGGTGACAGACTCAG</td>
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<tr>
<td>Dgat2</td>
<td>NM_001012345</td>
<td>TGCCCGCAGCGAGAACAAGAATAA</td>
<td>GCACGTTCCAGTCAAATGCCA</td>
</tr>
<tr>
<td>Dio2</td>
<td>NM_031720</td>
<td>ATGGGACTCTCTACGCGTAGA</td>
<td>GCACAGGCAAAGCTCAAGAAG</td>
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<tr>
<td>Fabp1</td>
<td>NM_012556.2</td>
<td>GCCAAGAGAACTTTGAGGCC</td>
<td>CTTGTAAGAGATGTACCCAGT</td>
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<td>Fas</td>
<td>NM_017332</td>
<td>GAGTCCGAGTCTGTCTCCGCTTG</td>
<td>GCCGTAGGTTGCTGTTGTTGCTTAG</td>
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<td>Glut4</td>
<td>NM_012751</td>
<td>AGTGACTGGGACACTGCTGCTT</td>
<td>ACATTGTGGGCGAGCATAGC</td>
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<td>Gpat3</td>
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<td>GCCCACTTGTCAATTACAGA</td>
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<tr>
<td>Hsl</td>
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<td>CCTCGATCTCCGGTGATATTCAGA</td>
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<tr>
<td>Pgc1a</td>
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<td>AACCCATAGCTGGTCCCATCCATCC</td>
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<tr>
<td>Ucp1</td>
<td>NM_012682</td>
<td>TGGTGAGGTCAGCAACTTCC</td>
<td>GTGGGCTGCAATGAGAATAC</td>
</tr>
</tbody>
</table>

Table 2. Effects of MTII infusion in MPO on blood variation of insulin, NEFA and TG in Sham- and DMH-lesioned rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Δ-Insulin (pmol/L)</th>
<th>Δ-NEFA (mmol/L)</th>
<th>Δ-TG (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF-Sham-lesioned</td>
<td>-28.29±5.99</td>
<td>-0.127±0.028</td>
<td>-0.168±0.248</td>
</tr>
<tr>
<td>MTII-Sham-lesioned</td>
<td>-25.37±10.62</td>
<td>-0.011±0.050</td>
<td>-0.104±0.071</td>
</tr>
<tr>
<td>aCSF-DMH-lesioned</td>
<td>-29.18±8.76</td>
<td>0.020±0.073</td>
<td>0.048±0.117</td>
</tr>
<tr>
<td>MTII-DMH-lesioned</td>
<td>-71.08±10.50*</td>
<td>-0.035±0.029</td>
<td>-0.002±0.150</td>
</tr>
</tbody>
</table>