A Major Role for Perifornical Orexin Neurons in the Control of Glucose Metabolism in Rats

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OBJECTIVE—The hypothalamic neuropeptide orexin influences (feeding) behavior as well as energy metabolism. Administration of exogenous orexin-A into the brain has been shown to increase both food intake and blood glucose levels. In the present study, we investigated the role of endogenous hypothalamic orexin release in glucose homeostasis in rats.

RESEARCH DESIGN AND METHODS—We investigated the effects of the hypothalamic orexin system on basal endogenous glucose production (EGP) as well as on hepatic and peripheral insulin sensitivity by changing orexinergic activity in the hypothalamus combined with hepatic sympathetic or parasympathetic denervation, two-step hyperinsulinemic-euglycemic clamps, immunohistochemistry, and RT-PCR studies.

RESULTS—Hypothalamic disinhibition of neuronal activity by the γ-aminobutyric acid receptor antagonist bicuculline (BIC) increased basal EGP, especially when BIC was administered in the perifornical area where orexin-containing neurons but not melanocortin-concentrating hormone–containing neurons were activated. The increased BIC-induced EGP was largely prevented by intracerebroventricular pretreatment with the orexin-1 receptor antagonist. Intracerebroventricular administration of orexin-A itself caused an increase in plasma glucose and prevented the daytime decrease of EGP. The stimulatory effect of intracerebroventricular orexin-A on EGP was prevented by hepatic sympathetic denervation. Plasma insulin clamped at two or six times the basal levels did not counteract the stimulatory effect of perifornical BIC on EGP, indicating hepatic insulin resistance. RT-PCR showed that stimulation of orexin neurons increased the expression of hepatic glucoregulatory enzymes.

CONCLUSIONS—Hypothalamic orexin plays an important role in EGP, most likely by changing the hypothalamic output to the autonomic nervous system. Disturbance of this pathway may result in unbalanced glucose homeostasis. Diabetes 58:1998–2005, 2009

The hypothalamic neuropeptide orexin is involved in arousal and energy homeostasis (1). Lack of orexin results in narcolepsy and hypophagia. Despite the reduced food intake, both narcoleptic patients with orexin deficiency and the animal model with genetic ablation of orexin neurons tend to be obese (2,3). These findings indicate that the link between orexin and energy homeostasis is not only via appetite stimulation (4,5) but also involves additional mechanisms in the control of energy metabolism. In keeping with this notion, orexin-ataxin-3 transgenic mice show reduced metabolic rate, independent of other behavioral changes induced by orexin, such as arousal, locomotion, and food intake (6).

Orexin-A can regulate plasma glucose concentrations via both central and peripheral mechanisms (7,8), but the neurotransmitter(s) responsible for controlling the endogenous activity of the orexin neurons is (are) not evident. Besides the glutamatergic input that is derived from a local neuronal network (9), orexin neurons also receive γ-aminobutyric acid (GABA)ergic inputs from a variety of brain areas such as arcuate nucleus (ARC) neurons (9), basal forebrain (10), and preoptic area (11). During the light period (i.e., the sleeping/fasting period of rats), orexin neurons in the perifornical area are inhibited by GABA inputs originating from the biological clock neurons located in the suprachiasmatic nucleus (SCN) (12). Interestingly, hypothalamic application of the GABAA receptor antagonist bicuculline (BIC) not only activates orexin neurons (13) but also increases plasma glucose concentrations (14–16).

To test the hypothesis that GABA acts as an inhibitory neurotransmitter in upstream brain areas to control the glucoregulatory function of orexin, we activated the orexin neurons in the perifornical orexin area (PF-Oa) of rats by retrodialysis of GABAA receptor antagonist and investigated its effect on endogenous glucose production (EGP), using the stable isotope technique. The effect of BIC on hepatic and peripheral insulin sensitivity was investigated by performing euglycemic clamps at two different levels of hyperinsulinemia. The specific involvement of orexin neurons in the response of EGP to BIC was confirmed in several ways: 1) by comparing the EGP response from BIC administration in the PF-Oa with that in two nearby brain areas that do not contain orexin neurons (i.e., the dorsal part of the dorsomedial hypothalamus [DDMH] and the hypothalamic paraventricular nucleus [PVN]), 2) by comparing EGP responses with and without intracerebroventricular coinjection of orexin-1 receptor (OX-1R) antagonist SB-408124 during BIC administration in the PF-Oa, and 3) by investigating the effects of intracerebroventricular administration of orexin-A and melanin-concentrating hormone (MCH) on EGP. To inves-
tigate the possible involvement of the autonomic nervous system in the glucoregulatory actions of orexin-A, we combined the intracerebroventricular administration of orexin-A with specific hepatic sympathetic or parasympathetic denervations. Furthermore, to elucidate the metabolic signaling pathway utilized by the orexin system to control hepatic insulin sensitivity, we also examined several hepatic glucoregulatory factors by quantitative real-time PCR.

RESEARCH DESIGN AND METHODS

All experiments were conducted under the approval of the animal care committee of the Royal Netherlands Academy of Arts and Sciences. Male Wistar rats weighing 300–350 g (Harlan Nederland, Horst, the Netherlands) were housed in individual cages (25 × 25 × 35 cm), with a 12/12-h light-dark schedule (lights on at 0700 h). Food and water were available ad libitum, unless stated otherwise.

Surgery preparation. Animals underwent surgeries according to the different experimental designs under anesthesia, with 0.8 ml/kg i.m. Hypnorm (Janssen, High Wycombe, Buckinghamshire, U.K.) and 0.4 ml/kg s.c. Dormicur (Roche, Ahnere, the Netherlands).

Silicon catheters were inserted into the right jugular vein and left carotid artery for intravenous infusions and blood sampling, respectively. With a standard Kopf stereotaxic apparatus, bilateral microdialysis probes (14) were placed into the PF-Oa, dDMH, and the PVN. Intracerebroventricular guiding probes were placed into the lateral cerebral ventricle. All coordinates were adapted from the atlas of Paxinos and Watson (17) (see the online appendix data 1 and Table 1 [available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0385/DC1]). All catheters and probes were fixed on top of the head and secured with dental cement.

Hepatic sympathetic or parasympathetic branches were denervated according to our previously published methods (14). The effectiveness of the hepatic sympathetic denervation was checked by measurement of norepinephrine content in the liver as described before (18). After recovery, animals were connected to a multichannel fluid-infusion swivel (Instech Laboratories, Plymouth Meeting, PA) 1 day before the experiment for adaption. Food was were connected to a multichannel fluid-infusion swivel (Instech Laboratories, Plymouth Meeting, PA) 1 day before the experiment for adaption. Food was

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significant increase in EGP as compared with their own equilibration, but both groups differed significantly from the Ringer's group at the end of their BIC administration period. Plasma insulin levels were not affected by the BIC treatment (Fig. 1D). In addition, BIC, but not Ringer's, significantly increased plasma corticosterone levels, without significant differences among the three groups (Fig. 1E). Moreover, no correlation was found between the area under the curve of the corticosterone response and the EGP increase in BIC-treated rats (r = 0.55, P = 0.10).

Immunohistochemical staining with the Fos antibody showed that in BIC-treated brains (n = 4–6), the major part of the activated neurons (as indicated by the expression of Fos in their nucleus) was limited to a restricted area around the dialysis probes (±500 μm spread from the edge of the dialysis probe). Double immunohistochemical stainings with Fos and orexin (n = 4–6) showed that when the Ringer's dialysis probes ended in the upper part of the PF-Oa, very few Fos positive nuclei (one to five per section) were observed and no colocalization of orexin and Fos was found. On the other hand, in animals with BIC dialysis probes ending in the upper part of the PF-Oa, double immunohistochemical staining showed that most of the orexin neurons (81 ± 14 single-labeled neurons per section) were activated (58 ± 9 Fos/orexin double-labeled per section [i.e., ~71%]) (Fig. 2A). With the same BIC stimulation, most of the MCH neurons that are inter-

**FIG. 1.** GABAa receptor antagonist BIC administration in the PVN, dDMH, or PF-Oa causes different EGP responses that are independent of changes in plasma insulin and corticosterone. **A:** Representative microdialysis probe placements in PVN, PF-Oa, and dDMH. The left and right panels of graph A1 are located at AP coordinates −3.30 and −1.88 mm, respectively. Graphs A2, A3, and A4 show a Nissl-stained example of each placement. PVN, DMH, and VMH are outlined by a white dotted line. **B:** Average EGP before (equilibration state) and after BIC (or vehicle) in different hypothalamic nuclei. **C:** Plasma corticosterone concentration before (equilibration state) and after BIC (or vehicle) in different hypothalamic nuclei. PVN, dDMH, and VMH are outlined by a white dotted line. **D:** Retrodialysis of BIC in the PVN, dDMH, and PF-Oa. E: Display the plasma glucose concentration, EGP, plasma insulin, and corticosterone concentration before (equilibration state) and after BIC (or vehicle) in different hypothalamic nuclei. (**A**), vehicle retrodialysis state; **B**, BIC (or vehicle) retrodialysis state. **F:** Average EGP before (○) and after a 2-h infusion of BIC in the PF-Oa (■) with intracerebroventricular vehicle or orexin-1 receptor antagonist SB-408124 (pre)treatment. Intracerebroventricular (pre)treatment with the SB-408124 blocks the BIC-induced EGP increase. **C**: Compact zone of DMH. DM, dorsomedial DMH; f, fornix; OT, optic tract; VL, ventrolateral DMH; II, third ventricle; IV, fourth ventricle. Scale bar: 400 μm. Data are presented as means ± SE. #P < 0.05 vs. equilibration state; *P < 0.05 vs. vehicle control; **P < 0.05 vs. dDMH and PVN.

Antagonizing the central orexin-1 receptor prevents the increase in EGP induced by BIC administration in the PF-Oa (experiment 2). Retrodialysis of BIC in the PF-Oa, combined with intracerebroventricular vehicle treatment, significantly increased EGP (Fig. 1F) as well as
plasma glucose concentration (6.1 ± 0.1 vs. 7.8 ± 0.3 mmol/l, P < 0.001) (i.e., similar to the results of experiment 1). However, intracerebroventricular (pre)treatment with SB-408124 prevented the BIC-induced increase in EGP. The BIC-induced increase in plasma glucose concentration was also attenuated, but it was still significantly higher than its own equilibration state (5.9 ± 0.1 vs. 6.9 ± 0.2 mmol/l, P = 0.002) and showed a trend to be significantly different from the BIC-induced increase in plasma glucose in the vehicle group (P = 0.055).

Central administration of orexin-A stimulates EGP (experiment 3). To confirm that orexin neurons are indeed involved in the BIC-induced increase in EGP, in experiment 3 we performed an intracerebroventricular infusion of either orexin-A or vehicle. Intracerebroventricular administration of vehicle had no effect on plasma glucose levels (Fig. 3A). However, a clear decline in EGP (Fig. 3B) and metabolic clearance rate (MCR) (EGP/plasma glucose concentration) (Fig. 3C) was apparent (i.e., similar to what was found in the Ringer’s animals of experiment 1). Intracerebroventricular infusion of orexin-A increased the plasma glucose level. In contrast to the vehicle group, no decrease in EGP and MCR was observed. EGP at the end of the orexin-A infusion was also significantly higher than that in the vehicle group at the end of the infusion state; thus, intracerebroventricular orexin-A prevented the endogenous decline of EGP. Plasma insulin concentrations did not change during intracerebroventricular infusion of either vehicle or orexin-A (Fig. 3D). In line with the absence of Fos immunoreactivity in MCH neurons after BIC stimulation, no significant effects of intracerebroventricular-administered MCH (n = 5) on plasma glucose levels, EGP, or MCR compared with the vehicle control animals were found (Fig. 3A–C).

Hepatic sympathetic, but not parasympathetic, denervation blocks the effect of intracerebroventricular orexin-A infusion on EGP (experiment 4). Successful hepatic sympathetic denervation (HSX) was validated by a significant decrease of liver norepinephrine concentrations compared with sham denervation (shamX) (Fig. 3E). After HSX, intracerebroventricular infusion of orexin-A no longer could prevent the endogenous decline of EGP and MCR as shown in experiment 3 (compare Fig. 3G and H with Fig. 3B and C). However, after hepatic parasympathetic denervation (HPX) or shamX the intracerebroventricular infusion of orexin-A was as effective as in liver-intact animals to prevent the endogenous drop in EGP and MCR (Fig. 3G and H). Plasma glucose changes, on the other hand, were not affected by any of the denervation procedures (i.e., plasma glucose levels increased after intracerebroventricular orexin-A infusion in all three groups [Fig. 3F]). No differences were found in plasma glucagon concentrations as a result of the intracerebroventricular orexin-A infusion or the denervation procedure (Fig. 3F).

Removal of the endogenous GABA inhibition of perifornical orexin neurons reduces hepatic insulin sensitivity (experiment 5). During clamp 1 (3 mU·kg\(^{-1}\)·min\(^{-1}\)), retrodialysis of BIC in the PF-Oa (n = 6) and dDMH (n = 4) after the equilibration state still induced a significant increase in EGP in both groups, despite the presence of peripheral hyperinsulinemia (Fig. 4A). Furthermore, EGP in the dDMH group was significantly lower

FIG. 2. A–D: Fos immunoreactivity around the microdialysis probes in the PF-Oa and dDMH area. Fos and orexin double stainings are shown for BIC administration in the PF-Oa (A) and dDMH (B). C: Fos and MCH double staining after BIC administration in the PF-Oa. D: Illustrates the Fos and orexin double staining in a Ringer’s control. After BIC administration in the PF-Oa, Fos immunoreactivity is also present in orexin target areas such as PVN (E), locus coeruleus (G), central amygdaloid nucleus (I), and intermediolateral cell column of the sacral spinal cord (K). F, H, J, and L: The panels on their respective right side show the absence of Fos-ir in these areas with Ringer’s dialysis in PF-Oa area. E–L: Double stained for Fos and orexin. The photos shown are representative for all the other animals in the same group, which are four to six rats depending on individual groups. Cc, central canal; f, fornix; III, third ventricle; IV, fourth ventricle; OT, optic tract. Scale bar: A–D, G, and H: 100 μm. E, F, I–L: 200 μm. (A high-quality digital representation of this figure is available in the online issue.)
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FIG. 4. Average EGP (A), insulin suppression of EGP (B), and Rd (C) under hyperinsulinemic-euglycemic clamp 1 (3 mU · kg⁻¹ · min⁻¹) and clamp 2 (9 mU · kg⁻¹ · min⁻¹) conditions. BIC administration in the PF-Oa induced the strongest increase in EGP as well as in Rd. Data are presented as means ± SE. # P < 0.05 vs. Ringer's control; * P < 0.05 vs. PF-Oa; #P < 0.05 vs. clamp 1. A: □, vehicle retrodialysis group; ■, BIC retrodialysis group.
glomerular genes, we also checked three genes that have previously been associated with the development of hepatic insulin resistance (25). However, the BIC-induced activation of orexin neurons had no significant effects on the expression level of either tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), or suppressor of cytokine signaling (SOCS)-3 (Fig. 5D–F).

DISCUSSION

Plasma glucose concentrations are kept within a narrow physiological range by dynamically balancing glucose production and utilization to avoid hypoglycemia and hyperglycemia and to guarantee substrate availability for energy production. Disturbances in the regulation of glucose metabolism can result in insulin resistance and type 2 diabetes. Accumulated animal data of recent years indicate that derangements of hypothalamic neural networks may act as a critical factor responsible for an unbalanced glucose metabolism (26). The present study identifies orexin as one of the critical players in such a glucoregulatory hypothalamic network. Activation of orexin neurons in the hypothalamic perifornical area increases blood glucose concentrations via a stimulation of EGP. For the stimulatory effect of orexin on glucose production to become apparent, an intact autonomic outflow from brain to liver via the hepatic sympathetic innervation is essential.

DMH and PVN have both been defined as being part of the endocrine and autonomic hypothalamic output network, with the general concept that the PVN functions as the hypothalamic integrating center for autonomic and endocrine information and serves as the final neuroendocrine and autonomic output nucleus from the hypothalamus (27), whereas the DMH has a more specific role in controlling the stress response and circadian rhythms of sleep (28). In comparison with the PVN, the separation between DMH and PF-Oa is less obvious. The rat DMH is a large complex nucleus, with its dorsomedial and ventrolateral parts divided by a compact central zone. Unlike the PVN, the different divisions of the DMH do not show a clear neurochemical separation. For instance, a large part of the PF orexin neurons extends into the ventrolateral DMH. In fact, in some (lesion) studies the DMH was considered as one area, often including part of the PF-Oa (29). In the present study, the glucoregulatory effects of BIC markedly differed between PF-Oa, dDMH, and PVN.

FIG. 5. Effects of BIC and hyperinsulinemia on the hepatic expression level of PEPCK, G6Pase, glucokinase, TNF-α, IL-6, and SOCS-3 mRNA. BIC administration in the perifornical orexin area counteracts the effects of hyperinsulinemia on the hepatic expression level of the PEPCK, G6Pase, and glucokinase genes and has no effects on that of the TNFα, IL-6, and SOCS-3 genes. □, Ringer's control; ■, BIC groups. Data are presented as means ± SE. #P < 0.05 vs. Ringer's; *P < 0.05 vs. nonclamp; ^P < 0.05 vs. clamp 1.
with respect to its effects on EGP and peripheral glucose uptake. Removal of the GABAergic inhibition clearly separated the PF-Oa, dDMH, and PVN.

Within the PF-Oa area, the distribution of orexin and MCH neurons strongly overlaps, without any colocalization. MCH and orexin both have been characterized as orexigenic peptides (30), and both orexin and MCH neurons receive GABAergic inputs (31), as well as being sensitive to other metabolic inputs such as leptin (32), NPY/agouti-related peptide, and proopiomelanocortin (33,34). Nevertheless, our and other studies (13) show that during the sleep period only orexin, but not MCH, neurons can be activated by removal of the GABAergic inhibition, indicating that the stimulatory effect of BIC on EGP probably involves activation of orexin, but not MCH, neurons. Indeed, intracerebroventricular orexin, but not intracerebroventricular MCH, was able to affect EGP, and intracerebroventricular administration of the orexin antagonist blocked a major part of the EGP stimulatory effect of BIC. Together, these data indicate that in the PF-Oa area, orexin neurons play a prominent role in the control of EGP.

Both neuronal and hormonal factors have been considered to relay the orexin signal to the periphery. Central administration of orexin can activate the hypothalamic-pituitary-adrenal axis and consequently increase plasma corticosterone levels (35). Corticosterone is able to stimulate hepatic glucose production, but in the present study EGP responses to BIC were site specific, whereas the corticosterone responses were not, suggesting that corticosterone is not the major factor responsible for the increase in EGP. Indeed, an altered pancreatic release of glucagon does not seem to be the determining factor. An alternative pathway for relaying orexin signaling to the liver is via the autonomic nervous system. Indeed, orexin can influence liver function via a multilevel sympathetic output pathway (36). Previous studies (14,21) have already shown that denervation of the sympathetic input to the liver abolishes a hypothalamic induced increase in EGP. Also in line with other studies (37,38), a purely hormonal effect of orexin on EGP is therefore not very likely. In the present study, we showed that central administration of orexin-A can only affect EGP when an intact sympathetic innervation of the liver is present, indicating a stimulatory effect of central orexin on sympathetic neuronal outflow. Remarkably, the sympathetic denervation of the liver did not prevent the stimulatory effect of intracerebroventricular orexin-A on plasma glucose concentrations. The separation of the plasma glucose and EGP responses indicates that increased central orexin signaling might affect plasma glucose concentrations not only through changes in the hepatic glucose production but also by affecting peripheral glucose uptake. A central control of peripheral glucose uptake, mediated by the autonomic nervous system, is also supported by previous studies showing that the increased glucose uptake in skeletal muscle and brown adipose tissue after the central administration of N-methyl-D-aspartate and leptin is abolished by sympathetic denervation (39,40). Since plasma insulin is not affected by orexin-A administration and hepatic sympathetic denervation (14), and our experiments were performed under fasting condition, the effect on glucose in experiment 4 seems to be mediated by a change in non–insulin-dependent glucose uptake. The GABAergic input to the orexin neurons is timed by the central biological clock located in the SCN, with the most prominent inhibition occurring during the sleep period (13,41). This timed GABA input probably is responsible for switching the orexin activity on and off in its target areas, thereby controlling the sleep/wake cycle (42,43). Since the SCN not only times glucose metabolism (44) but also controls sympathetic preautonomic neurons (45), very possibly the SCN utilizes GABA as a timing signal to control orexin activity and consequently influence sympathetic outflow and regulate glucose metabolism. On the other hand, it is well accepted that insulin signaling in the arcuate AGRP/NPY neurons accounts for ~40% of the insulin-mediated suppression of EGP via the autonomic output to liver (21,46,47). Therefore, it is possible that removal of GABA inhibition at the level of the PF-Oa could also block central insulin signaling by antagonizing the arcuate NPY/GABA projection to the orexin neurons or by activating the orexinergic projection to the arcuate NPY neurons (counteracting the suppressive effect of insulin on NPY neurons) (21,23,48). However, most of our experiments were performed under basal insulin conditions, making the contribution of such a pathway less prominent. Nevertheless, the present data support the conclusion from other studies using the orexin knockout mice model by showing that orexin is indeed an essential factor for maintaining hypothalamic insulin sensitivity for glucose metabolism (49). However, at which hypothalamic area and under which situation this interaction takes place needs further investigation.

In conclusion, we identified orexin as an important hypothalamic regulator of glucose homeostasis. The hypothalamic orexin system is possibly involved in incorporating timing information from the master brain clock into the control mechanism of EGP and also provides a possible molecular explanation for the previously observed correlation between short sleep duration and an increased risk for insulin resistance (50). The present results show that the inappropriate activation of orexin neurons during sleep deprivation will induce an increase in basal EGP and a reduction in hepatic insulin sensitivity.

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