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# Hypothalamic Orexin Stimulates Feeding-Associated Glucose Utilization in Skeletal Muscle via Sympathetic Nervous System

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## SUMMARY

**Hypothalamic neurons containing orexin (hypocretin) are activated during motivated behaviors and active waking. We show that injection of orexin-A into the ventromedial hypothalamus (VMH) of mice or rats increased glucose uptake and promoted insulin-induced glucose uptake and glycogen synthesis in skeletal muscle, but not in white adipose tissue, by activating the sympathetic nervous system. These effects of orexin were blunted in mice lacking β-adrenergic receptors but were restored by forced expression of the $\beta_2$-adrenergic receptor in both myocytes and nonmyocyte cells of skeletal muscle. Orexin neurons are activated by conditioned sweet tasting and directly excite VMH neurons, thereby increasing muscle glucose metabolism and its insulin sensitivity. Orexin and its receptor in VMH thus play a key role in the regulation of muscle glucose metabolism associated with highly motivated behavior by activating muscle sympathetic nerves and $\beta_2$-adrenergic signaling.**

## INTRODUCTION

Energy homeostasis, as determined by the balance between calorie intake and energy expenditure, is regulated by interconnected neuroendocrine and autonomic pathways emanating from and controlled by the central nervous system (Flier, 2004). The hypothalamus, a key component of the system for regulation of energy homeostasis, continuously monitors signals that reflect energy status and initiates appropriate behavioral and metabolic responses (Schwartz et al., 2000). It thus controls glucose utilization in insulin-sensitive organs, such as skeletal muscle, as well as whole-body energy metabolism (Sudo et al., 1991; Kamohara et al., 1997; Haque et al., 1999; Minokoshi et al., 1999).

Skeletal muscle is a principal site for glucose and fatty acid metabolism and is responsible for insulin resistance associated with obesity and type 2 diabetes mellitus (Kahn and Flier, 2000). Recent studies indicate that the hypothalamus regulates muscle glucose metabolism and its insulin sensitivity. We have previously shown that electrical stimulation of the ventromedial hypothalamus (VMH) increases glucose uptake in certain peripheral tissues, including skeletal muscle, brown adipose tissue (BAT), and heart of rats (Sudo et al., 1991). Intravenous (i.v.) or intracerebroventricular (i.c.v.) administration of leptin also induces an acute increase in both whole-body glucose turnover and glucose uptake by the same peripheral tissues in mice without any substantial change in plasma insulin or glucose level (Kamohara et al., 1997). We showed that injection of leptin into the medial hypothalamus (MH) increases glucose uptake in skeletal muscle, heart, and BAT via sympathetic nerves (Haque et al., 1999; Minokoshi et al., 1999) and in a manner that apparently is dependent on β-adrenergic receptor (β-AR) stimulation (Haque et al., 1999). Moreover, injection of leptin into the MH resulted in the stimulation of fatty acid oxidation in skeletal muscle through the activation of sympathetic nerves and of α2 subunit-containing AMP kinase (α2AMPK) in skeletal muscle (Minokoshi et al., 2002).

Orexin-A and -B (also known as hypocretin-1 and −2) are a pair of neuropeptides expressed by a specific population of neurons in the lateral (LH) and perifornical areas of the hypothalamus (Sakurai et al., 1998; de Lecea et al., 1998). They are

derived from a common precursor peptide that is the product of the prepro-orexin gene (Sakurai et al., 1998; de Lecea et al., 1998). The orexin-containing neurons are activated during motivated behaviors and active waking (Sakurai, 2007), and they regulate sympathetic tone, metabolic rate, and blood glucose concentration as well as food intake, wakefulness, and reward seeking (Harris et al., 2005; de Lecea et al., 2006; Sakurai, 2007; Tsuneki et al., 2008). The actions of the orexin peptides are mediated by orexin receptor type 1 (OX-R1) and OX-R2, which are widely expressed in the brain, including hypothalamic nuclei such as VMH, paraventricular nucleus (PVH), and ARH (Marcus et al., 2001). Human narcolepsy, which is accompanied by a specific loss of hypothalamic orexin neurons, is associated with metabolic abnormalities, including an increased incidence of type 2 diabetes and obesity (Schuld et al., 2000). Deficiency in orexin neurons as a result of expression of ataxin-3 leads to manifestation of obesity with hypophagia (Hara et al., 2001). Furthermore, orexin transgenic mice were recently found to exhibit enhanced insulin sensitivity with regard to whole-body glucose metabolism (Funato et al., 2009).

Because orexin increases sympathetic outflow (Shirasaka et al., 1999) and because OX-Rs are abundant in the MH (Marcus et al., 2001), we hypothesized that hypothalamic orexin and its receptors in the MH might stimulate muscle glucose metabolism via the sympathetic nervous system, similar to the effect of leptin. Here, we show that orexin neurons and its receptor in VMH play an important role in the regulation of muscle glucose metabolism by preferentially activating muscle sympathetic nerves. The orexin system is activated by a nonhomeostatic regulatory system associated with taste stimulation and regulates muscle glucose metabolism in response to feeding.

## RESULTS

### Orexin-A Injection into the MH Increases Muscle Glucose Uptake

We first examined the effect of orexin-A injection into the MH on whole-body glucose turnover in Sprague-Dawley rats. Orexin-A injection (5 pmol) into the MH resulted in an increase in basal whole-body glucose turnover without any glucose and insulin injection (Figure 1A, left panel). Plasma glucose level did not change after injection of orexin-A (Figure 1A, right panel), suggesting an increase in both glucose utilization and production. Injection of orexin-A did also not increase plasma insulin or epinephrine concentrations (or the plasma glucose concentration) for up to 4 hr after the injection (see Table S1 available online).

We next examined 2-deoxy-D-glucose (2DG) uptake in peripheral tissues. The rate constant of 2DG uptake in skeletal muscle was significantly increased at 2 hr after injection of orexin-A (5 pmol) into the MH (Figure 1B). This effect was more pronounced in the red type of muscle (2.7-fold increase in the soleus) than in the white type (1.7-fold increase in white gastrocnemius). In contrast, 2DG uptake in white adipose tissue (WAT) was not affected (Figure 1B). Uptake of 2DG in muscle was maximal at 2 hr after orexin-A injection and had returned to baseline levels by 4 hr (Figure 1C). Injection of lower dose of orexin-A (0.5 pmol) increased 2DG uptake in the soleus, but the effect was smaller than that of 5 pmol orexin-A (Figure S1A). Orexin-B (5 pmol) injection into VMH also increased 2DG uptake in the soleus but not in the other muscles examined or in WAT at 2 hr after injection (Figure S1B).

We injected orexin peptides into the VMH portion of the MH because we previously showed that the VMH is the most effective site for increasing glucose uptake in skeletal muscle in response to electrical stimulation or injection of leptin (Sudo et al., 1991; Minokoshi et al., 1999). Orexin-A remained largely restricted to VMH at 2 hr after the injection; a small amount of orexin-A was detected in the ARH, but this amount did not differ significantly from that in the control (uninjected) side of the ARH in the same animals (Figure 1D). Injection of orexin-A into the PVH or LH did not affect 2DG uptake in skeletal muscle or WAT (data not shown). These results thus suggest that VMH is the target site for orexin-A in its induction of 2DG uptake in muscle.

The i.c.v. injection of orexin-A or orexin-A injection into the ARH has been shown to stimulate feeding behavior and locomotion (Kotz, 2006). However, orexin-A injected into the VMH had no effect on food intake or locomotor activity (Figures 1E and 1F), as described elsewhere (Dube et al., 1999).

We next determined norepinephrine (NE) turnover in peripheral tissues by assessing the decrease in tissue NE content after inhibition of catecholamine (CA) synthesis. NE turnover was increased in skeletal muscle, but not in WAT, in response to orexin-A administration (Figure 1G). Furthermore, prior administration of guanethidine, a blocker of sympathetic nerve activity but not of CA secretion from the adrenal medulla (Lundberg et al., 1986), completely inhibited the stimulatory effect of orexin-A on 2DG uptake in the soleus and extensor digitorum longus (EDL) (Figure 1H). These results suggest that sympathetic nerve in skeletal muscle is activated preferentially by injection of orexin-A into the MH and regulates muscle 2DG uptake.

### Orexin-A-Induced Muscle Glucose Uptake Requires $\beta_2$-AR Signaling in Muscle Cells

In rats, leptin injection into the MH increases 2DG uptake in muscle via a β-adrenergic mechanism (Haque et al., 1999). We therefore examined the effect of orexin-A injected into the VMH on glucose uptake in $\beta_1$-, $\beta_2$-, and $\beta_3$-AR triple-knockout mice (β-less mice). Orexin-A did not increase muscle 2DG uptake in β-less mice, whereas it did so in red and mixed types of muscle, but not in white muscle or WAT, of wild-type (WT) mice (Figure 2A). Consistent with the results obtained from rats, orexin-A injection did not alter plasma glucose or insulin concentrations for 2 hr after the injection (Figure S2A). Orexin-A did also not affect food intake or plasma glucose and insulin levels in WT mice (Figures S2B and S2C). Neither locomotor activity nor the electromyogram of back muscle differed between WT mice injected with saline and those injected with orexin-A (Figures S2D and S2E). The activity of muscle α2AMPK, which is regulated by α-AR signaling (Minokoshi et al., 2002), was not affected by orexin-A in WT mice (Figure S2F). These results indicate that orexin-A–induced muscle glucose uptake is not attributable to muscle contraction, insulin secretion, or a change in AMPK activity.

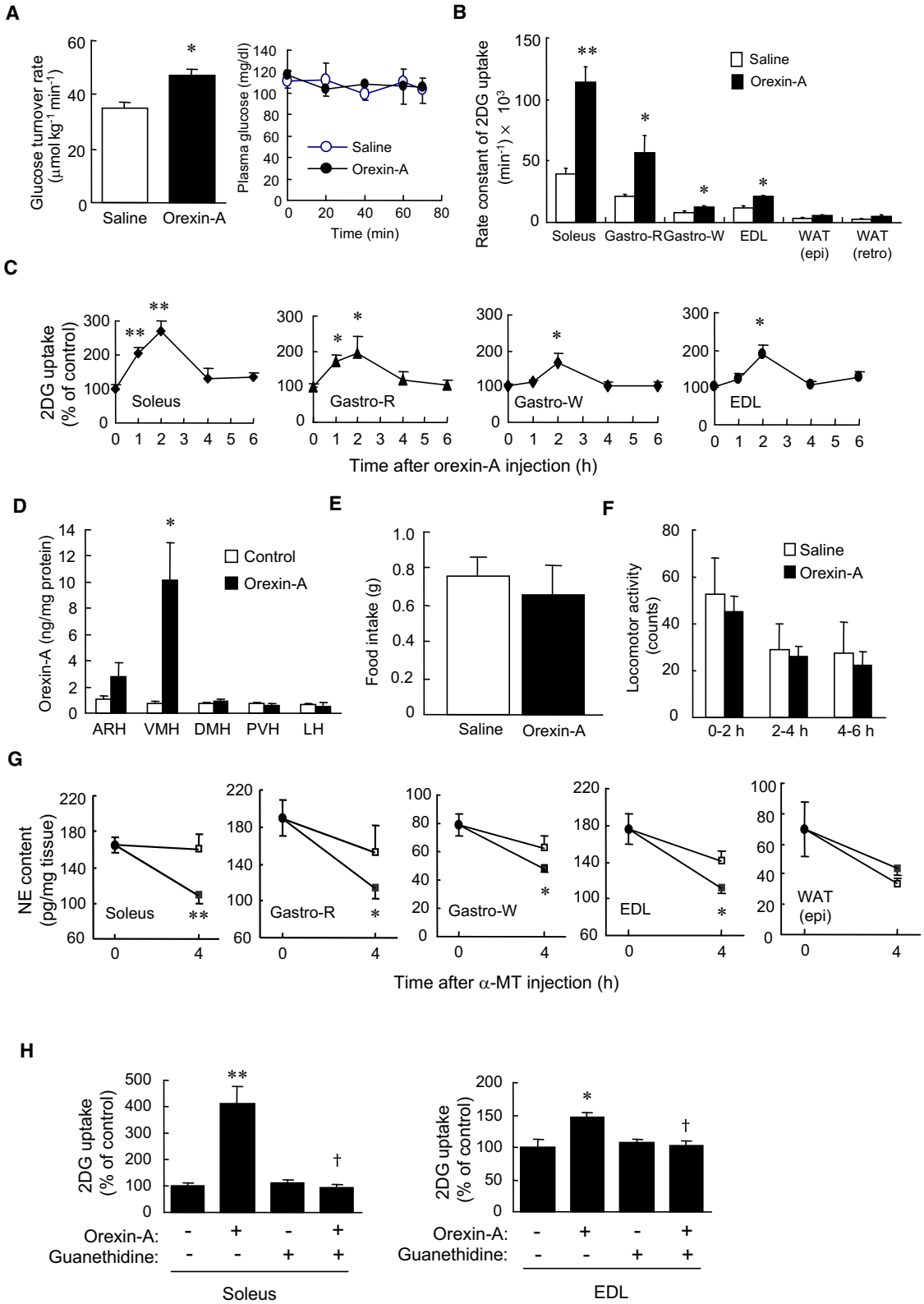



**Figure 1. Injection of Orexin-A into VMH of Rats Increases 2DG Uptake in Skeletal Muscle through Activation of the Sympathetic Nervous System**

(A) Glucose turnover rate (left, $n = 5$) and time course of plasma glucose concentration (right, $n = 5$) after orexin-A (5 pmol) or saline injection into VMH. $^{*}p < 0.05$, $^{**}p < 0.01$ versus saline-injected animals or other control.

We next examined whether orexin-A injection into VMH affects the insulin signaling pathway in skeletal muscle. Orexin-A increased the levels of phosphorylation of insulin receptor substrate–1 (IRS-1) at the YXXM (p85 binding) motif, of Akt on $Ser^{437}$, and of AS160 on $Thr^{642}$, but it did not affect that of the β subunit of the insulin receptor (IR) on $Tyr^{1146}$, in the soleus of WT mice. It had no effect on the phosphorylation of any of these proteins in β-less mice (Figure 2B). Furthermore, orexin-A increased the amount of phosphoinositide (PI) 3–kinase activity associated with IRS-1 in WT mice but not in β-less mice (Figure 2C). These results thus suggest that orexin-A increases glucose uptake in muscle by activating the insulin signaling pathway (but not IR) via muscle sympathetic nerves and β-ARs.

The soleus of mice preferentially expresses the $\beta_2$ isoform of β-ARs (Figure 2D). Intraperitoneal (i.p.) injection of the $\beta_2$-adrenergic antagonist ICI 118551, but not that of the $\beta_1$-adrenergic antagonist CGP 20712, inhibited orexin-A-induced glucose uptake in the soleus (Figure 2E). Those antagonists appear to be specific to $\beta_1$- and $\beta_2$-adrenergic receptors, respectively, because CGP 20712, but not ICI 118551, significantly reduced the heart rate (Figure S3A). ICI 118551 did also not affect the orexin-A-induced increase in NE turnover in the soleus (Figure S3B). These results suggest that ICI 118551 suppressed orexin-A-induced glucose uptake by inhibiting the $\beta_2$-adrenergic receptor in the soleus but not the sympathetic nerve activity.

To examine further the role of the $\beta_2$-AR in skeletal muscle, we restored expression of the $\beta_2$-AR in the soleus and red portion of the gastrocnemius in the right hind limb of β-less mice by electroporation with an expression construct controlled by the skeletal muscle–specific promoter of the human skeletal muscle actin (HSA) gene (Brennan and Hardeman, 1993) (Figures 2F–2H). RT-PCR analysis revealed the presence of $\beta_2$-AR mRNA in the right soleus (74.2% ± 13.3% of the amount in WT mice; $n = 5$) and red gastrocnemius (67.8% ± 20.4% of the amount in WT mice; $n = 5$), but not in the white gastrocnemius or EDL, of the modified mice (Figure 2F). Although injection of orexin-A into VMH increased 2DG uptake to similar extents in skeletal muscle on both sides of WT mice (data not shown), it significantly increased this parameter in the right soleus and red gastrocnemius of the modified animals, compared with that observed in the control muscle on the left that had been transfected with the empty vector alone (Figure 2G). Uptake of 2DG in these muscles of the engineered mice was not affected by injection of saline into VMH (Figure 2H). These results suggest that $\beta_2$-ARs expressed in skeletal muscle cells contribute to orexin-A-induced 2DG uptake in this tissue.

## VMH Is a Target of Orexin-A for Muscle Glucose Uptake

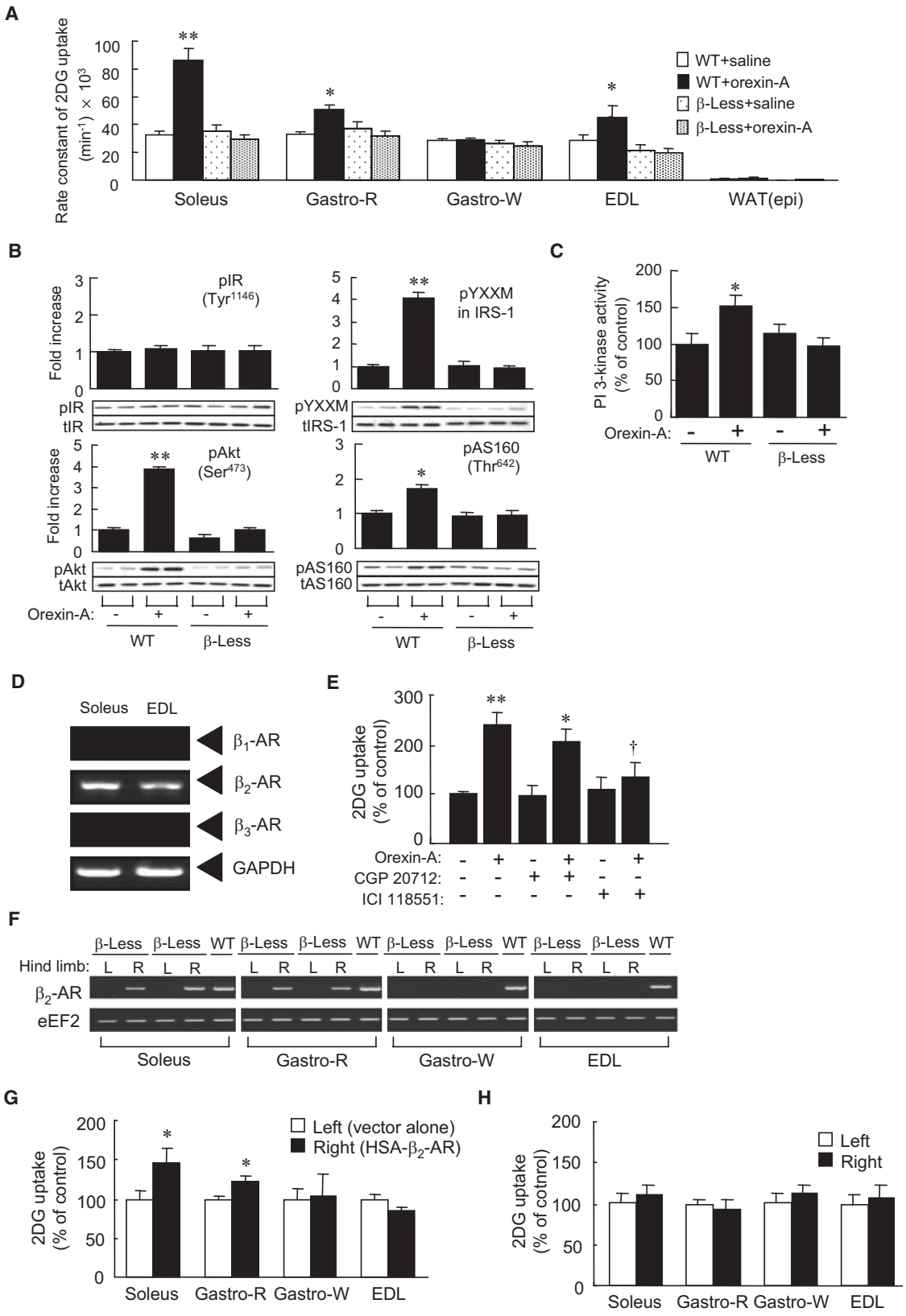
Measurement of orexin-A-like immunoreactivity in hypothalamic nuclei revealed that orexin-A was largely restricted to VMH of mice at 2 hr after its injection into this nucleus, with a small amount of the peptide also being detected in the dorsomedial hypothalamus (DMH) (Figure 3A). Together, our data from rats and mice thus implicate VMH as an important target within the MH for orexin-A in its induction of 2DG uptake in muscle. Immunohistofluorescence analysis revealed that the mouse VMH contains many orexin-positive processes (Figure 3B), some of which are located in close proximity to neurons positive for SF1/Ad4BP, a specific marker for VMH (Parker et al., 2002; Shima et al., 2005). Furthermore, electrophysiological recording of the membrane potential of mouse VMH neurons within brain slices (Figure 3C) revealed that bath application of orexin-A (500 nM) increased the firing rate of 73% (16/22) of these neurons and that this effect was reversible after washout of the peptide (Figure 3D). All neurons examined (3/3) depolarized (7.5 ± 0.8 mV) in response to orexin-A even in the presence of 1 μM tetrodotoxin, suggesting that orexin-A directly activates some VMH neurons (Figure 3E).

The stimulatory effects of orexin-A on NE turnover (Figure 3F) and 2DG uptake (Figure 3G) in the soleus and EDL were significantly inhibited by prior bilateral injection of the OX-R antagonist SB334867 (Smart et al., 2001) into VMH. Orexin-A activates neuropeptide Y (NPY)–containing neurons in the ARH (Horvath et al., 1999). However, i.c.v. injection of NPY or agouti-related peptide (AgRP) did not induce a significant increase in muscle glucose uptake (Figure S4), whereas the dose of the injections significantly increased food intake as reported previously by us (Fujimoto et al., 2007). These observations thus suggest that neurons in VMH and nearby areas, but not NPY- and AgRP-containing neurons, mediate the effect of orexin-A on glucose uptake in skeletal muscle.

## Orexin-A Enhances Insulin-Induced Muscle Glucose Uptake and Glycogen Synthesis

The effect of orexin-A on glucose uptake was maximal at ~2 hr and had declined by 4 hr after injection (Figure 1C). This time course differs from that of the effect of leptin on tissue 2DG uptake, which was observed at 6 hr (Kamohara et al., 1997; Haque et al., 1999; Minokoshi et al., 1999). We hypothesized that orexin-A in VMH might contribute to the short-term regulation of muscle glucose metabolism, such as that triggered by the transient increase in insulin secretion during feeding. We therefore injected mice with insulin (0.75 U/kg of body mass, i.v.) 90 min after injection of orexin-A into VMH and measured

(B) Rate constant of 2DG uptake in peripheral tissues 2 hr after injection of orexin-A or saline ($n = 5$–6). Gastro-R, red gastrocnemius; Gastro-W, white gastrocnemius; EDL, extensor digitorum longus; WAT (epi), epididymal WAT; WAT (retro), retroperitoneal WAT.
(C) Time course of 2DG uptake in skeletal muscle in response to orexin-A administration ($n = 5$).
(D) Concentration of orexin-A in hypothalamic nuclei at 2 hr after injection of the peptide into VMH ($n = 5$). DMH, dorsomedial hypothalamus.
(E) Food intake during the 2-h period after injection of orexin-A or saline ($n = 5$).
(F) Locomotor activity during the indicated time periods after injection of orexin-A or saline ($n = 4$).
(G) NE turnover in peripheral tissues after injection of orexin-A. The NE content of tissues ($n = 9$–10) was measured before (filled circles) and 4 hr after injection of α-methyl-*p*-tyrosine (α-MT, intraperitoneal) and either saline (open squares) or orexin-A (filled squares).
(H) Measurement of 2DG uptake in skeletal muscle 2 hr after injection of orexin-A or saline into VMH and 16 hr after i.p. injection of guanethidine (100 mg/kg) or saline ($n = 5$). $^{\dagger}p < 0.05$ versus orexin-A alone. Data are means ± SEM for the indicated numbers ($n$) of rats. Error bars are shown as SEM.


A
Rate constant of 2DG uptake (min-1) × 10³
WT+saline
WT+orexin-A
β-Less+saline
β-Less+orexin-A
Soleus
Gastro-R
Gastro-W
EDL
WAT(epi)
B
Fold increase
pIR (Tyr1146)
pIR
tIR
pYXXM in IRS-1
pYXXM
tIRS-1
pAkt (Ser473)
pAkt
tAkt
pAS160 (Thr642)
pAS160
tAS160
Orexin-A:
WT
β-Less
C
PI 3-kinase activity (% of control)
Orexin-A:
WT
β-Less
D
Soleus
EDL
β1-AR
β2-AR
β3-AR
GAPDH
E
2DG uptake (% of control)
Orexin-A:
CGP 20712:
ICI 118551:
F
β-Less
WT
Hind limb:
L
R
β2-AR
eEF2
Soleus
Gastro-R
Gastro-W
EDL
G
2DG uptake (% of control)
Left (vector alone)
Right (HSA-β2-AR)
Soleus
Gastro-R
Gastro-W
EDL
H
2DG uptake (% of cotnrol)
Left
Right
Soleus
Gastro-R
Gastro-W
EDL

2DG uptake and glycogen synthesis in the soleus 30 min later (Figure 4A); the blood glucose level was maintained constant by glucose injection (Figure S5).

Insulin increased the rates of 2DG uptake (Figure 4B) and glycogen synthesis from glucose (Figure 4C) in the soleus by factors of ~6 and 8, respectively. Although orexin-A injection alone increased 2DG uptake about two-fold and showed a tendency to increase glycogen synthesis, it markedly potentiated the insulin effects on both of these processes. Orexin-A also enhanced the insulin-induced tyrosine phosphorylation of the IR in the soleus (Figures 4D and 4E), whereas orexin-A injection alone had no effect on IR phosphorylation (Figures 4D and 4E; also see Figure 2B). The extents of phosphorylation of Akt on $Ser^{473}$ and of glycogen synthase kinase 3β (GSK3β) on $Ser^{9}$ in the soleus induced by insulin plus orexin-A were greater than those induced by either agent alone (Figures 4D and 4E). The activity of glycogen synthase *a* in the soleus was increased significantly by insulin plus orexin-A (Figure 4F), whereas that of glycogen phosphorylase *a* was not affected at 45 min (data not shown) and was only slightly decreased at 2 hr after orexin-A injection alone (Figure S6).

## Glycogen Synthesis by Orexin-A Plus Insulin Requires the $\beta_2$-AR in Nonmyocyte Cells

We next examined the effects of orexin-A and insulin on glycogen synthesis from glucose in the soleus of β-less mice in which the $\beta_2$-AR expression was restored in the right soleus under the control of the HSA gene promoter. Unexpectedly, the rate of glycogen synthesis in the right soleus of these animals treated with the combination of orexin-A and insulin was significantly smaller than that in the left soleus (Figure 5A). It was possible that sympathetic nerves innervating nonmyocyte cells of skeletal muscle, such as blood vessel cells, are necessary for induction of glycogen synthesis by orexin-A plus insulin. We therefore examined the effects of expression of the $\beta_2$-AR under the control of the CAG promoter, which confers a high level of gene expression in all tissues (Niwa et al., 1991). Expression of the $\beta_2$-AR under the control of the CAG promoter in the soleus muscle of β-less mice resulted in a receptor mRNA abundance that was 80.2% ± 11.2% ($n$ = 6) of that observed in WT mice (Figure 5B). Electroporation with a vector encoding enhanced green fluorescent protein (EGFP) under the control of the CAG promoter conferred EGFP expression not only in myocytes but also in nonmyocyte cells, including cells of blood vessels, whereas the HSA gene promoter yielded EGFP expression only in myocytes (Figure 5C).

The right soleus muscle of β-less mice expressing $\beta_2$-AR under the control of the CAG promoter manifested a significantly greater increase both in the rate of glycogen synthesis (Figure 5D) and in glycogen content (Figure 5E) in response to the administration of both orexin-A and insulin than did the control (left) muscle treated with the empty vector alone. The increase in glycogen synthesis in the control muscle in β-less mice in response to orexin-A plus insulin was almost similar to that in WT mice after insulin injection alone (Figure 4C). These results suggest that the stimulatory effect of orexin-A on insulin-induced muscle glycogen synthesis requires $\beta_2$-ARs in nonmyocyte cells of skeletal muscle.

## Conditioned Saccharin Drinking Activates the Orexin System and Muscle Glucose Metabolism

Orexin neurons are associated with highly rewarded and motivated behaviors, such as hedonic feeding, food seeking, and addiction (Harris et al., 2005; Sakurai, 2007). The calorie-free sweetener saccharin and glucose are often used as conditioned reinforcers in animal experiments. Moreover, saccharin or glucose ingestion increases the abundance of orexin mRNA in the hypothalamus (Furudono et al., 2006). Oral carbohydrate feeding also activates sympathetic outflow (Young and Landsberg, 1977; Welle et al., 1980). To examine whether orexin neurons stimulate muscle glucose metabolism in response to hedonic feeding motivated and conditioned with saccharin, we trained mice to drink 1 ml of saccharin solution (10 mM) spontaneously within 10 min.

On the day of the experiment, similar exposure to saccharin revealed that saccharin ingestion induced a significant increase in the level of c-Fos immunoreactivity in orexin-positive neurons in LH at 50 min after the onset of saccharin drinking (Figure 6A), without affecting that in melanin-concentrating hormone (MCH) neurons in LH (Figure 6B). We attempted to train orexin knockout mice (Chemelli et al., 1999) to drink saccharin solution, but the animals ingested only a small volume within 10 min (Figure 6C), consistent with the previous observation that these mice are not able to adapt to a restricted feeding schedule (Akiyama et al., 2004; Mieda et al., 2004).

**Figure 2. Orexin-A-Induced 2DG Uptake in Skeletal Muscle of Mice Requires Muscle $\beta_2$-AR Signaling**

(A) Rate constant of 2DG uptake in peripheral tissues of WT or β-less mice ($n$ = 6–8) at 2 hr after injection of orexin-A (5 pmol) or saline into VMH. *$p < 0.05$, **$p < 0.01$ versus saline-injected animals or other control.

(B) Effects of orexin-A injected into VMH on phosphorylation of insulin signaling molecules in the soleus muscle of WT or β-less mice ($n$ = 4–5) at 2 hr after injection. Representative immunoblots with antibodies to the phosphorylated (p) or total (t) forms of the proteins are shown below the quantitative data.

(C) IRS-1-associated PI 3-kinase activity in the red gastrocnemius muscle at 2 hr after injection of orexin-A or saline into VMH of WT or β-less mice ($n$ = 5).

(D) RT-PCR analysis of $\beta_1$-, $\beta_2$-, and $\beta_3$-AR mRNAs in the soleus and EDL muscles of WT mice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was examined as an internal control.

(E) Effects of prior i.p. administration of β-AR antagonists (1 mg/kg) on glucose uptake in the soleus of WT mice ($n$ = 6–8) measured 2 hr after injection of orexin-A or saline into VMH. $^{\dagger}p < 0.05$ versus orexin-A alone.

(F) RT-PCR analysis of $\beta_2$-AR mRNA in skeletal muscle of left (L) and right (R) hind limbs of β-less mice after forced expression of mouse $\beta_2$-AR in the right soleus and red gastrocnemius under the control of the muscle-specific HSA gene promoter (8 days after electroporation). The left soleus and red gastrocnemius were transfected with the empty vector alone. Data for untreated WT mice are shown for comparison. Eukaryotic translation elongation factor 2 (eEF2) mRNA was examined as an internal control.

(G and H) 2DG uptake in skeletal muscle of the left and right hind limbs of β-less mice ($n$ = 8) measured 8 days after electroporation as in (F) and at 2 hr after administration of orexin-A (G) or saline (H) into VMH. All quantitative data are means ± SEM for the indicated numbers ($n$) of mice. Error bars are shown as SEM.

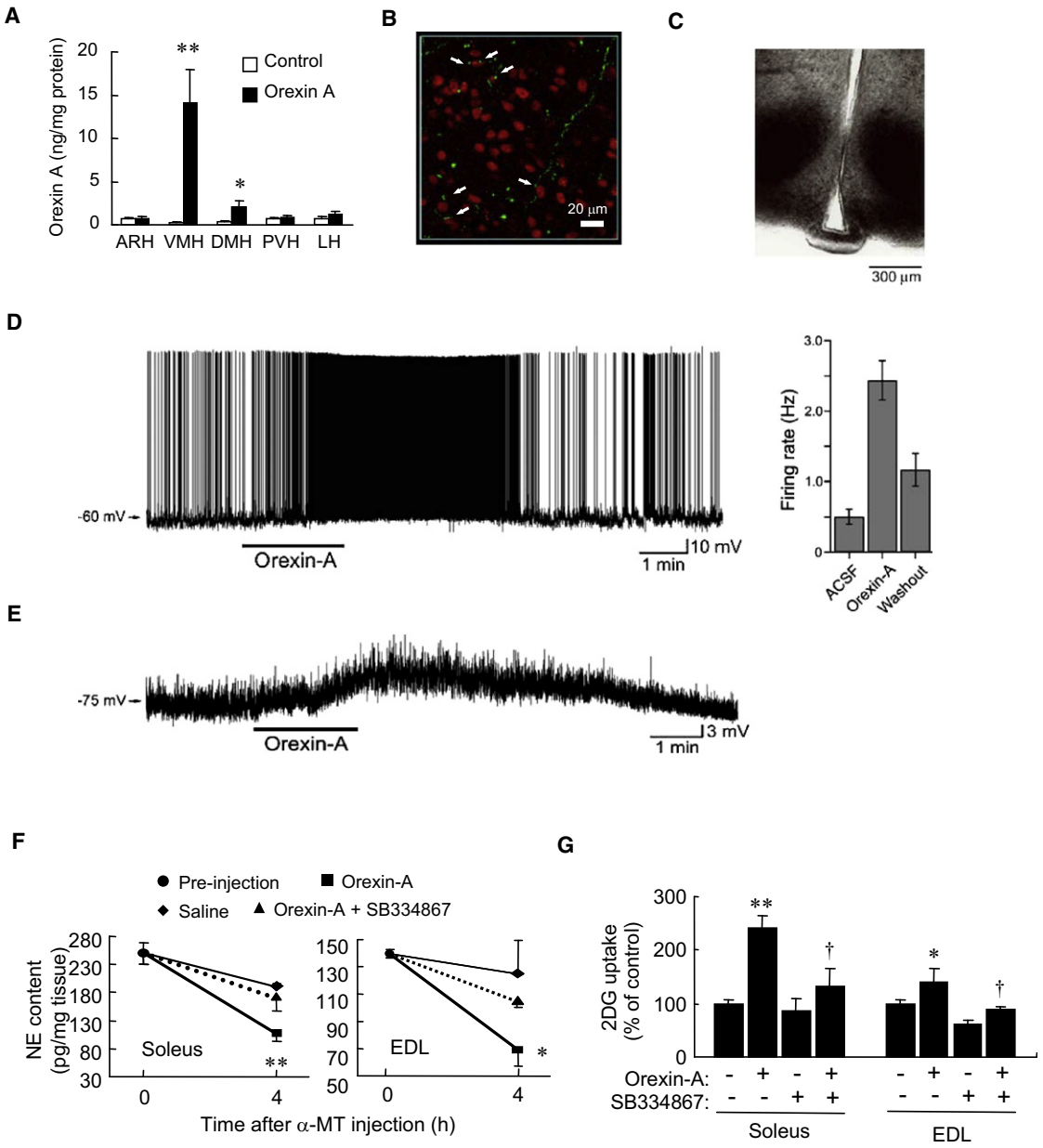



**Figure 3. Activation of VMH Neurons Mediates Orexin-A-Induced Muscle 2DG Uptake of Mice**

(A) Concentration of orexin-A in hypothalamic nuclei at 2 hr after injection of the peptide (5 pmol) into VMH of mice ($n = 5$). *$p < 0.05$, **$p < 0.01$ versus corresponding saline or other control.

(B) Immunohistofluorescence image of orexin neurons projecting to VMH of mice. Arrows indicate the close apposition of orexin-positive processes (green) and SF1-positive neurons (red). Scale bar, 20 μm.

(C) Differential interference contrast image of a coronal slice of mouse brain including VMH (dark bilateral oval-shaped areas).

(D) Voltage recording from a mouse VMH neuron exposed to orexin-A (500 nM) for the time indicated by the bar (left panel). The effects of addition and washout of orexin-A on the firing rate of orexin-sensitive neurons ($n = 16$) is also shown (right panel). ACSF, artificial cerebrospinal fluid.

(E) Orexin-A–induced depolarization of a VMH neuron in the presence of tetrodotoxin (1 μM).

(F) NE turnover in the soleus and EDL of mice ($n = 4$–5) measured as in Figure 1G after injection of orexin-A with or without prior injection of the OX-R antagonist SB334867 (250 pmol) into VMH.

(G) 2DG uptake in skeletal muscle of mice ($n = 5$–8) at 2 hr after injection of orexin-A with or without prior injection of SB334867 into VMH. †$p < 0.05$ versus orexin-A alone. All quantitative data are means ± SEM. Error bars are shown as SEM.

We next examined the effects of saccharin drinking on insulin-stimulated glucose metabolism in skeletal muscle. We injected insulin (0.75 U/kg, i.v.) 40 min after the onset of saccharin drinking and administered radioisotopes for measurement of 2DG uptake and glycogen synthesis 10 min after insulin injection (Figure 6D). Plasma glucose level was maintained constant

by glucose injection (Figure S7A). Spontaneous saccharin drinking significantly enhanced the stimulatory effects of insulin on both 2DG uptake (Figure 6E) and glycogen synthesis (Figure 6F) in the soleus, whereas it did not increase insulin-induced 2DG uptake in WAT (Figure S7B). Saccharin drinking alone (without insulin injection) showed a tendency to increase 2DG uptake and glycogen synthesis in the soleus muscle, but this effect was not statistically significant (data not shown). There were no differences in plasma insulin level between animals drinking saccharin or water before as well as after insulin injection (Figure S7C), probably as a result of the anticipatory response by training. In addition, plasma insulin concentration did not change by treatment with SB334867 or ICI118551 in animals drinking saccharin before and after insulin injection (data not shown). Consistent with the results shown in Figures 2E, 3G, and 3H, injection of SB334867 into both sides of VMH or i.p. injection of ICI 118551 abolished the effects of saccharin on insulin-stimulated 2DG uptake and glycogen synthesis in the soleus (Figures 6E and 6F) without affecting saccharin drinking behavior (data not shown). SB334867 and ICI 118551 did not affect insulin-induced or basal 2DG uptake or glycogen synthesis in the absence of saccharin drinking (data not shown). Manifestation of the saccharin effects on muscle glucose metabolism required the training of mice with saccharin for several days. Our results thus suggest that conditioned taste stimulation with saccharin activates an orexin-MH-sympathetic nervous system axis and thereby promotes insulin-induced muscle glucose uptake and glycogen synthesis.

### Conditioned Glucose Tasting Enhances Glucose Metabolism through OX-Rs in the MH

Finally, mice were allowed to lick and taste a small volume of glucose solution (2 g/kg) on each of three consecutive days, were deprived of food overnight, and then were injected with SB334867 or saline into both sides of VMH on the next day 10 min before the next scheduled exposure to glucose. The blood glucose level after glucose ingestion was significantly greater in mice injected with SB334867 than in those injected with saline, whereas the plasma insulin levels did not differ between the two groups (Figure 7A). These results suggest that OX-Rs in the VMH and nearby area regulate blood glucose level in response to oral glucose ingestion in a manner independent of plasma insulin. Manifestation of the SB334867 effect on muscle glucose metabolism required the training of mice with glucose for several days. In contrast, SB334867 injected into VMH had no effect on the plasma glucose, as well as insulin concentration, when glucose was administered i.p., even after training of the mice with glucose tasting (Figure 7B). Furthermore, oral glucose ingestion showed higher glucose level in orexin knockout mice than in WT mice, although plasma insulin level was significantly higher in orexin knockout mice (Figure 7C).

## DISCUSSION

Our present findings reveal that orexin-A and its receptors in the MH regulate glucose uptake and glycogen synthesis in skeletal muscle via stimulation of sympathetic nerves and the activation of $\beta_2$-ARs in both myocytes and nonmyocyte cells, triggering both direct activation of the insulin signaling pathway in myocytes and an increase in the availability of insulin to these cells. We also found that hedonic feeding conditioned with sweet taste stimulation activates the orexin-MH-sympathetic nervous system axis and thereby stimulates muscle glucose metabolism.

### Role of Sympathetic Nerves in the Orexin-A-Regulated Muscle Glucose Metabolism

Studies with catecholamine administration have suggested that the sympathetic nervous system inhibits the insulin signaling pathway in peripheral tissues. However, our present data show that the preferential stimulation of sympathetic nerves and subsequent $\beta_2$-AR signaling result in activation of the insulin signaling pathway in skeletal muscle in vivo. Our results thus suggest that the effects of sympathetic nerves on muscle glucose metabolism may differ in some instances from those of catecholamine administration. Consistent with this notion, a $\beta_2$-AR-specific agonist was previously shown to increase glucose uptake in L6 myocytes via activation of PI 3-kinase, with this effect being inhibited by protein kinase A (PKA) (Nevzorova et al., 2006). Indeed, orexin-A injection into VMH did not increase $\alpha$2AMPK activity in skeletal muscle, which is regulated via an $\alpha$-adrenergic mechanism. Furthermore, the activity of muscle glycogen phosphorylase *a*, which is increased by PKA or a high cytosolic free $Ca^{2+}$ concentration, was not significantly affected in response to orexin-A injection. Although humoral factors might be required for the orexin's effect, these results suggest that orexin-A injected into VMH enhances glucose uptake and glycogen synthesis through a specific $\beta_2$-AR signaling pathway but not PKA or AMPK. Recently, $\beta$-less mice have been shown to have better insulin sensitivity in insulin tolerance test than did WT mice (Asensio et al., 2005). This phenomenon may be due to the increased level of plasma epinephrine secreted from adrenal medulla in response to hypoglycemia, which inhibits muscle insulin signaling via activation of PKA-signaling pathway in WT mice.

Our data also indicate that $\beta_2$-ARs in nonmyocyte cells of skeletal muscle are required for the enhancement by orexin-A of insulin-induced glycogen synthesis. Insulin delivery to skeletal muscle cells via blood vessels plays a key role in whole-body glucose disposal (Clerk et al., 2006; Chiu et al., 2008). Both $\beta_2$-ARs in blood vessels (Guimarães and Moura, 2001) and insulin (Sowers, 2004) stimulate vascular relaxation. Our observation that orexin-A enhanced the insulin-induced tyrosine phosphorylation of the IR in skeletal muscle suggests that orexin-A injected into VMH might promote insulin delivery to muscle cells by activating $\beta_2$-ARs. Our findings thus suggest that an orexin-A-sympathetic nerve-$\beta_2$-AR signaling system regulates glucose metabolism in muscle as follows: first, in the presence of a low plasma concentration of insulin, orexin-A in the MH triggers activation of PI 3-kinase, Akt, and other downstream components of the insulin signaling pathway in skeletal muscle, at least in part through activation of $\beta_2$-ARs in myocytes; and second, in the presence of high plasma insulin levels, orexin-A enhances insulin delivery to myocytes through activation of $\beta_2$-ARs in blood vessels and thereby potentiates the effects of insulin on muscle glucose metabolism.

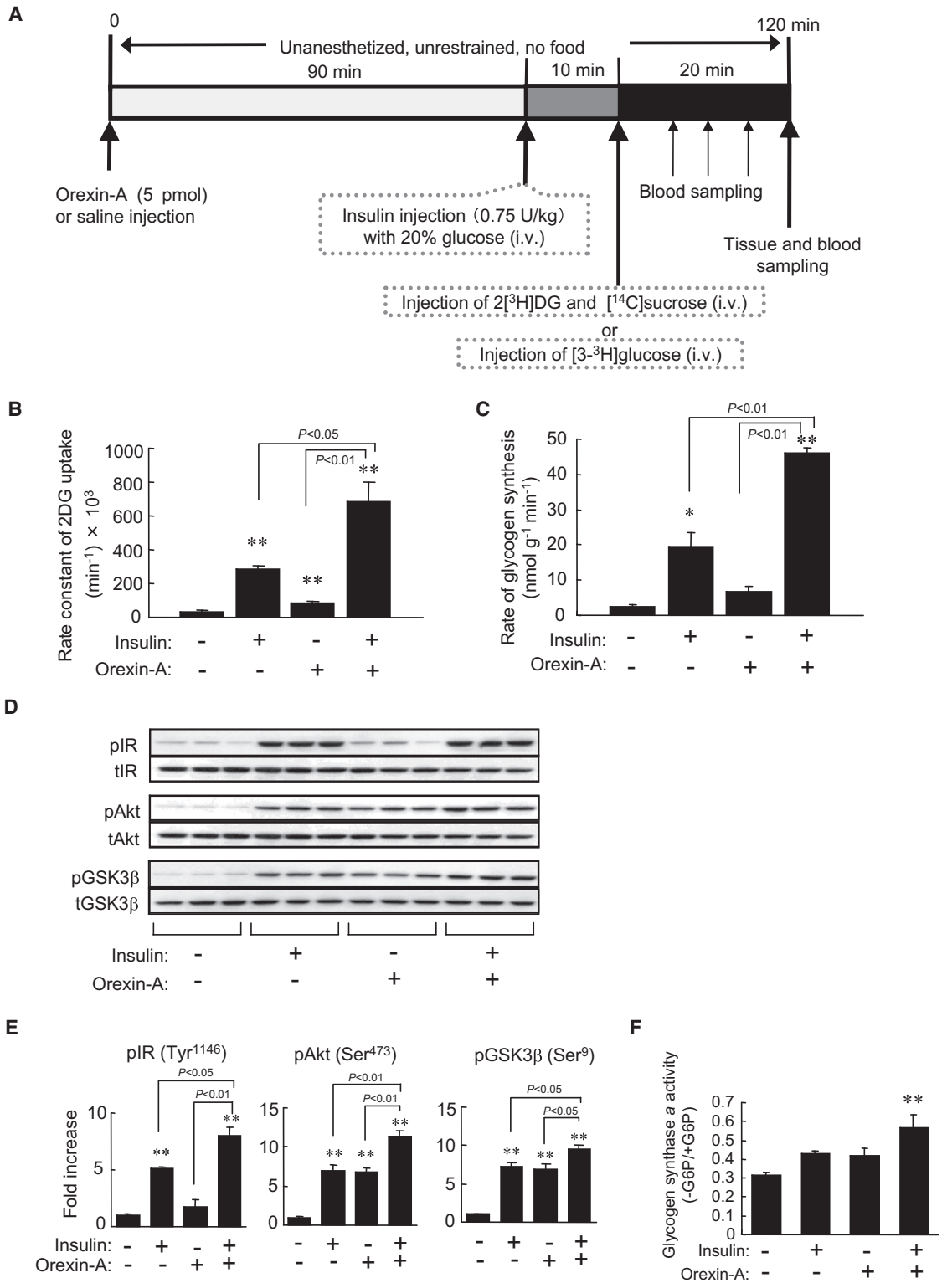



**Figure 4. Orexin-A Enhances Insulin-Induced 2DG Uptake and Glycogen Synthesis in Skeletal Muscle of Mice**

(A) Experimental protocol for measurement of glucose metabolism in skeletal muscle of mice treated with orexin-A and insulin.

(B and C) Rate constant of 2DG uptake ($n = 5$) and rate of glycogen synthesis ($n = 7$–$9$), respectively, in the soleus measured 2 hr after orexin-A injection into VMH and subsequent i.v. injection of insulin. *$p < 0.05$, **$p < 0.01$ versus corresponding saline value.

(D and E) Phosphorylation of the β subunit of the IR ($Tyr^{1146}$), Akt ($Ser^{473}$), and GSK3β ($Ser^{9}$) in the soleus ($n = 3$) at 2 hr after orexin-A injection into VMH and subsequent i.v. injection of insulin. Immunoblots are shown in (D) and quantitative data in (E).

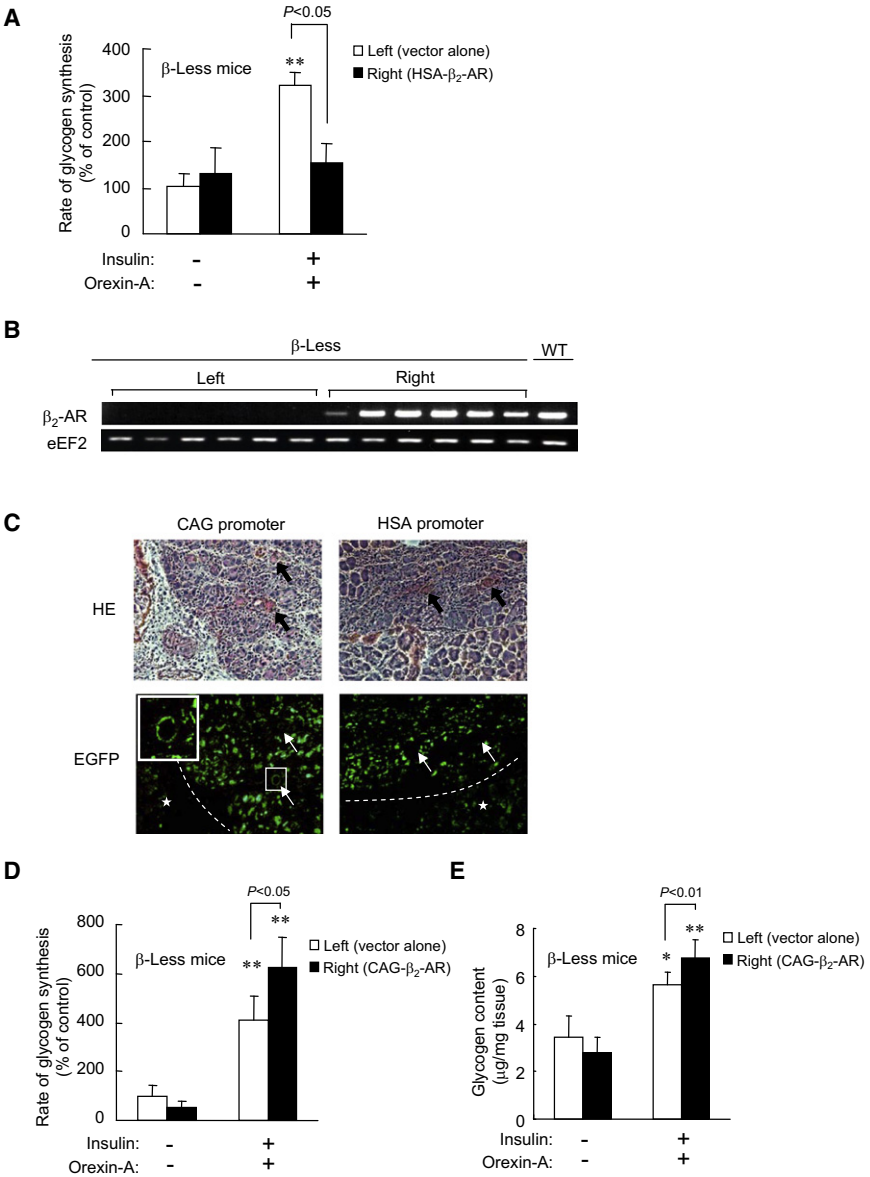



**Figure 5. Enhancement of Insulin-Induced Glycogen Synthesis by Orexin-A Requires $\beta_2$-ARs in Nonmyocyte Cells of Skeletal Muscle**

(A) Effects of orexin-A and insulin administered as in Figure 4A on the rate of glycogen synthesis in the left and right soleus muscles of β-less mice ($n$ = 5) expressing the $\beta_2$-AR in the right soleus under the control of the HSA gene promoter as in Figure 2F. *p < 0.05, **p < 0.01 versus corresponding control value.

(B) RT-PCR analysis of $\beta_2$-AR mRNA in the left and right soleus of β-less mice ($n$ = 6) expressing the $\beta_2$-AR under the control of the CAG promoter in the right soleus. Mice were examined at 5 days after electroporation.

(C) Hematoxylin-eosin (HE) staining (upper panels) and EGFP fluorescence analysis (lower panels) of skeletal muscle after electroporation with a vector encoding EGFP under the control of the CAG (left panels) or HSA gene (right panels) promoters. Arrows indicate blood vessels in the soleus muscle expressing EGFP or not; the inset shows at higher magnification the EGFP-positive blood vessel in the boxed region. Dotted lines demarcate the soleus and gastrocnemius muscles, with the asterisks indicating the gastrocnemius.

(D and E) Rate of glycogen synthesis and glycogen content, respectively, in the soleus of β-less mice ($n$ = 10) expressing the $\beta_2$-AR under the control of the CAG promoter in the right soleus. All quantitative data are means ± SEM. Error bars are shown as SEM.

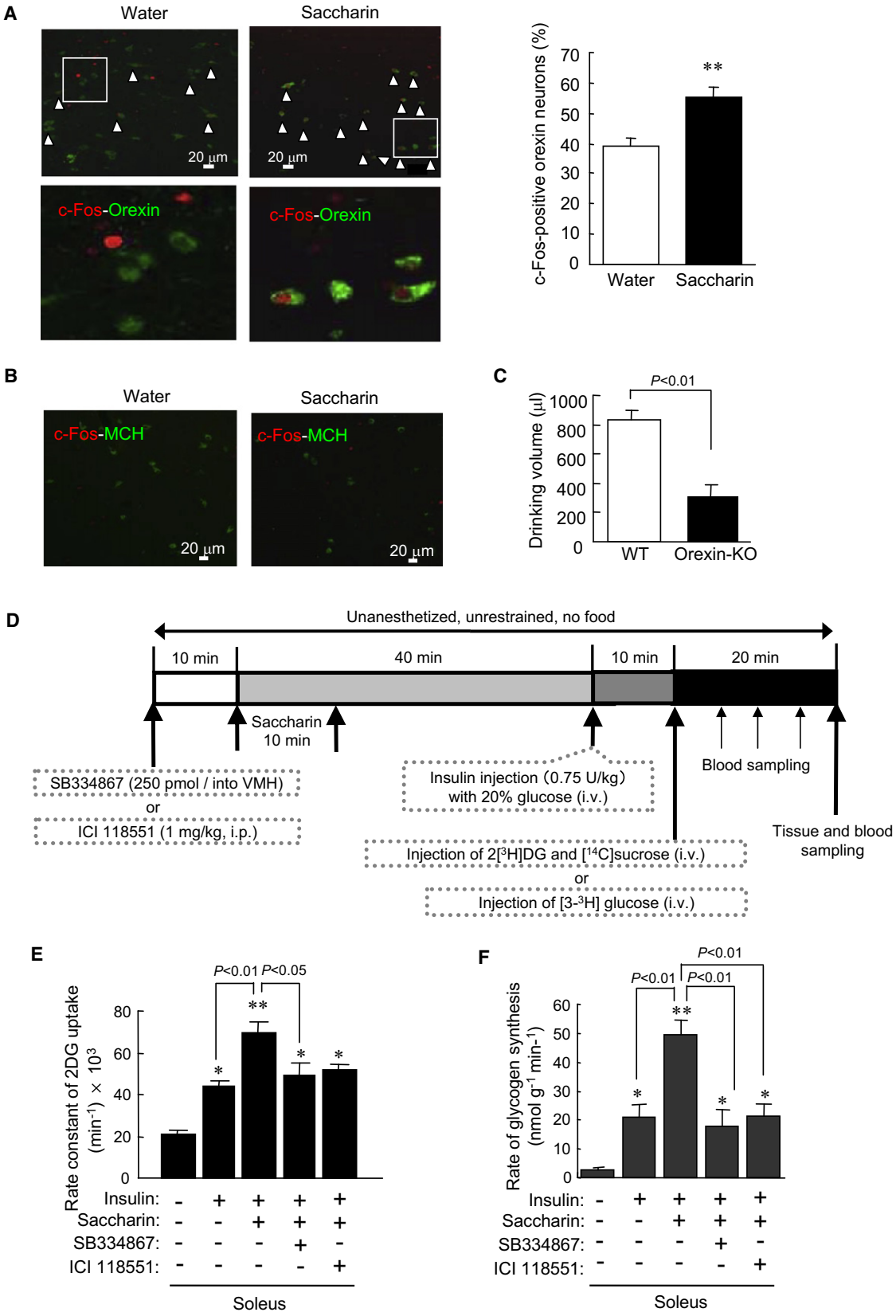
(F) Activity of glycogen synthase *a* in the soleus ($n$ = 7) at 2 hr after injection of orexin-A into VMH and subsequent i.v. injection of insulin. Data are presented as the ratio of activity measured in the absence to that measured in the presence of glucose 6-phosphate (G6P). All quantitative data are means ± SEM. Error bars are shown as SEM.

## VMH as a Key Target for Orexin-A in Its Effects on Skeletal Muscle

Our present data implicate VMH as a key target for orexin-A in its activation of glucose metabolism in skeletal muscle. VMH was found to contain many cells activated by orexin-A. The effects of orexin-A on the sympathetic nervous system and glucose metabolism differ from those of electrical stimulation or injection of leptin in part. Electrical stimulation of VMH elicits sympathetic nerve activity in most peripheral tissues, including the adrenal medulla (Saito et al., 1989a), and injection of leptin into VMH increases plasma catecholamine levels (Satoh et al., 1999), whereas injection of orexin-A into VMH did not increase epinephrine secretion from the adrenal medulla or affect sympathetic nerve activity in WAT. Our preliminary data also reveal that injection of orexin-A into the MH activates glucose metabolism and norepinephrine turnover in the heart, as well as in skeletal muscle, but not in BAT (authors' unpublished data). Thus, in contrast to the effects of leptin, orexin-A in VMH and nearby area selectively regulates some peripheral tissues, including skeletal muscle, through the stimulation of sympathetic nerves.

## Physiological Role of the Orexin System in Muscle Glucose Metabolism

Taste stimulation has been associated with meal-induced thermogenesis (LeBlanc and Brondel, 1985; Diamond et al., 1985; LeBlanc, 2000), and oral sucrose feeding activates the sympathetic nervous system (Young and Landsberg, 1977; Welle et al., 1980). The initial phase of a meal is thought to be the origin of an increase in heat production not necessarily associated with the absorption or digestion of nutrients and coincides


A
Water
Saccharin
20 μm
c-Fos-Orexin
c-Fos-positive orexin neurons (%)
**
Water
Saccharin
B
c-Fos-MCH
C
P<0.01
Drinking volume (μl)
WT
Orexin-KO
D
Unanesthetized, unrestrained, no food
10 min
40 min
10 min
20 min
Saccharin 10 min
SB334867 (250 pmol / into VMH)
or
ICI 118551 (1 mg/kg, i.p.)
Insulin injection (0.75 U/kg) with 20% glucose (i.v.)
Blood sampling
Tissue and blood sampling
Injection of 2[3H]DG and [14C]sucrose (i.v.)
or
Injection of [3-3H] glucose (i.v.)
E
P<0.01 P<0.05
Rate constant of 2DG uptake (min-1) × 10³
Insulin:
Saccharin:
SB334867:
ICI 118551:
Soleus
F
P<0.01
P<0.01
P<0.01
Rate of glycogen synthesis (nmol g-1 min-1)
Soleus

with a rapid increase in both plasma NE and insulin concentrations (Diamond et al., 1985; LeBlanc and Brondel, 1985). In humans, a highly palatable meal results in greater thermogenesis than does a nonpalatable meal consisting of the homogenized same palatable meal (LeBlanc and Brondel, 1985). After a carbohydrate-rich meal, forearm glucose uptake was reduced pronouncedly by β-adrenergic blockade in human (Astrup et al., 1990). In contrast, forced feeding of rats and humans by gavage resulted in a reduction in thermogenesis and subsequent obesity (Rothwell and Stock, 1978; LeBlanc et al., 1984; Saito et al., 1989b). Consistent with this notion, orexin-A injection into VMH showed greater effect on glucose uptake in red type of skeletal muscles than in white type of muscles. Red type of muscles has many mitochondria, a key organelle for thermogenesis.

We used saccharin and glucose as the conditioned reinforcers to activate orexin neurons in mice. The situation may be more complicated in humans as well as rodents, however, with other factors such as the palatability of foods, the environment during a meal, and habituation to the feeding regimen possibly contributing to activation of this system.

In the present study, we examined the effect of bolus injection of insulin and orexin-A on glucose metabolism and insulin signaling, maintaining blood glucose level. Our preliminary study showed that bolus injection of insulin was more effective than constant infusion of insulin (hyperinsulinemic-euglycemic camp) to manifest the synergic effect of hypothalamic orexin-A and insulin (data not shown). Furthermore, the action of orexin-A on glucose uptake is shorter than that of leptin. The activation of orexin neurons may also be terminated by high glucose concentration soon after the meal is consumed (Yamanaka et al., 2003). The orexin system is thus transiently activated by conditioned taste stimulation and enhances glucose utilization in skeletal muscle induced by a rapid increase in the circulating insulin level, rather than continuous hyperinsulinemia.

It is also possible that the orexin-MH–sympathetic nervous system axis regulates muscle glucose metabolism during fasting. Orexin neurons are activated by fasting (Diano et al., 2003) and are associated with food-seeking behavior (Sakurai et al., 1998) and adaptive augmentation of arousal level during fasting (Yamanaka et al., 2003). Although the activity of sympathetic nerves in certain tissues such as BAT decreases markedly in response to fasting, that in skeletal muscle does not (Dulloo et al., 1988). The orexin-MH system might thus support the metabolic demand required for fasting-related behaviors, such as food seeking or fight-or-flight, by maintaining glucose utilization in skeletal muscle.

Collectively, our present results suggest that the orexin-MH–sympathetic nervous system axis plays an important role in the regulation of glucose metabolism in skeletal muscle. The $\beta_2$-adrenergic receptor in both myocytes and nonmyocyte cells of skeletal muscle is involved in the regulation. Glucose metabolism in skeletal muscle is thus regulated by the nonhomeostatic regulatory system associated with taste stimulation, motivation, and anticipation. It is also possible that glucose metabolism in skeletal muscle is associated with sleep and wakefulness through the orexin-MH–sympathetic nervous system axis.

## EXPERIMENTAL PROCEDURES

### Animals

Male Sprague-Dawley rats (Nihon SLC, Hamamatsu, Japan), C57BL/6J mice (Nihon SLC), β-less mice (Bachman et al., 2002), and orexin knockout mice (Chemelli et al., 1999) were studied at 10 to 13 weeks of age, with the exception that electrophysiological studies were performed with 4-week-old mice. Animals were housed individually in plastic cages at 24 ± 1°C with lights on from 06:00 to 18:00 hr and were maintained with free access to a laboratory diet (MF; Oriental Yeast, Tokyo, Japan) and water.

All animal studies were reviewed and approved by the appropriate ethics committee in National Institute for Physiological Sciences and were performed according to institutional guidelines concerning the care and handling of experimental animals.

### Statistical Analysis

Data are presented as mean ± SEM. Data were evaluated by the unpaired or paired Student's *t* test or by analysis of variance followed by Bonferroni's multiple-range test. A $P$ value of $< 0.05$ was considered statistically significant.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two tables, and eight figures and can be found with this article online at http://www.cell.com/cell-metabolism/supplemental/S1550-4131(09)00309-X.

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**Figure 6. Conditioned Saccharin Drinking Activates the Hypothalamic Orexin System and Thereby Stimulates Muscle Glucose Metabolism**

(A) Immunohistofluorescence analysis of c-Fos expression (red) in neurons positive for orexin-like immunoreactivity (green) in the LH of mice at 50 min after the onset of conditioned drinking of saccharin solution or water. The boxed regions of the upper panels are shown at higher magnification in the lower panels. Arrowheads indicate c-Fos-positive orexin neurons. Scale bars, 20 μm. The percentage of c-Fos-positive cells among 120 orexin neurons in the LH and perifornical area in each of three mice is also shown. **$p < 0.01$ versus corresponding control values.

(B) Immunohistofluorescence analysis of c-Fos expression (red) in neurons positive for MCH-like immunoreactivity (green) within the LH of mice at 50 min after conditioned drinking of saccharin solution or water. Scale bars, 20 μm.

(C) Volume of saccharin solution drank during 10 min by prepro-orexin knockout (Orexin-KO) or WT mice ($n = 5$) subjected to the training protocol for saccharin drinking.

(D) Experimental protocol for analysis of muscle glucose metabolism after conditioned saccharin drinking. Mice were trained to drink saccharin solution during 10 min beginning at 10:00 hr for 3 days before experiments.

(E and F) Rate constant of 2DG uptake and rate of glycogen synthesis, respectively, in the soleus muscle of mice ($n = 7$–9) after conditioned saccharin drinking with or without prior injection of SB334867 (into VMH) or ICI 118551 (i.p.) or of subsequent injection of insulin (i.v.). All quantitative data are means ± SEM. Error bars are shown as SEM. **$p < 0.05$ versus corresponding control values.

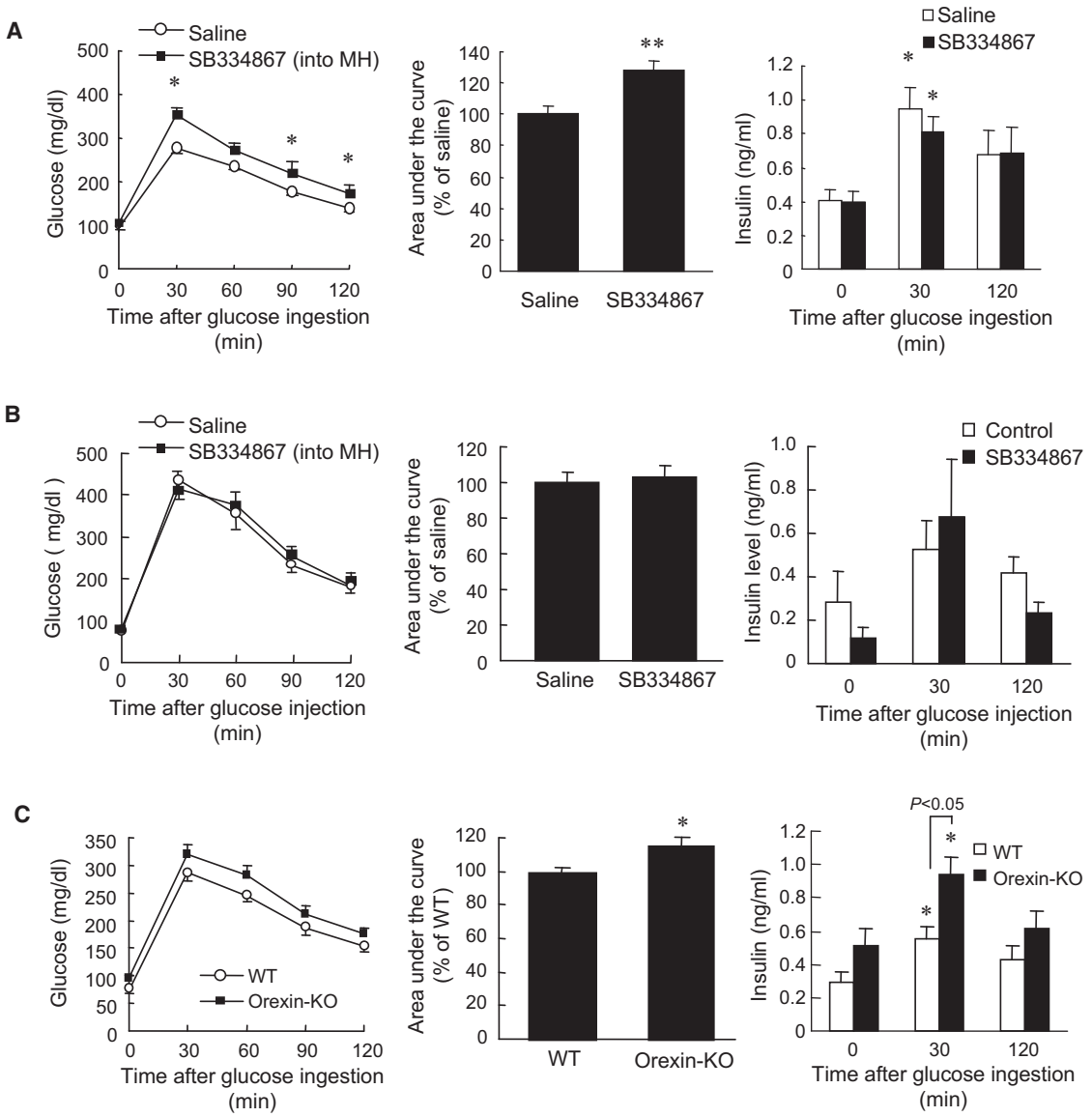


**Figure 7. Conditioned Tasting of Glucose Solution Enhances Whole-Body Glucose Metabolism through OX-Rs in VMH**

(A) Time course of blood glucose concentration after conditioned oral glucose ingestion (2 g/kg) in mice ($n$ = 7) deprived of food overnight and injected with SB334867 or saline into VMH (left panel). The area under the curves of blood glucose concentration versus time (middle panel): *p < 0.05, **p < 0.01 versus corresponding saline value. Time course of plasma insulin level (right panel): *p < 0.05 versus corresponding value for time 0.

(B) Time course of blood glucose concentration after i.p. glucose injection (2 g/kg) in mice ($n$ = 5) deprived of food overnight and injected with SB334867 or saline into VMH (left panel). The area under the curves of blood glucose concentration versus time (middle panel) and plasma insulin level (right panel) are also shown.

(C) Time course of blood glucose concentration after conditioned oral glucose ingestion (2 g/kg) in Orexin-KO mice ($n$ = 10–11) deprived of food overnight (left panel). The area under the curves of blood glucose concentration versus time (middle panel): *p < 0.05, versus corresponding saline value. Plasma insulin level (right panel): *p < 0.05 versus corresponding value for time 0. Data are means ± SEM. Error bars are shown as SEM.

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