

**Distinct effects of leptin and melanocortin agonist in medial  
hypothalamic nuclei on glucose uptake in peripheral tissues**

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## **ABSTRACT**

### **OBJECTIVE**

The medial hypothalamus mediates leptin-induced glucose uptake in peripheral tissues, and brain melanocortin receptors (MCR) mediate certain central effects of leptin. However, the contributions of the leptin receptor and MCR in individual medial hypothalamic nuclei to regulation of peripheral glucose uptake have remained unclear. In the present study, 1) I examined effects of injection of leptin into medial hypothalamic nuclei on glucose uptake in peripheral tissues. 2) I also examined the effect of anti-leptin antibody on glucose uptake in peripheral tissues in response to intraperitoneal (i.p.) injection of leptin. Finally, 3) I explored the role of brain MCR in leptin-induced glucose uptake in peripheral tissues: I examined, a) the effects of the injection of the MCR agonist MT-II into the lateral ventricle (i.c.v.) and individual medial hypothalamic nuclei on tissue glucose uptake, and b) effects of the MCR antagonist SHU9119 injected into i.c.v. on leptin-induced tissue glucose uptake.

### **RESEARCH DESIGN AND METHODS**

Leptin was injected into the VMH, dorsomedial (DMH), arcuate (ARC), paraventricular (PVH) hypothalamus, the lateral ventricle (i.c.v.) or i.p. in freely moving mice. MT-II was also injected into the medial hypothalamic nuclei, or i.c.v. in mice. The anti-leptin antibody

was injected into VMH. The MCR antagonist SHU9119 was injected i.c.v. Glucose uptake was measured by the 2-[<sup>3</sup>H]deoxy-D-glucose (2DG) method.

## **RESULTS**

Leptin injection either into VMH or i.p. increased 2DG uptake in skeletal muscle, brown adipose tissue (BAT) and heart, but not spleen or white adipose tissue, whereas that into ARC increased glucose uptake in BAT alone and that into DMH or PVH had no effect. Injection of anti-leptin antibody into VMH inhibited 2DG uptake in peripheral tissues in response to i.p. injection of leptin. Injection of MT-II either into VMH or i.c.v. also increased 2DG uptake in skeletal muscle, BAT, and heart, whereas that into PVH increased 2DG uptake in BAT alone and that into DMH or ARC had no effect. Injection of SHU9119 into i.c.v. abolished the effects of leptin injection into VMH.

## **CONCLUSIONS**

VMH is an important site for leptin-induced glucose uptake in skeletal muscle, BAT, and heart. The effects of leptin are dependent on MCR activation. The VMH also mediates MT-II-induced glucose uptake in skeletal muscle, BAT, and heart. The leptin receptor in ARC and MCR in PVH regulate glucose uptake in BAT. Medial hypothalamic nuclei thus play distinct roles in leptin- and MT-II-induced glucose uptake in peripheral tissues.

## INTRODUCTION

Leptin is an adipocyte hormone that inhibits food intake and increases energy expenditure (1).

The hypothalamus is a principal target of leptin in its regulation of energy metabolism (2–6).

The arcuate nucleus (ARC) is the most well characterized of hypothalamic nuclei in terms of its role in the central effects of leptin (2–6). ARC contains two populations of leptin-responsive neurons: pro-opiomelanocortin (POMC)-expressing neurons that release the potent anorexic peptide  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH), and neurons that release two potent orexigenic peptides, agouti-related peptide (AgRP) and neuropeptide Y (NPY) (2–6).  $\alpha$ -MSH activates the melanocortin receptor (MCR), whereas AgRP competitively inhibits this receptor and NPY functionally antagonizes MCR signaling (7). Both sets of neurons project to second-order MCR-expressing neurons within the hypothalamus, including the paraventricular (PVH), ventromedial (VMH), dorsomedial (DMH), and lateral hypothalamus, as well as to other brain regions such as the brain stem (2, 5, 8, 9). Leptin inhibits food intake through reciprocal regulation of POMC and AgRP/NPY neurons in ARC and consequent activation of MCR in hypothalamic nuclei including PVH (7, 10). Mice lacking the melanocortin 3 (MC3R) or 4 (MC4R) receptor show increased adiposity and feeding efficiency (5). Restoration of MC4R expression in certain sets of PVH neurons

prevented hyperphagia and reduced body weight in MC4R-null mice (10). In addition to that in ARC, the leptin receptor Ob-Rb in other hypothalamic nuclei has also been shown to regulate energy intake and adiposity. Neurons positive for steroidogenic factor 1 (SF1, also known as Ad4BP) (11, 12) are largely restricted to VMH in the adult brain. Leptin depolarizes these neurons, and specific ablation of the leptin receptor in SF1-positive cells induced obesity and increased susceptibility to a high-fat diet in mice (13).

The leptin receptor in the brain also regulates glucose metabolism in certain peripheral tissues (14–18). Treatment with leptin ameliorates diabetes in lipodystrophic mice and humans (19, 20). Intravenous or intracerebroventricular (i.c.v.) administration of leptin markedly increased whole-body glucose turnover and glucose uptake by certain tissues in mice without any substantial change in plasma insulin or glucose levels (14). Our group have also previously shown that microinjection of leptin into the medial hypothalamus, such as into VMH, but not into the lateral hypothalamus, preferentially increased glucose uptake in skeletal muscle, heart, and brown adipose tissue (BAT) (16, 17). Moreover, leptin injection into the medial hypothalamus enhanced insulin sensitivity of peripheral tissues in a manner dependent on the sympathetic nervous system and  $\beta$ -adrenergic signaling (15). Restoration of Ob-Rb expression in ARC and VMH of the Ob-Rb-mutated Koletsky rat by adenovirus- or

adeno-associated virus-mediated gene transfer improved peripheral insulin sensitivity and reduced plasma glucose concentration (18, 21). Ablation of suppressor of cytokine signaling 3 (SOCS3) in SF1-positive cells (11, 12) also improved glucose homeostasis in mice fed a high-fat diet (22). Furthermore, i.c.v. injection of the MCR agonist (MT-II) increased whole-body glucose turnover and expression of GLUT4 in skeletal muscle (23). The leptin receptor in ARC and VMH as well as the brain melanocortin pathway are thus implicated in the regulation of glucose uptake in peripheral tissues as well as in energy metabolism. However, relatively little is known about the contributions of the leptin receptor and MCR in individual medial hypothalamic nuclei to regulation of glucose uptake in peripheral tissues, as opposed to their roles in the regulation of food intake and leanness.

I have now examined the acute effects of microinjection of leptin and MT-II into VMH, ARC, DMH, and PVH, all of which express Ob-Rb, MC3R, and MC4R at a high level (4–6, 8, 24–27), on glucose uptake in peripheral tissues of mice in vivo. The results suggest that VMH mediates stimulatory actions of leptin and MT-II on glucose uptake in skeletal muscle, heart, and BAT, whereas the leptin receptor in ARC as well as MCR in PVH regulate glucose uptake in BAT. The medial hypothalamic nuclei thus appear to have different roles in the regulation of glucose uptake in peripheral tissues by leptin and MT-II, and VMH plays an important role

in glucose uptake in peripheral tissues.

## **METHODS**

### **Animals**

Male FVB mice (CLEA Japan, Tokyo, Japan) were studied at 12 to 16 weeks of age. The animals were housed individually in plastic cages at  $24^{\circ} \pm 1^{\circ}\text{C}$  with lights on from 06:00 to 18:00 hours, and they were maintained with free access to a laboratory diet (Oriental Yeast, Tokyo, Japan) and water. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg per kilogram of body mass) and xylazine (10 mg/kg), and a chronic double-walled stainless steel cannula was implanted stereotaxically and unilaterally into the right side of VMH, ARC, DMH or PVH, or into the lateral ventricle according to the atlas of Franklin and Paxinos (28). The stereotaxic coordinates were AP 1.3 (1.3 mm anterior to the bregma), L 0.3 (0.3 mm lateral to the bregma), and H 5.8 (5.8 mm below the bregma on the surface of the skull) for VMH; AP 1.6, L 0.2, and H 6.1 for ARC; AP 1.6, L 0.3, and H 5.5 for DMH; AP 0.75, L 0.2, and H 4.9 for PVH; and AP 0.3, L 1.0, and H 2.25 for the lateral ventricle. Cannulas were anchored firmly to the skull with acrylic dental cement. In experiments for administration of anti-leptin antibody, cannulas were implanted bilaterally into VMH. In experiments featuring administration of the MCR antagonist SHU9119, cannulas were implanted both into VMH and i.c.v. for injections of leptin and SHU9119, respectively.

Three days before determination of tissue glucose uptake, a silicone catheter was implanted into the right atrium of mice through the external jugular vein. Animals were handled repeatedly during the recovery period (2 weeks) after brain cannula implantation to habituate them to the injection and blood sampling procedures. Correct placement of the cannula tips was verified microscopically in brain sections in every experiment, with >90% of animals manifesting correct placement. Tyrosine phosphorylation of signal transducer and activator of transcription 3 (STAT3) in each medial hypothalamic nucleus was examined after injection of leptin, as described below. All experiments were performed with mice in the fed condition.

All animal experiments were performed in accordance with institutional guidelines for the care and handling of experimental animals, and they were approved by the ethics committee for animal experiments of the National Institute for Physiological Sciences.

#### **Administration of leptin, MT-II, SHU9119, and anti-leptin antibody**

Leptin (5 ng) (National Hormones & Pituitary Program, Torrance, CA) or MT-II (10 ng) (Phoenix Pharmaceuticals, Burlingame, CA) dissolved in 0.1  $\mu$ l of physiological saline was injected with the use of a Hamilton microsyringe into the right side of VMH, DMH, PVH, or ARC of freely moving mice through the unilateral cannula implanted into the corresponding

nucleus. MT-II (3  $\mu$ g) dissolved in 0.5  $\mu$ l of saline was injected into the lateral ventricle. The concentrations of leptin (3 $\mu$ M) and MT-II (100 $\mu$ M) injected into the medial hypothalamic nuclei are pharmacologically maximum to activate leptin receptor and MCR respectively (29-31). Anti-leptin-neutralizing antibody (0.3 ng) (R&D systems, Inc., Mineapolis, MN) (32) dissolved in 0.1  $\mu$ l of saline was injected bilaterally into VMH 30 minutes before i.p. injection of leptin (5mg/kg). SHU9119 (1  $\mu$ g) (Phoenix Pharmaceuticals) dissolved in 0.5  $\mu$ l of saline was injected i.c.v. immediately before leptin injection. Control animals received 0.1  $\mu$ l of saline delivered into the various nuclei or 0.5  $\mu$ l of saline delivered i.c.v., respectively. Food was removed just before the administrations.

#### **Measurement of the rate constant of 2[<sup>3</sup>H]DG uptake in peripheral tissues**

The rate constant of net tissue uptake of 2-[<sup>3</sup>H]deoxy-D-glucose (2[<sup>3</sup>H]DG) in peripheral tissues was determined as described previously (33-35). A mixture of 6.25  $\mu$ Ci of 2[<sup>3</sup>H]DG (10 Ci/mmol) and 1.25  $\mu$ Ci of [<sup>14</sup>C]sucrose (10 Ci/mmol) (American Radiolabeled Chemicals, St. Louis, MO) dissolved in 50  $\mu$ l of saline was injected through the jugular vein catheter 3 or 6 h after the microinjection of leptin or MT-II. Blood was collected 0, 10, 15, and 20 min after injection of the radioactive tracers. Immediately after collection of the final blood sample (20 min), an overdose of pentobarbital sodium (100 mg/kg) was injected through the jugular vein

catheter and the mice were rapidly decapitated. Correct placement of the cannula was verified microscopically in brain sections, and hypothalamic nuclei were then rapidly dissected as described below and frozen in liquid nitrogen for subsequent immunoblot analysis. Skeletal muscle [soleus, red and white portions of the gastrocnemius, and extensor digitorum longus (EDL)] as well as interscapular BAT, heart, spleen, and epididymal white adipose tissue (WAT) were rapidly dissected and weighed. The tissue samples were then homogenized, the homogenates were centrifuged to remove debris, and the resulting supernatants as well as plasma samples were assayed for radioactivity. Plasma samples were also analyzed for glucose (Glucose CII Test; Wako, Osaka, Japan) and insulin [Mouse Insulin ELISA Kit (U-Type); Shibayagi, Gunma, Japan] concentrations. The rate constant of net tissue uptake of  $2[{}^3\text{H}]\text{DG}$  was calculated as described previously (34, 35). The radioactivity of  $[{}^{14}\text{C}]\text{sucrose}$  in tissues was used to calculate the  $2[{}^3\text{H}]\text{DG}$  radioactivity remaining in the extracellular space (34, 35).

### **Sampling of medial hypothalamic nuclei**

Medial hypothalamic nuclei were isolated as described previously (36). The accuracy of the dissection was assessed by measurement (as described below) of mRNAs for neuropeptides or transcription factors: corticotropin-releasing factor (CRF) mRNA for PVH (37), POMC and

NPY mRNAs for ARC (38, 39), SF1 mRNA for VMH (11, 12), and the absence of these various mRNAs for DMH. The right side of PVH, ARC, VMH, or DMH was dissected from a 1-mm-thick sagittal section prepared from the midline of the fresh brain (Supplemental Figure 1). PVH was collected as the square area with an anterior margin of the border with the posterior region of the anterior commissure, a dorsal margin of the border with the thalamus, a ventral margin 1 mm ventral to the border with the thalamus, and a posterior margin of the white matter separating PVH from VMH-DMH. ARC was isolated as the ventral portion of the medial hypothalamus with a dorsal margin of the border with the ventral part of VMH and DMH. VMH and DMH were collected from the triangular area with an anterior-dorsal margin of the white matter separating PVH and the anterior hypothalamus from VMH-DMH, a ventral margin of the border with ARC, and a posterior margin of the border with the mammillary body.

### **Immunoblot analysis**

Hypothalamic nuclei were homogenized at 4°C in phosphate-buffered saline containing 1% Nonidet P-40. The homogenates were centrifuged at  $14,000 \times g$  for 20 min at 4°C, and the resulting supernatants (5 µg of protein) were fractionated by SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane, which was then exposed

to 5% dried skim milk in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 (TBST) before incubation for 16 h at 4°C in TBST containing 5% bovine serum albumin and primary antibodies (1 µg/ml) including those to the Tyr<sup>705</sup>-phosphorylated or total forms of STAT3 (Cell Signaling Technology, Danvers MA), to c-FOS (Santa Cruz Biotechnology, Santa Cruz, CA), or to β-actin (Cell Signaling Technology). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and enhanced chemiluminescence reagents (GE healthcare, Tokyo, Japan). Protein bands were quantified using Image J software (National Institutes of Health, <http://rsbweb.nih.gov/ij/>).

### **RNA extraction and RT-PCR analysis**

Total RNA was isolated from hypothalamic tissue with the use of Isogen (Nippon Gene, Wako, Japan), and portions of the RNA (300 ng) were subjected to reverse transcription (RT) with an oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (Takara, Shiga, Japan). The resulting cDNA was subjected to the polymerase chain reaction (PCR) with LA Taq (Takara) and primers obtained from Sigma Genosys (Ishikari, Japan). The primers (forward and reverse, respectively) included 5'-GCTAACTTTTTCCGCGTGTT-3' and 5'-GGTGAAGGTGAGATCCAGA-3' for CRF, 5'-ACCACGGAGAGCAACCTGCT-3' and

5'-CATGGAGTAGGAGCGCTTGC-3' for POMC, 5'-CTAGGTAACAAGCGAATGGG-3' and 5'-AATCAGTGTCTCAGGGCT-3' for NPY, 5'-GCCAGGAGTTCGTCTGTCTC-3' and 5'-ACCTCCACCAGGCACAATAG-3' for Ad4BP/SF1, and 5'-TTCATGATGTGACCCTGCAT-3' and 5'-CACTTGCTCAGGACACTGGA-3' for eukaryotic elongation factor 2 (eEF2).

### **Statistical analysis**

Data are presented as means  $\pm$  standard error of the mean (SEM). Statistical analysis of STAT3 phosphorylation and c-FOS expression was performed by Student's *t* test, and that for other experiments was performed by analysis of variance followed by Dunnett's test. A *P* value of  $<0.05$  was considered statistically significant.

## RESULTS

### Effects of leptin injection into medial hypothalamic nuclei on glucose uptake in peripheral tissues

Microinjection of leptin (5 ng) into VMH induced a significant increase in the rate constant of  $2[{}^3\text{H}]\text{DG}$  uptake in the red type of skeletal muscle (soleus and red portion of gastrocnemius), mixed type of skeletal muscle (EDL), BAT, and heart, but not in spleen or epididymal WAT (Fig. 1).  $2[{}^3\text{H}]\text{DG}$  uptake in red or mixed skeletal muscle was significantly increased at 6 h after leptin injection (Fig. 1A), whereas that in BAT and heart was increased at both 3 and 6 h (Fig. 1B).  $2[{}^3\text{H}]\text{DG}$  uptake in the white portion of the gastrocnemius showed a tendency to increase in response to leptin, but the change was not statistically significant.  $2[{}^3\text{H}]\text{DG}$  uptake in peripheral tissues at 3 h after saline injection into VMH did not differ from that apparent at 6 h (data not shown).

Injection of leptin into ARC induced a small but significant increase in the rate constant of  $2[{}^3\text{H}]\text{DG}$  uptake in BAT at 6 h after injection, but it had no effect on  $2[{}^3\text{H}]\text{DG}$  uptake in skeletal muscle, heart, spleen, or WAT (Fig. 2). Injection of leptin into DMH or PVH had no effect on  $2[{}^3\text{H}]\text{DG}$  uptake in peripheral tissues (Fig. 2).  $2[{}^3\text{H}]\text{DG}$  uptake in peripheral tissues after saline injection into DMH or PVH did not differ from that apparent

after saline injection into ARC (data not shown). Plasma glucose and insulin concentrations were not affected by leptin injection into VMH or other hypothalamic nuclei (Table 1), consistent with previous observations (14, 16).

To determine whether leptin activated Ob-Rb in the hypothalamic nuclei, I examined the tyrosine phosphorylation of STAT3 in tissue samples therefrom. RT-PCR analysis confirmed that the isolated PVH, ARC, and VMH specimens were enriched in CRF mRNA, POMC and NPY mRNAs, and SF1 mRNA, respectively, and that DMH was largely devoid of these mRNAs (Fig. 3A). The medial hypothalamic nuclei were thus accurately dissected. Microinjection of leptin into VMH, DMH, or PVH preferentially increased the tyrosine phosphorylation of STAT3 in the corresponding nucleus at 6 h after injection (Fig. 3B–D). These results confirmed that leptin was correctly injected into these various hypothalamic nuclei. Injection of leptin into ARC increased the tyrosine phosphorylation of STAT3 in DMH as well as in ARC (Fig. 3E), possibly as a result of leakage of leptin into DMH through the surface of the injection cannula, given that the ARC cannula passed through DMH. Together, these results suggested that VMH is a key target of leptin in its regulation of glucose uptake in skeletal muscle, heart, and BAT, whereas the leptin receptor in ARC mediates stimulation of glucose uptake in BAT.

I next examined whether leptin injection into VMH might increase neuronal activity in other hypothalamic nuclei by measuring expression of the transcription factor c-FOS. Leptin injection into VMH significantly increased c-FOS expression in ARC as well as in VMH at 6 h after injection (Fig. 3F).

### **Effects of anti-leptin antibody injected into VMH on i.p. leptin-induced glucose uptake in peripheral tissues**

To examine the role of VMH in tissue glucose uptake induced by peripheral leptin, I injected anti-leptin antibody (3 ng) into VMH 30 minutes before i.p. injection of leptin (5mg/kg). I.p. injection of leptin increased  $2[{}^3\text{H}]\text{DG}$  uptake in soleus muscle, BAT and heart but not spleen or epididymal WAT at 7 h after the injection (Fig. 4), similar to those of leptin injection into VMH (Fig. 1). Pretreatment of anti-leptin antibody into VMH blunted the effects of leptin (Fig. 4). Injection of anti-leptin antibody alone did not alter  $2[{}^3\text{H}]\text{DG}$  uptake in the peripheral tissues (Fig. 4). Plasma glucose and insulin levels did not change in response to i.p. injection of leptin or intra-VMH injection of anti-leptin antibody (Table 1).

### **Effect of i.c.v. injection of an MCR antagonist on glucose uptake in peripheral tissues in response to leptin injection into VMH**

I next examined the role of MCRs in glucose uptake in peripheral tissues induced by injection

of leptin into VMH (Fig. 5). Injection of the MCR antagonist SHU9119 (1  $\mu$ g) into the lateral ventricle (i.c.v.) abolished the increase in 2[<sup>3</sup>H]DG uptake in peripheral tissues normally apparent at 6 h after the injection of leptin into VMH. Injection of SHU9119 alone did not affect 2[<sup>3</sup>H]DG uptake in peripheral tissues. Plasma glucose and insulin levels were also not changed in response to i.c.v. injection of SHU9119 (Table 1).

#### **Effects of i.c.v. injection of an MCR agonist on glucose uptake in peripheral tissues**

I tested the effects of i.c.v. injection of the MCR agonist MT-II on glucose uptake in peripheral tissues (Fig. 6). The i.c.v. injection of MT-II (3  $\mu$ g) increased 2[<sup>3</sup>H]DG uptake in BAT, heart, and all types of skeletal muscle, including the white portion of the gastrocnemius, but not in spleen or epididymal WAT, at 3 or 6 h after injection. 2[<sup>3</sup>H]DG uptake in peripheral tissues at 3 h after saline injection did not differ from that apparent at 6 h (data not shown). The i.c.v. injection of MT-II increased the plasma glucose level at both 3 and 6 h after injection (Table 1). These results thus suggested that i.c.v. injection of MT-II promotes glucose production as well as glucose uptake in certain peripheral tissues. In contrast, plasma insulin concentration did not change after i.c.v. injection of MT-II, despite the associated hyperglycemia, suggesting MT-II inhibits insulin secretion from pancreatic  $\beta$  cells.

#### **Effects of MT-II injected into medial hypothalamic nuclei on glucose uptake in**

### **peripheral tissues**

Finally, I examined the effects of direct injection of MT-II into the individual medial hypothalamic nuclei on glucose uptake in peripheral tissues (Fig. 7). Microinjection of MT-II (10 ng) into VMH increased  $2[{}^3\text{H}]\text{DG}$  uptake in BAT, heart, and all types of skeletal muscle, but not in spleen or epididymal WAT. In contrast, injection of MT-II into PVH increased  $2[{}^3\text{H}]\text{DG}$  uptake only in BAT, and that into DMH or ARC did not affect  $2[{}^3\text{H}]\text{DG}$  uptake in any of the peripheral tissues examined. Plasma glucose and insulin levels did not change in response to injection of MT-II into any of the hypothalamic nuclei (Table 1).

## DISCUSSION

Leptin is a physiologically and clinically important hormone that regulates glucose metabolism in peripheral tissues. The medial hypothalamus is implicated as a key target of leptin in its regulation of glucose uptake in peripheral tissues (15–17). I have now shown that activation of the leptin receptor specifically in VMH, as detected by measurement of the tyrosine phosphorylation of STAT3, resulted in a marked increase in glucose uptake in skeletal muscle, heart, and BAT, similar to the effects of i.c.v. or peripheral administration of leptin (14, 40 and Fig. 4). In contrast, injection of leptin into ARC increased glucose uptake only in BAT. Injection of leptin into DMH or PVH had no effect on glucose uptake in any of the peripheral tissues examined. Furthermore, pretreatment of anti-leptin antibody into VMH bunted the leptin (i.p.)-induced glucose uptake in peripheral tissues. These data thus suggest that the leptin receptor in VMH and ARC regulates glucose uptake in different peripheral tissues and that VMH plays an important role in the regulation of leptin-induced glucose uptake in peripheral tissues.

My present results also indicate that MCR activation is necessary for the increase in glucose uptake in peripheral tissues induced by injection of leptin into VMH. The i.c.v. injection of SHU9119 thus abolished the effect of leptin injected into VMH on peripheral

glucose uptake, whereas i.c.v. injection of MT-II increased glucose uptake in peripheral tissues. Moreover, injection of MT-II into VMH, but not that into DMH or ARC, increased glucose uptake in skeletal muscle, BAT, and heart, whereas injection of MT-II into PVH increased glucose uptake preferentially in BAT. VMH and PVH have been shown to express MC3R and MC4R abundantly (7-9, 24, 26, 27) and innervated by  $\alpha$ -MSH neurons (7, 9). These data suggest that MCR in VMH and PVH plays an important role in the regulation of glucose uptake in peripheral tissues.

POMC neurons in ARC receive strong excitatory input from the dorsomedial region of VMH (41). The restoration of Ob-Rb expression in VMH by adeno-associated virus-mediated gene transfer in Koletsky rats increased the amount of POMC mRNA in ARC (21). I have now shown that injection of leptin into VMH increased c-FOS expression in ARC without an effect on STAT3 phosphorylation in ARC. Furthermore, the effect of leptin injected into VMH on glucose uptake in peripheral tissues was found to be dependent on MCRs in the brain. I therefore propose that stimulation of VMH neurons by leptin results in activation of a set of POMC neurons in ARC and thereby increases glucose uptake in skeletal muscle, heart, and BAT (Fig. 8). MCRs in VMH and PVH may contribute to the up-regulation of glucose uptake in peripheral tissues induced by leptin injection into VMH. In VMH, leptin receptor Ob-Rb

expresses in the dorsomedial region abundantly (25), while axons and boutons immunoreactive  $\alpha$ -MSH were observed in the ventrolateral region of the VMH and the VMH shell (9). Thus, leptin and MT-II-responsive neurons in the VMH that regulate glucose uptake in peripheral tissues may be different. Although c-FOS expression did not increase in PVH in response to leptin injection into VMH, this may have been due to the operation of  $\gamma$ -aminobutyric acid (GABA)-mediated neurotransmission in PVH (7).

The mechanism by which injection of leptin into ARC increased glucose uptake specifically in BAT remains unclear. One possible explanation for this observation is that the injection of small dose of leptin (5 ng) into ARC activated a selective set of POMC neurons in ARC that regulate glucose uptake in BAT alone, with POMC neurons being abundant in both the anterior and posterior regions of ARC (42). It is also possible that POMC neurons in ARC that regulate glucose uptake in skeletal muscle and heart require excitatory input as well as leptin for their full activation. In support of this, preferential ablation of leptin receptor in the VMH neurons expressing SF1 transcriptional factor exhibited impaired glucose metabolism before becoming obese (43), while POMC neuron-specific leptin receptor knock out mice did not reveal any disturbance of plasma insulin and glucose level (44). Recently, restoration of leptin receptor in POMC neurons into leptin receptor-deficient mice (db/db/ mice) was shown

to normalize glucose metabolism and locomotor activity in the mice (45). The improved glucose metabolism appears to be dependent on the improved locomotor activity in db/db mice.

The i.c.v. injection of MT-II increased glucose uptake in BAT to a markedly greater extent than did injection of MT-II into VMH or PVH. MC4R-expressing neurons in several regions of the brain stem as well as in the hypothalamic nuclei connect polysynaptically with interscapular BAT (46). Direct injection of MT-II into the raphe pallidus induces a thermogenic response in interscapular BAT (47). Glucose uptake in BAT might thus be regulated by MCRs in multiple brain regions such as brain stems, as well as VMH and PVH. To date, there is little histological evidence of a connection between VMH neurons and interscapular BAT, although electrical stimulation of VMH elicits sympathetic nerve activity in BAT (48) and injection of leptin into VMH increases plasma catecholamine levels more effectively than does that into other hypothalamic regions (49).

DMH is an important hypothalamic nucleus in the regulation of thermogenesis in BAT (50). Injection of a GABA type A receptor antagonist into DMH thus increased thermogenic activity in BAT (51). Moreover, injection of a GABA<sub>A</sub> receptor agonist into DMH blocked sympathetic, thermogenic, and cardiovascular responses induced either by injection of

prostaglandin E<sub>2</sub> into the medial preoptic area (52) or by skin cooling (53). Both Ob-Rb and MC4R are abundant in DMH (8, 25). Furthermore, DMH neurons, including those expressing MC4R, connect polysynaptically with interscapular BAT (46). However, I have now shown that injection of leptin or MT-II into DMH did not increase glucose uptake in the peripheral tissues examined. Although I cannot exclude the possibility that injection of leptin or MT-II activated only a subset of DMH neurons expressing Ob-Rb or MCR, my results suggest that the leptin receptor and MCR in DMH have other roles, such as the regulation of food intake or modulation of BAT thermogenesis in response to cold stimuli.

Skeletal muscle is a central player in glucose homeostasis in mammals. My present results indicate that VMH regulates glucose uptake in skeletal muscle as well as in heart and BAT. Our group previously showed that injection of leptin into the medial hypothalamus increased glucose uptake in peripheral tissues through the activation of sympathetic nerves (15, 16). Injection of leptin into the medial hypothalamus also increased insulin sensitivity in peripheral tissues by a  $\beta$ -adrenergic mechanism (15). Whereas the molecular mechanism remains elusive, a direct effect of  $\beta$ -adrenergic receptors expressed in peripheral tissues as well as a  $\beta$ -adrenergic receptor-dependent increase in blood flow appear to contribute to leptin-induced glucose uptake in these tissues (15).

The present results showed that injection of leptin or MT-II in some medial hypothalamic nuclei increased glucose uptake in peripheral tissues including skeletal muscle and/or BAT, while it did not alter plasma glucose level. These results suggest that leptin and MT-II stimulates hepatic glucose production as well as glucose utilization in peripheral tissues. The result was supported by the previous report showing that leptin increased plasma glucose turnover in mice at 6 h after the injection (14). Furthermore, Rossetti's group has shown that i.c.v. infusion of melanocortin receptor agonist increased hepatic glucose production in lean rats at 6 h after the start of the infusion (54), while it inhibited hepatic glucose production at 7 days (55). These results suggest that the effects of leptin and melanocortin receptor agonist on hepatic glucose production alter time-dependently.

Together, the present data have shown that injection of leptin into VMH, but not that into DMH or PVH, increased glucose uptake in skeletal muscle, heart, and BAT in a manner dependent on MCR activation. Leptin injection into ARC increased glucose uptake specifically in BAT. MT-II injection into VMH increased glucose uptake in skeletal muscle, heart, and BAT, whereas that into PVH increased glucose uptake selectively in BAT. MT-II injection into DMH or ARC did not affect glucose uptake in these peripheral tissues. Our results thus suggest that VMH plays a key role in leptin- and MT-II-induced glucose uptake in

skeletal muscle, heart, and BAT, whereas the leptin receptor in ARC and MCR in PVH regulate glucose uptake in BAT. The medial hypothalamic nuclei thus appear to play distinct roles in the regulation of glucose uptake in peripheral tissues by leptin and MT-II.

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## REFERENCES

1. Friedman JM, Halaas JL: Leptin and the regulation of body weight in mammals. *Nature* 395:763-770, 1998
2. Porte D Jr, Baskin DG, Schwartz MW: Leptin and insulin action in the central nervous system. *Nutr Rev* 60:S20-S29, 2002
3. Cowley MA, Cone RD, Enriori P, Louiselle I, Williams SM, Evans AE: Electrophysiological actions of peripheral hormones on melanocortin neurons. *Ann NY Acad Sci* 994:175-186, 2003
4. Elmquist JK, Coppari R, Balthasar N, Ichinose M, Lowell BB: Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. *J Comp Neurol* 493:63-71, 2005
5. Cone RD: Studies on the physiological functions of the melanocortin system. *Endocr Rev* 27:736-749, 2006
6. Myers MG, Cowley MA, Munzberg H: Mechanisms of leptin action and leptin resistance. *Annu Rev Physiol* 70:537-556, 2008

7. Cowley MA, Pronchuk N, Fan W, Dinulescu DM, Colmers WF, Cone RD: Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat. *Neuron* 24:155-163, 1999
8. Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB, Elmquist JK: Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. *J Comp Neurol* 457:213-235, 2003
9. Fu LY, van den Pol AN: Agouti-related peptide and MC3/4 receptor agonists both inhibit excitatory hypothalamic ventromedial nucleus neurons. *J Neurosci* 28:5433-5449, 2008
10. Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, Christiansen LM, Edelstein E, Choi B, Boss O, Aschkenasi C, Zhang CY, Mountjoy K, Kishi T, Elmquist JK, Lowell BB: Divergence of melanocortin pathways in the control of food intake and energy expenditure. *Cell* 123:493-505, 2005
11. Morohashi KI, Omura T: Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. *FASEB J* 10:1569-1577, 1996

12. Zhao L, Bakke M, Hanley NA, Majdic G, Stallings NR, Jeyasuria P, Parker KL: Tissue-specific knockouts of steroidogenic factor 1. *Mol Cell Endocrinol* 215:89-94, 2004
13. Dhillon H, Zigman JM, Ye C, Lee CE, McGovern RA, Tang V, Kenny CD, Christiansen LM, White RD, Edelman EA, Coppari R, Balthasar N, Cowley MA, Chua S Jr, Elmquist JK, Lowell BB: Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* 49:191-203, 2006
14. Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ: Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* 389:374-377, 1997
15. Haque MS, Minokoshi Y, Hamai M, Iwai M, Horiuchi M, Shimazu T: Role of the sympathetic nervous system and insulin in enhancing glucose uptake in peripheral tissues after intrahypothalamic injection of leptin in rats. *Diabetes* 48:1706-1712, 1999
16. Minokoshi Y, Haque MS, Shimazu T: Microinjection of leptin into the ventromedial hypothalamus increases glucose uptake in peripheral tissues in rats. *Diabetes* 48:287-291, 1999

17. Shiuchi T, Nakagami H, Iwai M, Takeda Y, Cui T, Chen R, Minokoshi Y, Horiuchi M: Involvement of bradykinin and nitric oxide in leptin-mediated glucose uptake in skeletal muscle. *Endocrinology* 142:608-612, 2001
18. Morton GJ: Hypothalamic leptin regulation of energy homeostasis and glucose metabolism. *J Physiol (Lond)* 583:437-443, 2007
19. Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL: Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* 401:73-76, 1999
20. Oral EA, Simha V, Ruiz E, Andewelt A, Premkumar A, Snell P, Wagner AJ, DePaoli AM, Reitman ML, Taylor SI, Gorden P, Garg A: Leptin-replacement therapy for lipodystrophy. *N Engl J Med* 346:570-578, 2002
21. Keen-Rhinehart E, Kalra SP, Kalra PS: AAV-mediated leptin receptor installation improves energy balance and the reproductive status of obese female Koletsky rats. *Peptides* 26:2567-2578, 2005
22. Zhang R, Dhillon H, Yin H, Yoshimura A, Lowell BB, Maratos-Flier E, Flier JS: Selective inactivation of Socs3 in SF1 neurons improves glucose homeostasis without affecting body weight. *Endocrinology* 149:5654-5661, 2008

23. Heijboer AC, van den Hoek AM, Pijl H, Voshol PJ, Havekes LM, Romijn JA, Corssmit EP: Intracerebroventricular administration of melanotan II increases insulin sensitivity of glucose disposal in mice. *Diabetologia* 48:1621-1626, 2005
24. Roselli-Reh fuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, Entwistle ML, Simerly RB, Cone RD: Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci U S A* 90:8856–8860, 1993
25. Elmquist JK, Bjørbaek C, Ahima RS, Flier JS, Saper CB: Distributions of leptin receptor mRNA isoforms in the rat brain. *J Comp Neurol* 395:535-547, 1998
26. Harrold JA, Widdowson PS, Williams G: Altered energy balance causes selective changes in melanocortin-4 (MC4-R), but not melanocortin-3 (MC3-R), receptors in specific hypothalamic regions: further evidence that activation of MC4-R is a physiological inhibitor of feeding. *Diabetes* 48:267-271, 1999
27. Liu H, Kishi T, Roseberry AG, Cai X, Lee CE, Montez JM, Friedman JM, Elmquist JK: Transgenic mice expressing green fluorescent protein under the control of the melanocortin-4 receptor promoter. *J Neurosci* 23:7143-7154, 2003

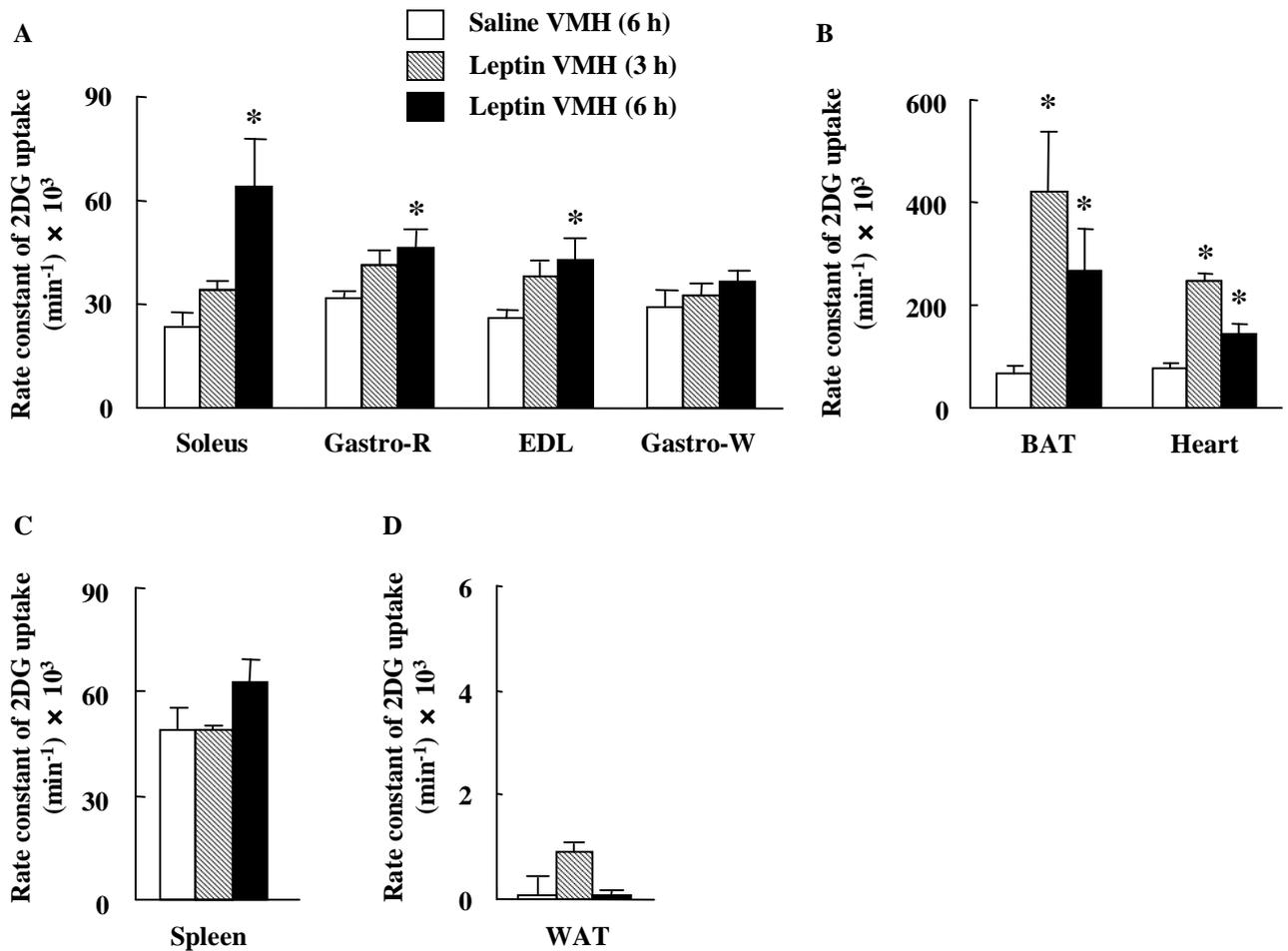
28. Franklin KBJ, Paxinos G: *The Mouse Brain in Stereotaxic Coordinates*. 3rd ed. San Diego, CA, Academic Press, 2007
29. Fong TM, Huang RR, Tota MR, Mao C, Smith T, Varnerin J, Karpitskiy VV, Krause JE, Van der Ploeg LH: Localization of leptin binding domain in the leptin receptor. *Mol Pharmacol* 53:234–240, 1998
30. Haskell-Luevano C, Cone RD, Monck EK, Wan YP: Structure activity studies of the melanocortin-4 receptor by in vitro mutagenesis: identification of agouti-related protein (AGRP), melanocortin agonist and synthetic peptide antagonist interaction determinants. *Biochemistry* 40:6164-6179, 2001
31. Irani BG, Le Foll C, Dunn-Meynell A, Levin BE: Effects of leptin on rat ventromedial hypothalamic neurons. *Endocrinology* 149: 5146–5154, 2008
32. Sung YY, Lee YS, Jung WH, Kim HY, Cheon HG, Yang SD, Rhee SD: Glucose intolerance in young TallyHo mice is induced by leptin-mediated inhibition of insulin secretion. *Biochem Biophys Res Commun* 338:1779-1787, 2005
33. Sokoloff L, Reivich M, Kennedy C, Des Rosiers MH, Patlak CS, Pettigrew KD, Sakurada O, Shinohara M: The [<sup>14</sup>C]deoxyglucose method for the measurement of

- local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J Neurochem* 28:897-916, 1977
34. Hom FG, Goodner CJ, Berrie MA: A [<sup>3</sup>H]2-deoxyglucose method for comparing rates of glucose metabolism and insulin responses among rat tissues in vivo. Validation of the model and the absence of an insulin effect on brain. *Diabetes* 33:141-152, 1984
35. Sudo M, Minokoshi Y, Shimazu T: Ventromedial hypothalamic stimulation enhances peripheral glucose uptake in anesthetized rats. *Am J Physiol* 261:E298-E303, 1991
36. Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, Mu J, Fougère F, Ferre P, Birnbaum MJ, Stuck BJ, Kahn BB: AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* 428:569-574, 2004
37. Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG: Identification of targets of leptin action in rat hypothalamus. *J Clin Invest* 98:1101-1106, 1996
38. Gehlert DR, Chronwall BM, Schafer MP, O'Donohue TL: Localization of neuropeptide Y messenger ribonucleic acid in rat and mouse brain by in situ hybridization. *Synapse* 1:25-31, 1987

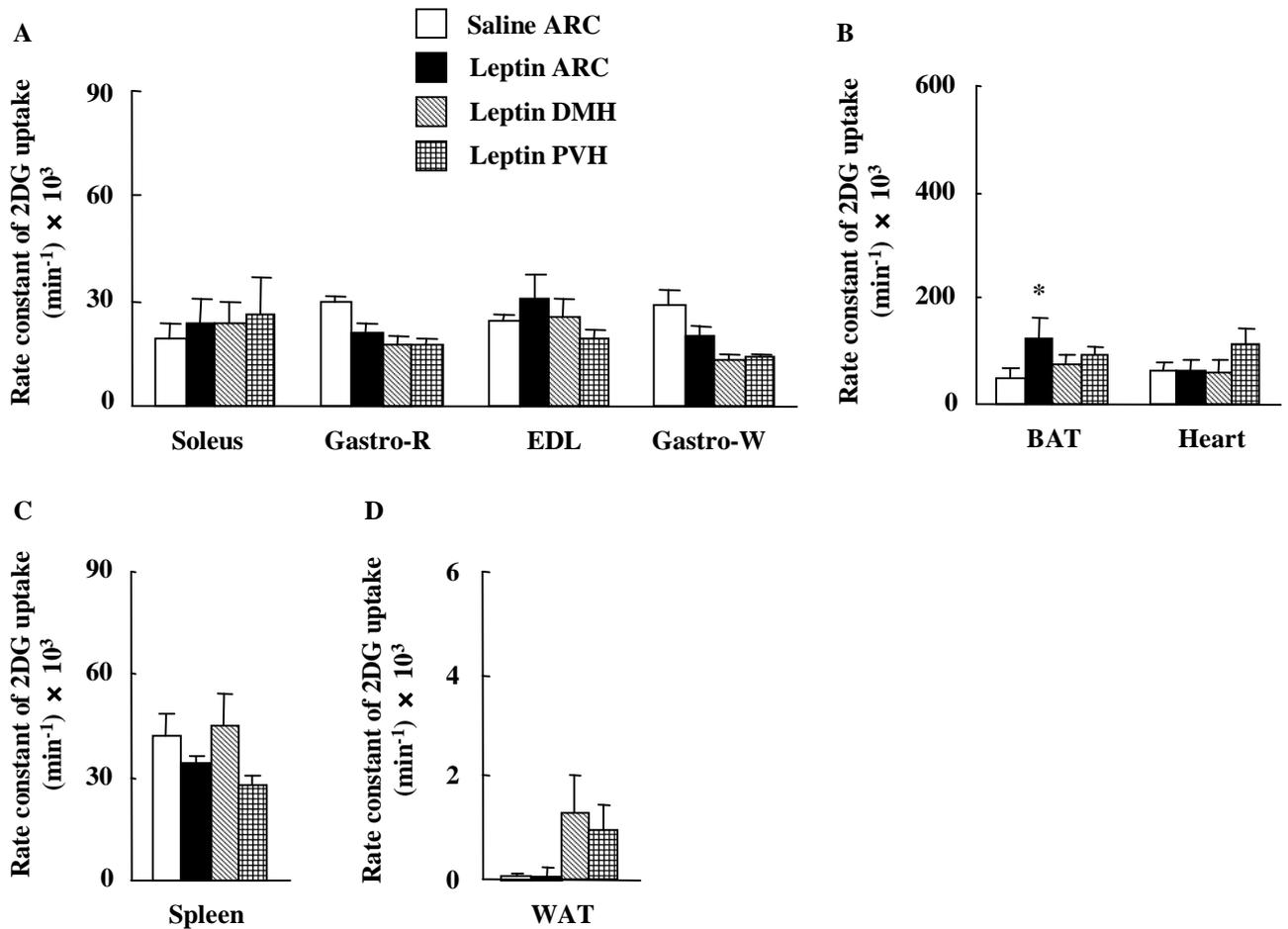
39. Cowley MA, Smart JL, Rubinstein M, Cerdán MG, Diano S, Horvath TL, Cone RD, Low MJ: Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411:480-484, 2001
40. Wang JL, Chinookoswong N, Scully S, Qi M, Shi ZQ: Differential effects of leptin in regulation of tissue glucose utilization in vivo. *Endocrinology* 140:2117-2124, 1999
41. Sternson SM, Shepherd GM, Friedman JM: Topographic mapping of VMH→arcuate nucleus microcircuits and their reorganization by fasting. *Nat Neurosci* 8:1356-1363, 2005
42. Munzberg H, Huo L, Nillni EA, Hollenberg AN, Bjorbaek C: Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin. *Endocrinology* 144:2121–2131, 2003
43. Bingham NC, Anderson KK, Reuter AL, Stallings NR, Parker KL: Selective loss of leptin receptors in the ventromedial hypothalamic nucleus results in increased adiposity and a metabolic syndrome. *Endocrinology* 149:2138-2148, 2008
44. Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, Tang V, Kenny CD, McGovern RA, Chua SC Jr, Elmquist JK, Lowell BB: Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron* 42:983-991, 2004

45. Huo L, Gamber K, Greeley S, Silva J, Huntoon N, Leng XH, Bjørbaek C: Leptin-dependent control of glucose balance and locomotor activity by POMC neurons. *Cell Metab* 9: 537-547, 2009
46. Voss-Andreae A, Murphy JG, Ellacott KL, Stuart RC, Nillni EA, Cone RD, Fan W: Role of the central melanocortin circuitry in adaptive thermogenesis of brown adipose tissue. *Endocrinology* 148:1550-1560, 2007
47. Skibicka KP, Grill HJ: Energetic responses are triggered by caudal brainstem melanocortin receptor stimulation and mediated by local sympathetic effector circuits. *Endocrinology* 149:3605-3616, 2008
48. Saito M, Minokoshi Y, Shimazu T: Accelerated norepinephrine turnover in peripheral tissues after ventromedial hypothalamic stimulation in rats. *Brain Res* 481:298-303, 1989
49. Satoh N, Ogawa Y, Katsuura G, Numata Y, Tsuji T, Hayase M, Ebihara K, Masuzaki H, Hosoda K, Yoshimasa Y, Nakao K: Sympathetic activation of leptin via the ventromedial hypothalamus: leptin-induced increase in catecholamine secretion. *Diabetes* 48:1787-1793, 1999

50. Dimicco JA, Zaretsky DV: The dorsomedial hypothalamus: a new player in thermoregulation. *Am J Physiol Regul Integr Comp Physiol* 292:R47-R63, 2007
51. Zaretskaia MV, Zaretsky DV, Shekhar A, DiMicco JA: Chemical stimulation of the dorsomedial hypothalamus evokes non-shivering thermogenesis in anesthetized rats. *Brain Res* 928:113-125, 2002
52. Madden CJ, Morrison SF: Excitatory amino acid receptors in the dorsomedial hypothalamus mediate prostaglandin-evoked thermogenesis in brown adipose tissue. *Am J Physiol Regul Integr Comp Physiol* 286:R320-R325, 2004
53. Nakamura K, Morrison SF: Central efferent pathways mediating skin cooling-evoked sympathetic thermogenesis in brown adipose tissue. *Am J Physiol Regul Integr Comp Physiol* 292:R127-R136, 2007
54. Gutierrez-Juarez R, Obici S, Rossetti L: Melanocortin-independent effects of leptin on hepatic glucose fluxes. *J Biol Chem* 279:49704-49715, 2004
55. Obici S, Feng Z, Tan J, Liu L, Karkanias G, Rossetti L: Central melanocortin receptors regulate insulin action. *J Clin Invest* 108:1079-1085, 2001



**Figure 1.** Effects of leptin injection into VMH on glucose uptake in peripheral tissues. The rate constant of 2[<sup>3</sup>H]DG uptake was measured in skeletal muscle (A), BAT and heart (B), spleen (C), and epididymal WAT (D) at 3 and 6 h after injection of leptin or saline (control) into VMH of mice. Gastro-R, red portion of gastrocnemius; Gastro-W, white portion of gastrocnemius. Data are means  $\pm$  SEM for five to seven mice. \* $P < 0.05$  versus the corresponding value for saline-injected controls.



**Figure 2.** Effects of leptin injection into ARC, DMH, or PVH on glucose uptake in peripheral tissues. The rate constant of  $2[{}^3\text{H}]\text{DG}$  uptake was measured in skeletal muscle (A), BAT and heart (B), spleen (C), and epididymal WAT (D) at 6 h after injection of leptin into the hypothalamic nuclei of mice. Results obtained after injection of saline into ARC are shown as a control. Data are means  $\pm$  SEM for six or seven mice. \* $P < 0.05$  versus the corresponding value for control animals injected with saline into ARC.

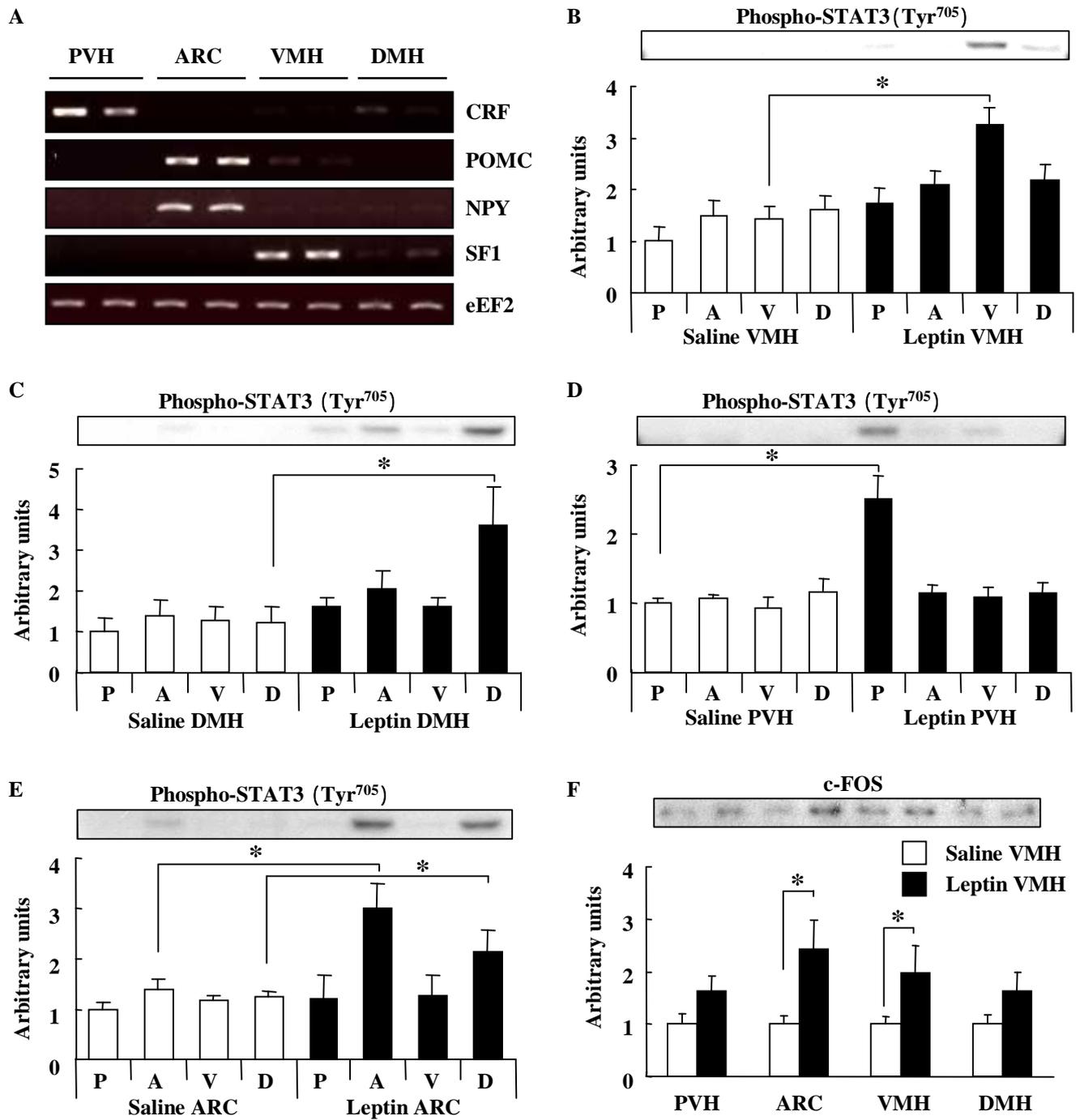
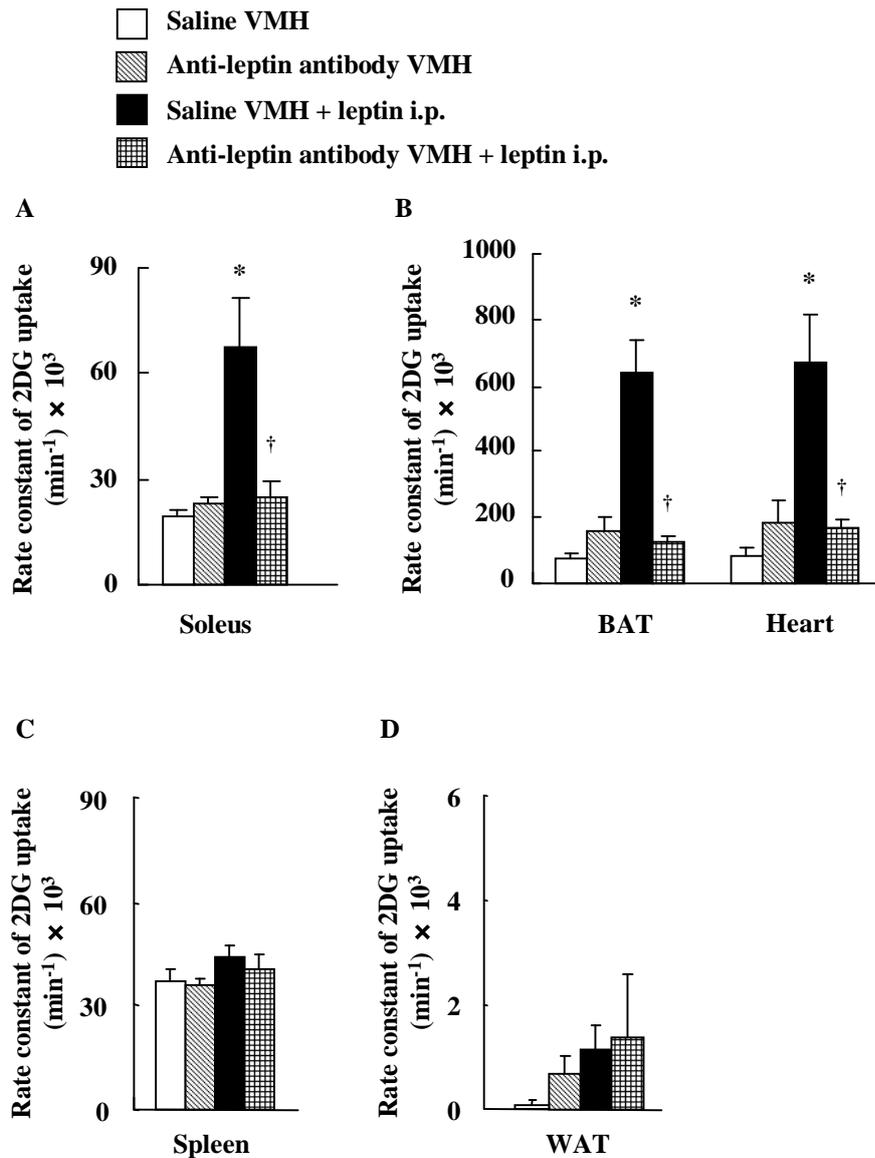
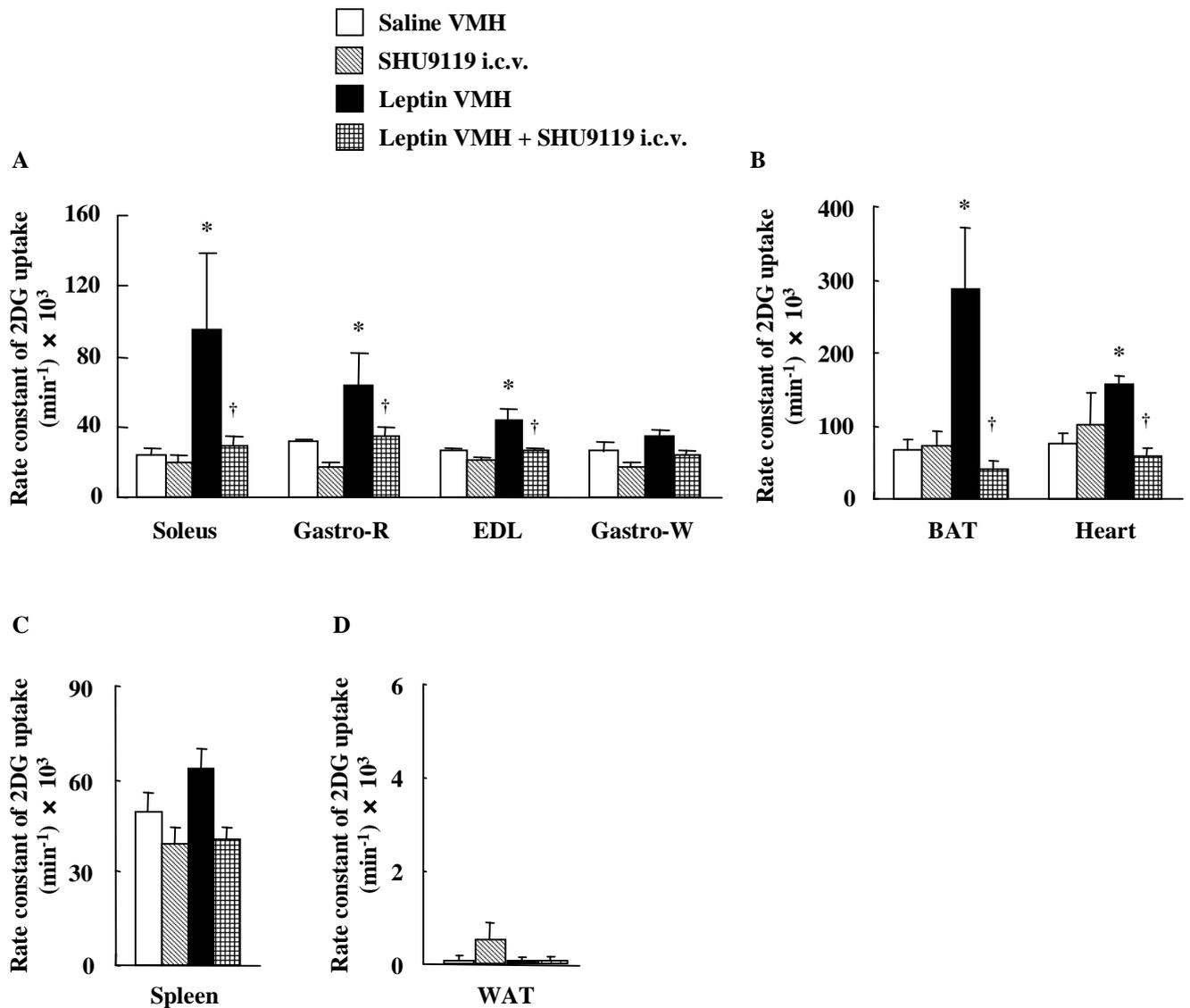


Figure 3.

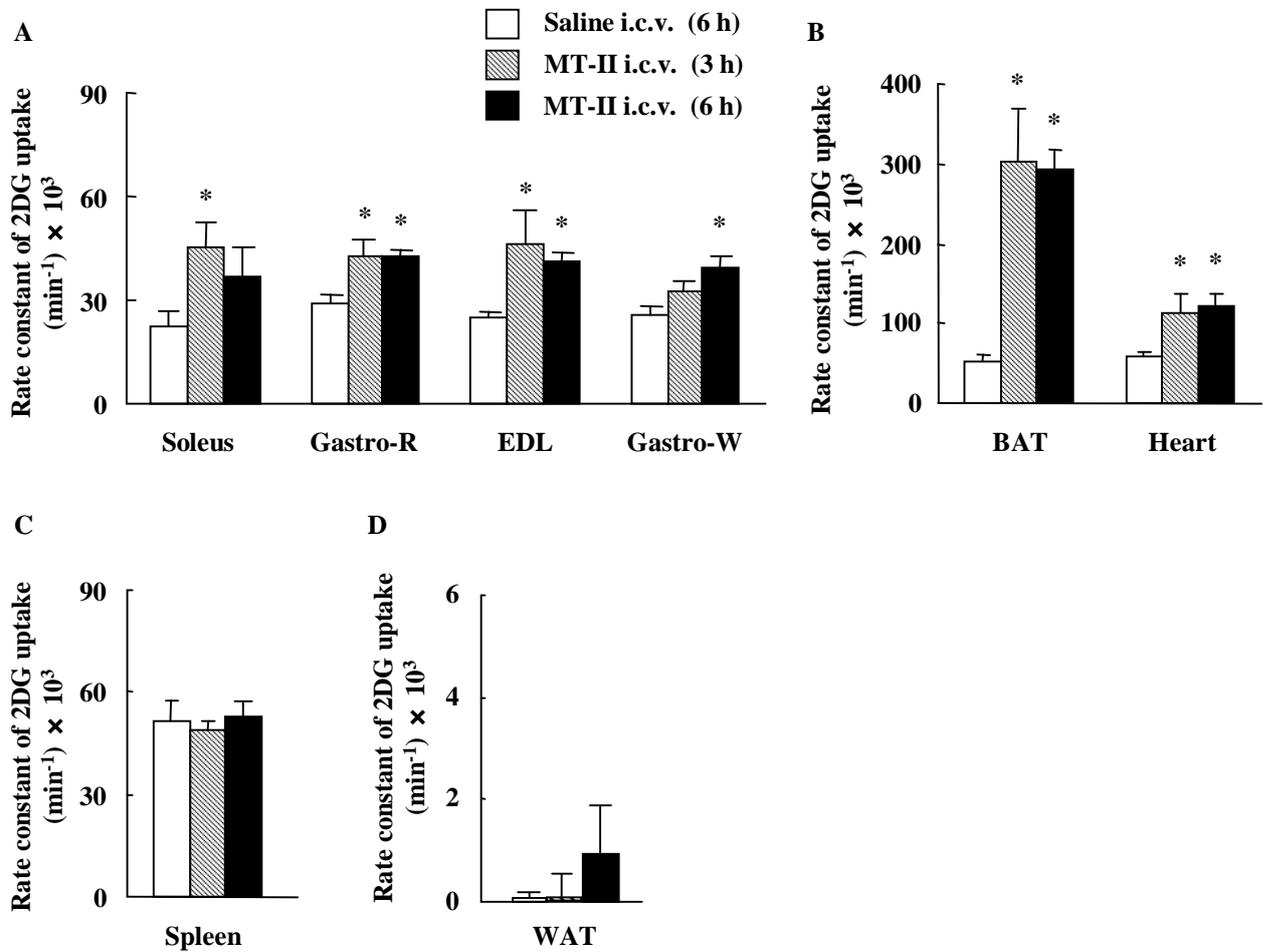
**Figure 3.** Effects of leptin injection into medial hypothalamic nuclei on tyrosine phosphorylation of STAT3 and c-FOS expression. (A) RT-PCR analysis of CRF, POMC, NPY, SF1, and eEF2 (loading control) mRNAs in PVH, ARC, VMH, and DMH. Data are from two animals in a representative experiment. (B–E) Immunoblot analysis of the phosphorylation of STAT3 on Tyr<sup>705</sup> in the medial hypothalamic nuclei at 6 h after leptin injection into VMH (B), DMH (C), PVH (D), or ARC (E). Leptin was injected into the right side of the hypothalamic nuclei, and the same side of PVH (P), ARC (A), VMH (V), and DMH (D) was collected. Injection of saline into VMH was used as a control. Figure shows separate saline controls for each nucleus. Representative blots of Tyr<sup>705</sup>-phosphorylated STAT3 are shown in the upper panels, and quantitative data (means  $\pm$  SEM) from four to six mice are shown in the lower panels. The amount of Tyr<sup>705</sup>-phosphorylated STAT3 was normalized by that of total STAT3, and the normalized values were expressed relative to the corresponding value for PVH of control mice. \* $P < 0.05$  for the indicated comparisons. (F) Immunoblot analysis of c-FOS expression in the medial hypothalamic nuclei after leptin injection into VMH. The right side of PVH, ARC, VMH, and DMH was collected at 6 h after injection of leptin or saline into the same side of VMH. A representative blot is shown in the upper panel, and quantitative data (means  $\pm$  SEM) from four to six mice are shown in the lower panel. The amount of c-FOS was normalized by that of  $\beta$ -actin, and the normalized values were expressed relative to the corresponding value for PVH of control mice. \* $P < 0.05$ .



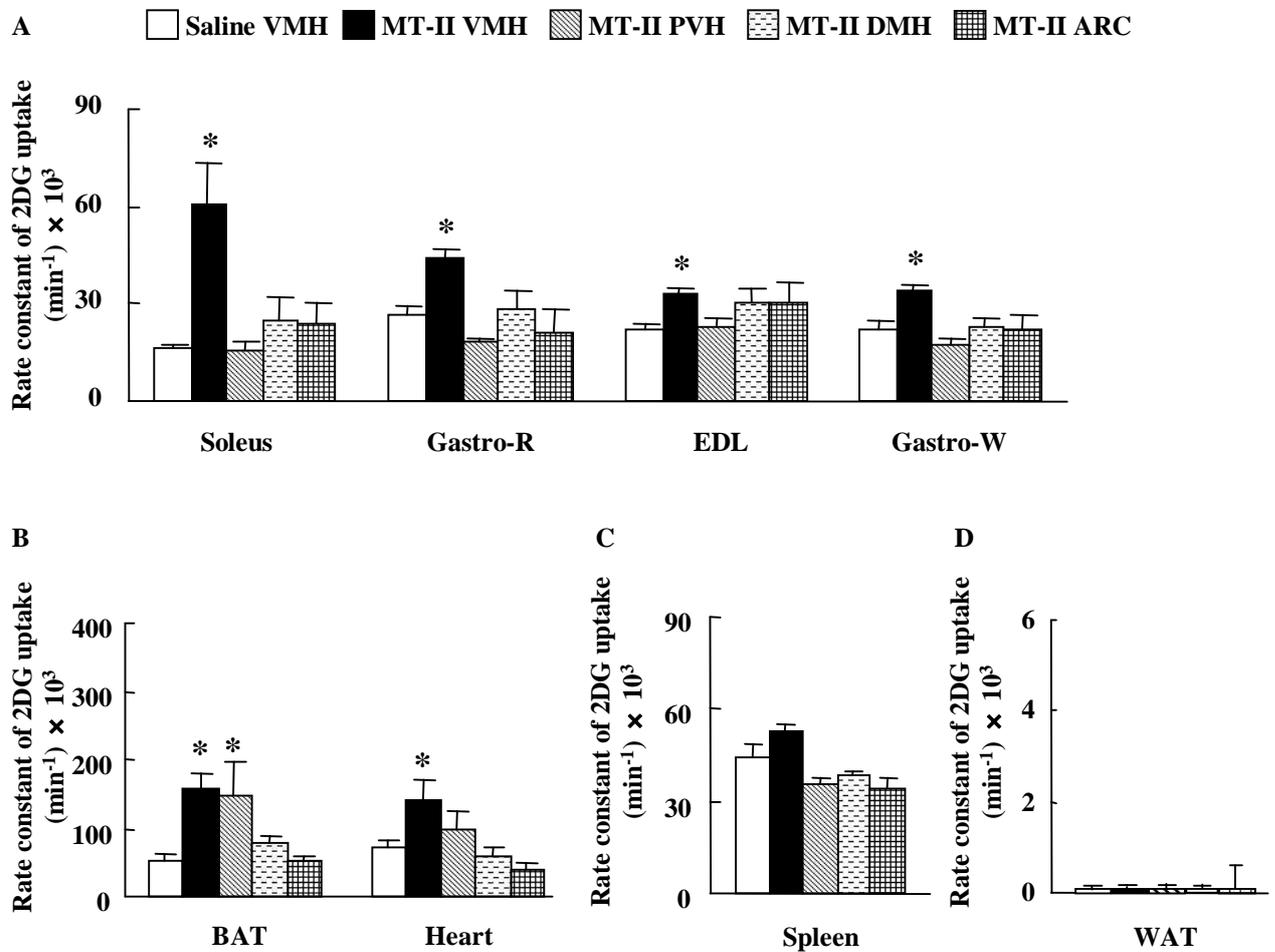
**Figure 4.** Effect of intra-VMH injection of anti-leptin antibody on glucose uptake in peripheral tissues induced by i.p. injection of leptin. The rate constant of 2<sup>[3]H</sup>JDG uptake in soleus muscle (A), BAT and heart (B), spleen (C), and epididymal WAT (D) was measured 7 h after i.p. injection of leptin (5mg/kg) or saline (control). The anti-leptin antibody was injected into VMH bilaterally 30 minutes before the i.p. injection of leptin. Data are means  $\pm$  SEM for five or six mice. \* $P < 0.05$  versus the corresponding value for saline-injected control animals. † $P < 0.05$  versus the corresponding value for leptin-injected animals.



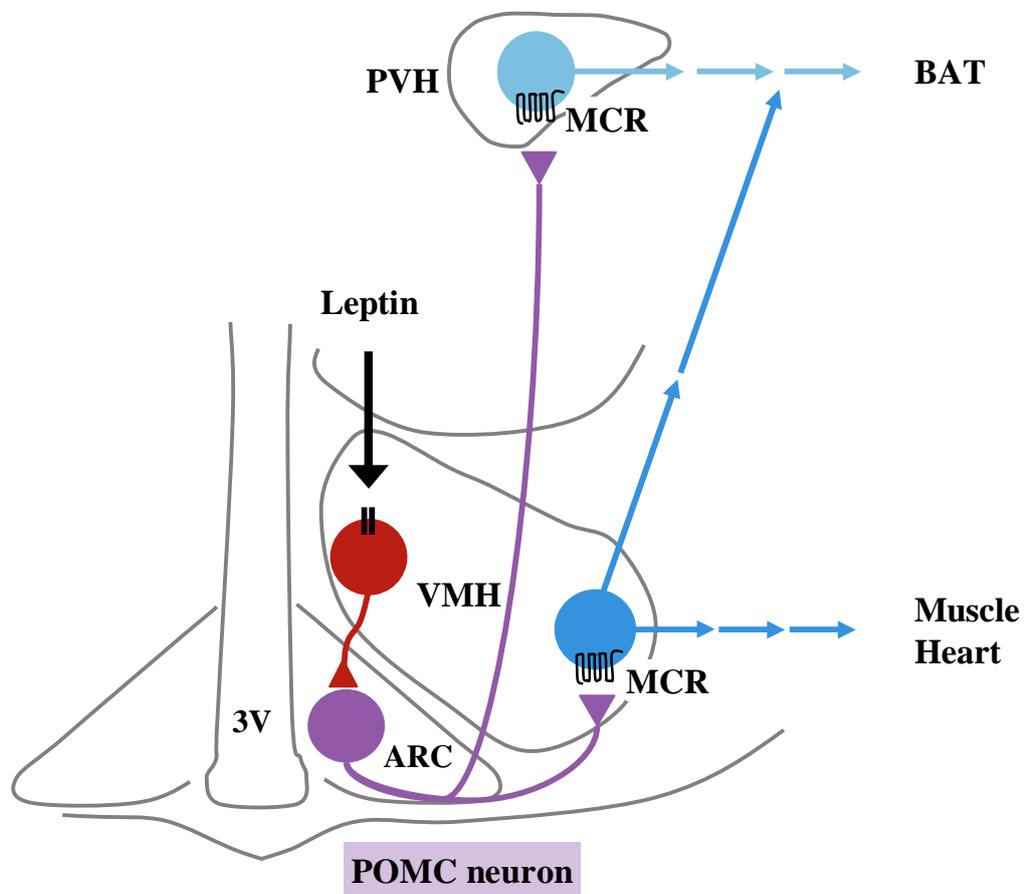
**Figure 5.** Effect of i.c.v. injection of SHU9119 on glucose uptake in peripheral tissues induced by injection of leptin into VMH. The rate constant of 2[<sup>3</sup>H]DG uptake in skeletal muscle (A), BAT and heart (B), spleen (C), and epididymal WAT (D) was measured 6 h after injection of leptin or saline (control) into VMH. The effect of SHU9119 was determined by i.c.v. injection immediately before injection of leptin. Data are means ± SEM for six or seven mice. \**P* < 0.05 versus the corresponding value for saline-injected control animals. †*P* < 0.05 versus the corresponding value for leptin-injected animals.



**Figure 6.** Effects of i.c.v. injection of MT-II on glucose uptake in peripheral tissues. The rate constant of  $2[{}^3\text{H}]\text{DG}$  uptake in skeletal muscle (A), BAT and heart (B), spleen (C), and epididymal WAT (D) was measured at 3 or 6 h after i.c.v. injection of MT-II or saline (control). Data are means  $\pm$  SEM for six or seven mice.  $*P < 0.05$  versus the corresponding value for saline-injected control animals.



**Figure 7.** Effects of MT-II injection into VMH, PVH, DMH, or ARC on glucose uptake in peripheral tissues. The rate constant of 2<sup>[3]H</sup>DG uptake in skeletal muscle (A), BAT and heart (B), spleen (C), and epididymal WAT (D) was measured at 6 h after the injection of MT-II into the individual hypothalamic nuclei. Injection of saline into VMH was used as a control. Data are means  $\pm$  SEM for six or seven mice. \* $P < 0.05$  versus the corresponding value for saline-injected control animals.

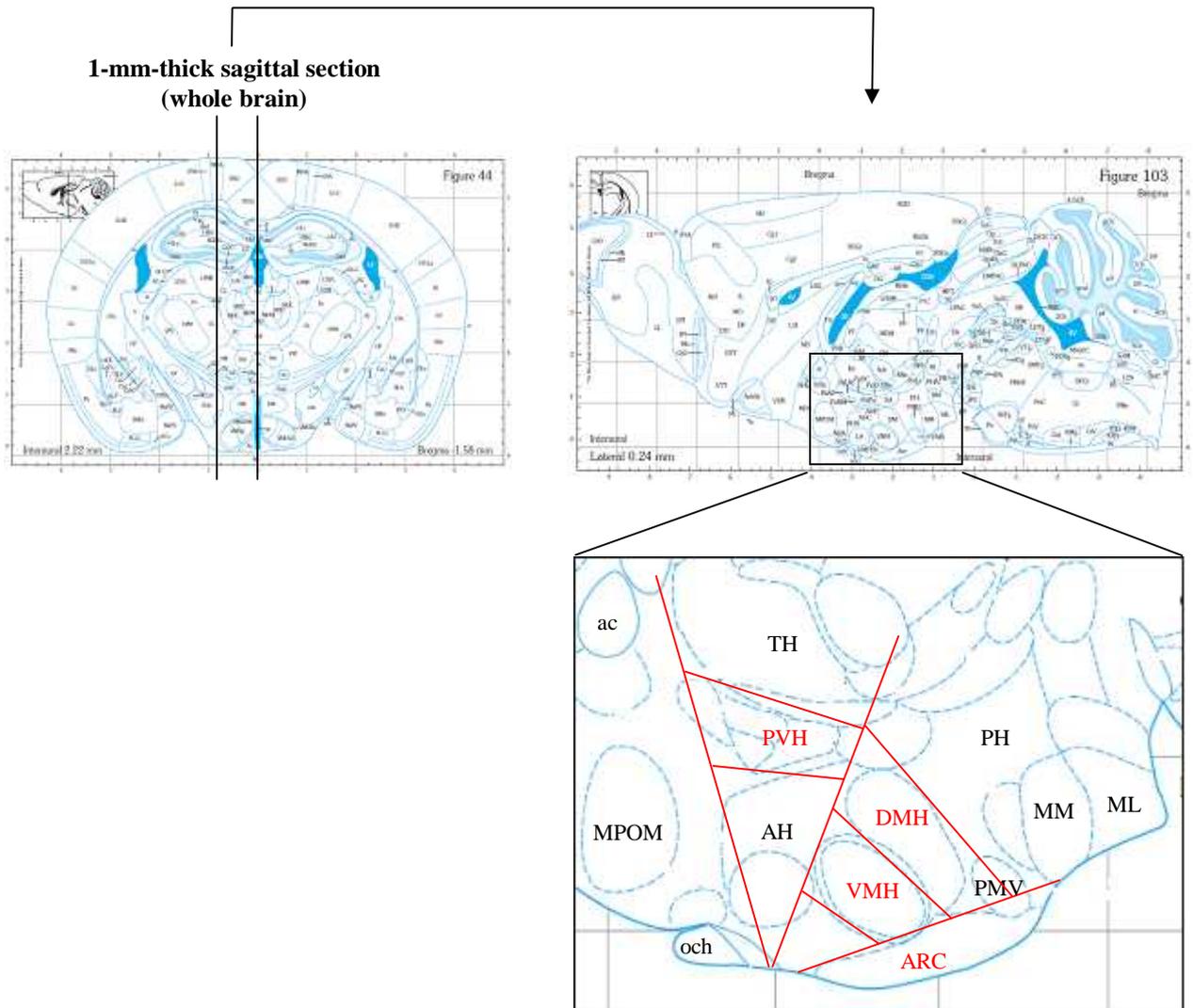


**Figure 8.** Proposed neural pathway in the hypothalamus regulating leptin-induced glucose uptake in peripheral tissues.

**Table 1.** Effects of leptin, MT-II, and SHU9119 on plasma glucose and insulin concentrations.

	Glucose (mg/dl)	Insulin (ng/ml)
Fig. 1		
Saline VMH (6 h)	141 ± 9.0	0.76 ± 0.23
Leptin VMH (3 h)	174 ± 14.8	0.80 ± 0.19
Leptin VMH (6 h)	147 ± 8.8	0.52 ± 0.13
Fig. 2		
Saline ARC	135 ± 4.0	0.86 ± 0.30
Leptin ARC	140 ± 4.2	0.73 ± 0.21
Leptin DMH	137 ± 19.9	0.72 ± 0.22
Leptin PVH	156 ± 6.0	0.83 ± 0.19
Fig. 4		
Saline VMH	126 ± 5.4	0.95 ± 0.28
Antibody VMH	135 ± 18.7	0.72 ± 0.26
Leptin i.p.	123 ± 7.2	0.65 ± 0.23
Antibody VMH + leptin i.p.	110 ± 5.3	0.41 ± 0.07
Fig. 5		
Saline VMH	136 ± 5.2	0.79 ± 0.22
SHU9119 i.c.v.	145 ± 7.7	0.90 ± 0.42
Leptin VMH	147 ± 8.8	0.52 ± 0.13
Leptin VMH + SHU9119 i.c.v.	131 ± 11.7	0.61 ± 0.19
Fig. 6		
Saline i.c.v. (6h)	148 ± 7.3	0.87 ± 0.29
MT-II i.c.v. (3h)	184 ± 12.8*	0.88 ± 0.22
MT-II i.c.v. (6h)	183 ± 7.0*	0.70 ± 0.12
Fig. 7		
Saline VMH	150 ± 8.7	0.88 ± 0.29
MT-II VMH	168 ± 8.7	0.67 ± 0.24
MT-II PVH	150 ± 12.7	0.85 ± 0.20
MT-II DMH	152 ± 8.5	0.73 ± 0.21
MT-II ARC	139 ± 11.2	0.70 ± 0.24

Plasma glucose and insulin concentrations were measured at 3 or 6 hours after injection of the compounds by using glucose CII test (Wako pure chemical, Osaka, Japan) and mouse insulin ELISA KIT (U-type) (Shibayagi, Gunma, Japan), respectively. Results are the means ± SEM for 5 to 7 mice. \*Significantly different from saline injection at P<0.05.



**Supplemental figure 1.** Sampling of medial hypothalamic nuclei.

The hypothalamus present in 1-mm-thick sagittal sections of the entire mouse brain was dissected into PVH, ARC, VMH, and DMH along the indicated red lines as described in Research Design and Methods. ac, anterior commissure; MPOM, medial preoptic nucleus, medial part; TH, thalamus; AH, anterior hypothalamic area; och, optic chiasm; PH, posterior hypothalamic nucleus; PMV, preammillary nucleus, ventral part; MM, medial mammillary nucleus, medial part; ML, medial mammillary nucleus, lateral part. Figures are modified from (28).