Hypothalamic neural networks in control of glucose homeostasis
Yi, C.X.

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Chapter 6

A major role for perifornical orexin neurons in the control of glucose metabolism in rats

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Chun-Xia Yi, Mireille J. Serlie, Mariëtte T. Ackermans, Ewout Foppen, Ruud M. Buijs, Hans P. Sauerwein, Eric Fliers & Andries Kalsbeek

**Abstract**

**Objective** The hypothalamic neuropeptide orexin influences (feeding) behaviour 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 hepatic and peripheral insulin sensitivity by changing orexergic activity in the hypothalamus combined with hepatic sympathetic or parasympathetic denervation, two step hyperinsulinemic-euglycemic clamps, and RT-PCR studies.

**Results** Hypothalamic disinhibition of neuronal activity by GABA-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 i.c.v. pretreatment with the orexin-1 receptor antagonist. I.c.v. administration of orexin-A itself caused an increase in plasma glucose and prevented the daytime decrease of EGP. The stimulatory effect of i.c.v. orexin-A on EGP was prevented by hepatic sympathetic denervation. Plasma insulin clamped at 2x or 6x 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. I.c.v. administration of SB-408124 suppressed the diurnal increase of glucose appearance only around dawn.

**Conclusions** Hypothalamic orexin plays an important role in EGP, most likely by changing the hypothalamic output to the autonomic nervous system. It also indicates that activation of perifornical orexin neurons is a necessary link in the control of the biological clock over the daily rhythm in glucose production. Disturbance of this pathway may result in unbalanced glucose homeostasis.
Introduction

The hypothalamic neuropeptide orexin is involved in arousal and energy homeostasis. 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. These findings indicate that the link between orexin and energy homeostasis is not only via appetite stimulation, 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 behavioural changes induced by orexin, such as arousal, locomotion and food intake.

Orexin-A can regulate plasma glucose concentrations via both central and peripheral mechanisms, but the neurotransmitter(s) responsible for controlling the endogenous activity of the orexin neurons is not evident. Besides the glutamatergic input that is derived from a local neuronal network, orexin neurons also receive GABAergic inputs from a variety of brain areas such as arcuate nucleus (ARC) NPY neurons, basal forebrain, and preoptic area. 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). Interestingly hypothalamic application of the GABAa receptor antagonist bicuculline (BIC) not only activates orexin neurons, but also increases plasma glucose concentrations.

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 i.c.v. co-infusion of orexin-1 receptor (OX-1R) antagonist SB-408124 during BIC administration in the PF-Oa, 3) by investigating the effects of i.c.v. administration of orexin-A and melanin-concentrating hormone (MCH) on EGP. To investigate the possible involvement of the autonomic nervous system in the glucoregulatory actions of orexin-A, we combined the i.c.v. administration of orexin-A with specific hepatic sympathetic or parasympathetic denervations. Furthermore, to elucidate the metabolic signalling pathway utilized by the orexin system to control
hepatic insulin sensitivity, we also examined several hepatic glucoregulatory factors by quantitative real time-PCR (RT-PCR). Both GABA signaling and orexin activity seem to be timed by the central pacemaker in the suprachiasmatic nuclei (SCN), as on the one hand GABAergic input to the orexin neurons is most pronounced during the daytime \(^{490, 492}\), and on the other hand the content and release of orexin in the central nervous system shows a pronounced day/night rhythm \(^{495-496}\). To investigate the involvement of endogenous orexin release in the daily rhythm of plasma glucose concentrations, we performed a loss-of-function experiment by blocking the OX-1R from the middle of the light period until the early dark period and measured the rate of glucose appearance.

### Research design and methods

#### Animals

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 (25x25x35 cm), with a 12/12-h light-dark schedule (lights on at 07.00 h). Food and water were available *ad libitum*, unless stated otherwise.

#### Surgery preparation

Animals underwent surgeries according to the different experimental designs under anaesthesia with 0.8 ml/kg Hypnorm (Janssen, High Wycombe, Buckinghamshire, UK), i.m., and 0.4 ml/kg Dormicum (Roche, Almere, The Netherlands), s.c.

Silicon catheters were inserted into the right jugular vein and left carotid artery for i.v. infusions and blood sampling, respectively. With a standard Kopf stereotaxic apparatus, bilateral microdialysis probes \(^{48}\) were placed into the PF-Oa, dDMH and the PVN. I.c.v. guiding probes were placed into the lateral cerebral ventricle. All coordinates were adapted from the atlas of Paxinos and Watson \(^{497}\) (see supplemental data.1, table.1). 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 \(^{48}\). The effectiveness of the hepatic sympathetic denervation was checked by measurement of noradrenaline content in the liver as described before \(^{196}\).

After recovery, animals were connected to a multi-channel fluid infusion swivel (Instech Laboratories, PA, USA) one day before the experiment for adaption. Food was removed at the beginning of the light period to exclude intestinal glucose absorption. The first experimental blood samples were collected 4 hours later.
Tracer dilution study

To study glucose kinetics \([6.6-{^2}H_2]\) glucose (as a primed (8.0 μmol in 5 min)-continuous (16.6 μmol/h) infusion) was used as tracer (>99% enriched; Cambridge Isotopes, Andover, USA). In Experiment 1 to 4, blood samples were taken at \(t=-5\) min for measuring background enrichment of \([6.6-{^2}H_2]\) glucose, at \(t=90, t=95\) and \(t=100\) for determining enrichment during the equilibration state, and at \(t=210, 220, 230, 240\) and \(250\) for determining enrichment during the retrodialysis and/or i.c.v. infusion of different drugs according to the different experimental designs.

In Experiment 1, Ringer’s dialysis (vehicle, 3μl/min) was started together with \([6,6-{^2}H_2]\) glucose infusion in both BIC and vehicle groups. After the \(t=100\) blood sample, the Ringer’s solution was changed to a BIC solution (100μmol/L, 3μl/min, Sigma, USA) or Ringer’s (vehicle control, 3μl/min) again. After \(t=250\) blood sampling, to validate the placement of the microdialysis probes, animals were perfused by 4% paraformaldehyde and went through Nissl and immunohistochemical staining.

In Experiment 2, the primed-continuous \([6,6-{^2}H_2]\) glucose infusion and Ringer’s dialysis (vehicle) were started (after primary background blood sampling) together with OX-1R antagonist 1-(6,8-difluoro-2-methyl-quinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea (SB-408124), (50mmol/L, 5μl/hr, Sigma-Aldrich Corp., St. Louis, MO, USA) or 20% DMSO (vehicle, 5μl/hr), the dose of SB-408124 was adapted from SB-334867 in previously reported data. At the end of the equilibration state, BIC retrodialysis was applied in both groups. After the \(t=250\) min sampling animals were perfused, i.c.v. and microdialysis probe placements were validated as described above.

In Experiment 3 with liver nerve intact rats and in Experiment 4 with liver sympathetic, parasympathetic or sham denervated rats, after the \(t=100\) blood sample, a continuous i.c.v. infusion of orexin-A (1mmol/L, 5μl/hr, Bachem, Germany), MCH (1mmol/L, 5μl/hr, Bachem, Germany) (only in Experiment 3) or purified water (Milli-Q water, vehicle, 5μl/hr) (only in Experiment 3) was started. After \(t=250\) blood sampling, animals were then deeply anaesthetized and 2 μl colored dye was injected via the i.c.v. guiding probe to validate the probe placement.

In Experiment 5, both hyperinsulinemic clamp-1 and clamp-2 consisted of a basal Ringer’s (vehicle) equilibration period (\(t=0\) – \(t=100\)), a primary Ringer’s – hyperinsulinemic - euglycemic clamp period (\(t=110\) – \(t=140\)) and a secondary BIC – hyperinsulinemic - euglycemic clamp period (\(t=140\) – \(t=250\)). At \(t=100\), insulin was administered in a primed (7.2mU/kg-min in 4min for clamp-1, and 21.6mU/kg-min in 4min for clamp-2) - continuous (3mU/kg-min for clamp-1 and 9mU/kg-min for clamp-2) i.v. infusion. A variable infusion of a 25% glucose solution (containing 1% \([6,6-{^2}H_2]\) glucose) was used to maintain euglycemia (5.5±0.2mmol/L), as determined by 10 min carotid catheter blood sampling. Thirty min after the start of the primary insulin infusion, Ringer’s perfusion of the microdialysis probes was replaced by the
BIC solution in BIC group. At the end of the clamp, five blood samples were taken with a 10 min interval from t=210 to t=250. Liver tissue was then collected under deep anaesthesia for RT-PCR study, and animals were perfused for Nissl and immunohistochemical staining.

In Experiment 6, animals provided with catheters and i.c.v. probe were divided into two groups, with *ad libitum* access to food and water. Group 1 received i.c.v. infusion with SB-408124 (50mmol/L, 5μl/hr). Group 2 received 20% dimethyl sulfoxide (DMSO, 5μl/hr, as vehicle and volume control for the SB-408124). Both groups had a primed-continuous [6,6-2H2] glucose i.v. infusion started at ZT2 and 0.1ml blood samples were taken every 60 min from ZT4 until ZT15. The i.c.v. infusion of SB-408124 or vehicle was applied from ZT6 till ZT12. Food intake was measured every hour (after each blood sampling).

*Immunohistochemistry*

For immunohistochemical staining, animals were sacrificed by an intra-atrial perfusion with saline, followed by a solution of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C. After post-fixation and equilibration for 48hrs with 30% sucrose in 0.1M Tris-buffered saline (TBS), the brain tissue was cut into 30 μm sections and divided into three equal groups for Fos and orexin or MCH immunostaining (Supplemental data.2).

*Quantitative real-time PCR*

Total RNA from liver tissue was isolated with Trizol (Invitrogen) and single-stranded complementary DNA was synthesized. One μl of each cDNA was incubated in a final volume of 20μl RT-PCR reaction containing 1X SYBR-green master mix (Applied Biosystems) and 3 pmol reverse and 3 pmol forward primers (Supplemental data.3, table 2). Efficiency of primer was determined on the basis of a cDNA dilution series. Quantitative RT-PCR was performed in an Applied Biosystems (Model ABI7300 Prism Sequence Detection System). Standard PCR conditions were used. The data were acquired and processed automatically by Sequence Detection Software 33 (Applied Biosystems Inc).

*Analytical methods*

Blood samples were immediately chilled on ice in tubes containing a 5μl heparin solution and centrifuged at 4°C. Plasma was then stored at -20°C for analysis. Plasma glucose concentrations were determined using a glucose/glucose oxidase-perid method (Boehringer Mannheim, Mannheim, Germany). Plasma insulin, glucagon and corticosterone concentrations were measured using radioimmunoassay kits [LINCO Research (St. Charles, MO, USA and ICN Biomedicals, Costa Mesa, CA, respectively).
Plasma \([6,6-^2\text{H}_2]\) glucose enrichment was measured by gas chromatography-mass spectrometry (GCMS), EGP was calculated by the methods of Steele.

**Statistics**

From Experiment 1 to 5, results are expressed by averaging values from three plasma samples at the end of the equilibration state, and five plasma samples at the end of the experimental period. In Experiment 6 the results are expressed by averaging values from four different time periods. ANOVA and Student’s \(t\)-test was used to test orexin-A or BIC effects on EGP, by comparing the mean of the equilibration state with the mean of the experimental period, and were also performed to evaluate the BIC effects of different hypothalamic nuclei and to compare the different states of (hyper)insulinemia, as well as evaluate the OX-1R effects on profile of glucose metabolism during different time periods in Experiment 6.

**Results**

**Removal of the endogenous GABA inhibition of perifornical orexin neurons stimulates endogenous glucose production (Experiment 1)**

Experiment 1 was performed to investigate whether activation of orexin neurons during the light period would affect glucose metabolism. Histological analysis of the probe placements showed that the tip of the microdialysis probes was located either in the lateral part of the PVN (Fig. 1A2), at the upper borders of the PF-Oa (Fig. 1A3), or in the dDMH (Fig. 1A4).

→ Figure 1  GABAa receptor antagonist BIC administration in the paraventricular (PVN), dorsal part of the dorsomedial (dDMH) or perifornical orexin area (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 dorsal DMH. The left and right panels of graph A1 are located at AP coordinates -3.30mm and -1.88mm, 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, C, D and E display the plasma glucose concentration, EGP, plasma insulin and corticosterone concentration before (equilibration state, light gray bars) and after BIC (or vehicle, dark gray bars) in different hypothalamic nuclei. F, Average EGP before (light gray bars) and after a 2-h infusion of BIC in the PF-Oa (dark gray bars), with i.c.v. vehicle or orexin-1 receptor antagonist SB-408124 (pre)treatment. I.c.v. (pre)treatment with the SB-408124 blocks the BIC-induced EGP increase. c: compact zone of DMH, DM: dorsomedial DMH, f: fornix, VL: ventrolateral DMH, III: third ventricle. Data are presented as mean±SEM. # P<0.05 vs. equilibration state; *P<0.05 vs. Vehicle control; ^P<0.05 vs. dorsal DMH and PVN.
Rats with Ringer’s retrodialysis (n=6, vehicle) showed no changes in plasma glucose levels as compared with their own equilibration state (Fig. 1B), however, it showed a clear decline in EGP (Fig. 1C). This steady decline most likely is due to absence of food in the cages, and not to the different brain infusions, as it was not observed in animals that remained in *ad libitum* conditions (supplemental data.4, supplemental Fig. 1).
Figure 2  Fos immunoreactivity around the microdialysis probes in the PF-Oa and dDMH area (A,B,C,D). Fos and orexin double stainings are shown for BIC administration in the PF-Oa (A) and dDMH (B). Fos and MCH double staining after BIC administration in the PF-Oa is shown in C. 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). F shows the absence of Fos-ir in PVN with Ringer’s dialysis in PF-Oa area. The photos shown are representative for all the other animals in the same group, which are 4 to 6 rats depending on individual groups. III, third ventricle, f: fornix, Scale bar: A-D: 100 μm, E, F: 200 μm.
Figure 3  Fos immunoreactivity is present in extra-hypothalamic orexin target areas. Sections A through F are double stained for Fos and orexin. Fos immunoreactivity is present in locus coeruleus (A), central amygdaloid nucleus (C) and intermediolateral cell column of the sacral spinal cord (E). The panels on their respective right side show the absence of Fos-ir in these areas with Ringer’s dialysis in PF-Oa area (B, D and F). IV: fourth ventricle, OT: optic tract. Scale bar: A, B: 100μm, C-F: 200 μm.
Retrodialysis of BIC in the PF-Oa (n=5) caused a significant hyperglycemic response and a pronounced increase in EGP as compared to its own equilibration state as well as to that of the Ringer’s infusion. Retrodialysis of BIC into the dDMH (n=6) and PVN (n=4) also caused a significant, albeit smaller hyperglycemic response. Different from the PF-Oa group, both the dDMH and PVN group did not show a significant increase in EGP as compared to 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 between 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 (±500μm 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 (1-5/sections) were observed, 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 stainings showed that most of the orexin neurons (81±14 single labelled neurons/section) were activated (58±9 Fos/orexin double labelled/section, i.e. ~71%; Fig. 2A). With the same BIC stimulation, most of the MCH neurons that are interspersed among the orexin neurons were not activated (Fig. 2C). However, when dialysis probes were placed in the dDMH, only a few orexin neurons (104±6 single labelled neurons/section) were activated (21±2 Fos/orexin double labelled neurons/section, i.e. ~20%; Fig. 2B) (p<0.001 vs. PF-Oa group). Fos expression after BIC administration into the PVN did not engage the orexin neurons. This specific activation of orexin neurons by BIC is consistent with a previous study that targeted BIC to the perifornical area 492. In addition, when most of the orexin neurons were Fos positive, Fos immunoreactivity was also found in orexin targeting areas, such as PVN (Fig. 2E), locus coeruleus (Fig. 3A), central amygdaloid nucleus (Fig. 3C) and even as far as the intermediolateral cell column of the sacral spinal cord (Fig. 3E) 501, confirming the intensive activation of orexin neurons. Although the ARC has been suggested to be involved in the appetite stimulating actions of orexin 502, 503, BIC administration into the PF-Oa did not result in different numbers of Fos immunoreactive neurons in this nucleus as compared to the Ringer’s control group (6.6±1.5 vs 3.3±0.5 per/section, p=0.14).
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 i.c.v. 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, i.c.v. (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 endogenous glucose production (Experiment 3)

To confirm that orexin neurons are indeed involved in the BIC induced increase in EGP, in Experiment 3 we performed an i.c.v. infusion of either orexin-A or vehicle. I.c.v. administration of vehicle had no effect on plasma glucose levels (Fig. 4A). However, a clear decline in EGP (Fig. 4B) and metabolic clearance rate (MCR) (EGP/plasma glucose concentration) (Fig. 4C) was apparent, i.e. similar to what was found in the Ringer’s animals of Experiment 1. I.c.v. 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 i.c.v. orexin-A prevented the endogenous decline of EGP. Plasma insulin concentrations did not change during i.c.v. infusion of either vehicle or orexin-A (Fig. 4D). In line with the absence of Fos immunoreactivity in MCH neurons after BIC stimulation, no significant effects of i.c.v. administered MCH (n=5) on either plasma glucose levels, EGP or MCR as compared to the vehicle control animals were found (Fig. 4A, B and C).

Hepatic sympathetic but not parasympathetic denervation blocks the effect of i.c.v. orexin-A infusion on EGP (Experiment 4).

Successful hepatic sympathetic denervation (HSX) was validated by a significant decrease of liver noradrenaline concentrations as compared to sham denervation (shamX) (Fig. 4E). After HSX, i.c.v. infusion of orexin-A no longer could prevent the endogenous decline of EGP and MCR as shown in Experiment 3 (compare 4H and I with 4B and C). However, after hepatic parasympathetic denervation (HPX) or sham denervation (ShamX) the i.c.v. infusion of orexin-A was as effective as in liver-intact animals to prevent the endogenous drop in EGP and MCR (Fig. 4H and I). Plasma glucose changes on the other hand, were not affected by any of the denervation procedures, i.e. plasma glucose levels increased after i.c.v. orexin-A infusion in all three
Figure 4 Involvement of the autonomic nervous system in the EGP increase induced by bicuculline administration in the PF-Oa. A-C, Average plasma glucose concentration, EGP and MCR before (light gray bars) and after a 2-h infusion of vehicle, orexin-A or MCH into the lateral cerebral ventricle (dark gray bars). D, Average plasma insulin concentration before (equilibration state, light gray bars) and after i.c.v. vehicle or orexin-A infusion (dark gray bars). E, Hepatic sympathetic denervation abolishes 96% of the noradrenaline content in the liver. F-I, Plasma glucagon concentration, EGP , MCR and plasma glucose concentration before (light gray bars) and after i.c.v. orexin-A infusion (dark gray bars) with HSX, HPX and shamX. Data are presented as mean±SEM. # P<0.05 Infusion state vs. Equilibration state (or shamX vs. HSX in E); *P<0.05 vs. Vehicle control in A and B and vs. HSX in H; ^P<0.05 MCH vs. orexin-A group.
groups (Fig. 4G). No differences were found in plasma glucagon concentrations as a result of the i.c.v. orexin-A infusion or the denervation procedure (Fig. 4F).

**Removal of the endogenous GABA inhibition of perifornical orexin neurons reduces hepatic insulin sensitivity (Experiment 5)**

During clamp-1 (3mU/kg.min) 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. 5A). Furthermore, EGP in the dDMH group was significantly lower than in the PF-Oa group. Thus the insulin mediated suppression of EGP, i.e. hepatic insulin sensitivity, was significantly attenuated in both BIC groups as compared to their vehicle control groups (Fig. 5B), but the effect was significantly more pronounced in the PF-Oa group than in the dDMH group. The rate of glucose disappearance (Rd) was significantly increased by BIC in the PF-Oa group, but not in the dDMH group (Fig. 5C) as compared to their respective control groups.

To explore the effect of BIC on peripheral insulin sensitivity, clamp-2 with a higher plasma insulin level was performed. Despite the higher insulin levels, which resulted in a significant further decrease in EGP in the two Ringer’s control groups, BIC still caused a significant increase in EGP in both the PF-Oa (n=7) and the dDMH (n=4) group as compared to their respective control groups (Fig. 5A). Again the effect was more pronounced in the PF-Oa group than in the dDMH group. With regard to Rd, the two Ringer’s groups showed a significant higher Rd as compared to clamp-1 and both BIC groups showed a significantly higher Rd than the control groups (Fig. 5C). The RT-PCR study on the liver tissue revealed that BIC treatment significantly di-

**Figure 5** Average EGP (A), insulin suppression of EGP (B) and Rd (C) under hyperinsulinemic-euglycemic clamp-1 (3 mU/kg.min) and clamp-2 (9 mU/kg.min) conditions. BIC administration in the PF-Oa induced the strongest increase in EGP as well as in Rd. Light gray bars, vehicle retrodialysis group; dark gray bars, BIC retrodialysis group. Data are presented as mean±SEM. # P<0.05 vs. Ringer’s control; *P<0.05 vs. PF-Oa; ^P<0.05 vs. clamp-1.
minimized the inhibitory effect of hyperinsulinemia on the expression of phosphoenolpyruvate carboxykinase (Pepck) and Glucose-6-Phosphatase (G6Pase), as well as its stimulatory effect on the expression of glucokinase (Fig. 6A-C). In addition to these glucoregulatory genes, we also checked three genes that have previously been associated with the development of hepatic insulin resistance. However, the BIC-induced activation of orexin neurons had no significant effects on the expression level of either tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), or suppressor of cytokine signaling-3 (SOCS-3) (Fig. 6D-F).

**Figure 6** Effects of BIC and hyperinsulinemia on the hepatic expression level of Pepck, G6Pase, glucokinase, TNF-alpha, 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. Light gray bars: Ringer’s control, dark gray bars, BIC groups. Data are presented as mean±SEM. # P<0.05 vs. Ringer’s, * P<0.05 vs. non-clamp, ^ P<0.05 vs. clamp-1.
Antagonizing the orexin-1 receptor suppresses the daily increase of glucose appearance at dawn (Experiment 6)

If an increased activity of orexin neurons results in an increase in EGP, a suppression of the endogenous increase in central orexin activity is expected to change glucose metabolism in the opposite direction. The rhythmic activity of orexin along the light/dark cycle, with lowest levels during the light period and an acrophase during the dark period in rats, is well known\(^{495,496}\). In Experiment 6 we administered the OX-1R antagonist SB-408124 i.c.v. from the early light period until the early dark period and measured the 12 hours profile of plasma glucose concentration and rate of glucose appearance (Ra). Since long term fasting is known to activate orexin neurons\(^{212}\), this study was carried out under *ad libitum* conditions in order not to disturb the physiological release pattern of orexin. Starting with a similar basal Ra (P=0.90, Fig. 7A), the OX-1R antagonist SB-408124 or vehicle was infused i.c.v. from ZT6 onwards.

**Figure 7** I.c.v. infusion of OX-1R antagonist SB-408124 suppresses the increase of Ra and MCR from dawn (ZT11-12) until the first hours of the dark period (ZT12-15), without influencing the plasma glucose and insulin concentration. Data are presented as mean±SEM. # P<0.05 vs. ZT4-6, ^ P<0.05 vs. ZT6-11, *P<0.05 vs. the same period in the vehicle group.

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continuously. From basal until the dark period, repeated-measures ANOVA detected significant effects of *Time* in the vehicle group on Ra ($F(3,19)=13.0, p<0.001$). During both dawn and dark period Ra was significantly higher than basal rates ($p=0.002$ and $p=0.003$ respectively) and during the light period ($p=0.002$ for both periods), but this effect was not found in the i.c.v. SB-408124 group ($F(3,23)=1.47, p=0.25$). Between the two groups, no significant effects of the OX-1R antagonist on Ra were detected ($p=0.90$ and $p=0.58$) during the basal and light period (i.e. ZT6-ZT11). The first and also most significant difference was seen just before the dark period (i.e. ZT11-ZT12), with the SB-408124 group having a significantly lower Ra than the vehicle group ($P<0.001$). This difference extended into the dark period from ZT12 ($P=0.03$). Plasma glucose levels, however, showed no *Time* difference between any of the time points within either the vehicle ($F(3,19)=1.15, p=0.36$) or SB-408124 group ($F(3,23)=1.11, p=0.34$). Furthermore, no difference was found between vehicle and SB-408124 groups ($F(1.9)=1.0, p=0.33$) (Fig. 7B). Consequently, repeated-measures ANOVA detected significant effects of *Time* in the vehicle group on MCR ($F(3,19)=9.76, p=0.001$), with both the dawn and dark period showing a significantly higher MCR than the basal ($p=0.002$ and $p=0.003$ respectively) and light period ($p=0.002$ for both periods). No effect of *Time* was detected in the i.c.v. SB-408124 group ($F(3,23)=1.22, p=0.33$). Moreover the SB-408124 group had a significantly lower MCR than the vehicle group during the dawn ($P=0.003$) and dark periods ($P=0.02$, Fig. 7C).

These results show a significant inhibitory effect of i.c.v. SB-408124 on a physiological increase in Ra and MCR starting around the light-dark transition (dawn) period and extending in the first part of the dark period. Furthermore, no differences were detected between the two groups with regard to their total food intake during this 12 h study ($8.95\pm1.64$ vs. $7.93\pm1.76$, $p=0.68$) and plasma insulin concentration (Fig. 7D), thus excluding an indirect effect of the OX-1R antagonist on Ra via a reduction in food intake or influence on insulin level. The fact that the time related effects of i.c.v. OX-1R antagonist were only seen shortly before and during arousal indicates that under physiological conditions the orexin system affects glucose metabolism in a time-dependent manner.

**Discussion**

Plasma glucose concentrations are kept within a narrow physiological range by dynamically balancing glucose production and utilization to avoid hypoglycaemia and hyperglycemia and to guarantee substrate availability for energy production. Disturbances in the regulation of glucose metabolism can result in insulin resistance and diabetes mellitus type 2. 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. 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 endogenous glucose production. Moreover, daily rise in endogenous orexin activity is involved in an increased blood glucose appearance at dawn. 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 64, whereas the DMH has a more specific role in controlling the stress response and circadian rhythms of sleep 274. 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 505. In the present study, the glucoregulatory effects of BIC markedly differed between PF-Oa, dDMH and PVN 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 co-localization. MCH and orexin both have been characterized as orexigenic peptides 506 and both orexin and MCH neurons receive GABAergic inputs 507, as well as being sensitive to other metabolic inputs such as leptin 508, NPY/AGRP and POMC 509, 510. Nevertheless, our and other studies 492 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 i.c.v. orexin, but not i.c.v. MCH, was able to affect EGP and i.c.v. 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 endogenous glucose production.

Both neuronal and hormonal factors have been considered to relay the orexin signal to the periphery. Central administration of orexin can activate the hypothalamo-pituitary-adrenal axis and consequently increase plasma corticosterone levels 511. 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. In addition, also 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. Previous studies 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, 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 i.c.v. orexin-A on plasma glucose concentrations. The separation of the plasma glucose and EGP responses indicate that increased central orexin signalling 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 NMDA and leptin is abolished by sympathetic denervation. Since plasma insulin is not affected by orexin-A administration and hepatic sympathetic denervation, 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 suprachiasmatic nucleus (SCN), with the most prominent inhibition occurring during the sleep period. 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. Since the SCN not only times glucose metabolism, but also controls sympathetic pre-autonomic neurons, 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 signalling in the arcuate AGRP/NPY neurons accounts for approximately 40% of the insulin mediated suppression of EGP via the autonomic output to liver. Therefore it is possible that removal of GABA inhibition at the level of the PF-Oa could also block central insulin signalling 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). 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 knock-out mice model, by showing that orexin is indeed an essential factor for maintaining hypothalamic insulin sensitivity for glucose metabolism. However, at which hypothalamic area and under which situation this interaction takes place needs further investigation.

GABAergic input to the orexin neurons is timed by the central biological clock located in the suprachiasmatic nucleus (SCN), with the most prominent inhibition occurring during the sleep period. 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. Glucose metabolism in the rat is also well timed by the SCN output. Data obtained from both human and rat studies strongly suggest that the rise in plasma glucose concentrations during the dawn period is primarily caused by an increased hepatic glucose production controlled by the endogenous clock located in the central nervous system.

With the present data, we propose that the orexin system in the PF area is an important intermediate between the biological clock and at least one aspect of the dawn-phenomenon, i.e. increased glucose production. Although the hypothalamic administration of BIC also clearly enhanced peripheral glucose uptake, we have not been able to link it to a specific nucleus or neurotransmitter system and the intermediate neural mechanism between the biological clock and peripheral glucose uptake is therefore still unknown at present.

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 endogenous glucose production and also provides a possible molecular explanation for the previously observed correlation between short sleep duration and an increased risk for insulin resistance. The present results show that the inappropriate activation of orexin neurons during sleep deprivation will induce an increase in basal endogenous glucose production and a reduction in hepatic insulin sensitivity.
Supplemental data.1

Table 1 Stereotactic coordinates for placements of microdialysis and i.c.v. probes.

<table>
<thead>
<tr>
<th>(mm)</th>
<th>Anteroposterior</th>
<th>Lateral</th>
<th>Ventral</th>
<th>Angle</th>
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<tbody>
<tr>
<td>PF-Oa</td>
<td>-2.4</td>
<td>2.5</td>
<td>-8.1</td>
<td>10</td>
</tr>
<tr>
<td>dDMH</td>
<td>-2.4</td>
<td>2.0</td>
<td>-7.9</td>
<td>10</td>
</tr>
<tr>
<td>PVN</td>
<td>-1.6</td>
<td>2.0</td>
<td>-7.9</td>
<td>10</td>
</tr>
<tr>
<td>i.c.v.</td>
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<td>2.0</td>
<td>-3.2</td>
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</tr>
</tbody>
</table>

Tooth bar was set as -3.2 mm. The ventral coordinates were standardized for 300g BW, every additional 25g BW will be placed 0.1 mm deeper.

Supplemental data.2

Immunocytochemistry

Two groups of brain sections were incubated overnight at 4°C with goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-orexin or rabbit anti-MCH (1:2000; Phoenix Pharmaceuticals, Belmont, CA) primary antibodies. Sections were then rinsed in 0.1M TBS, incubated 1 hr in biotinylated horse anti-goat IgG, and then 1 h in avidin–biotin complex (ABC, Vector Laboratories, Inc., Burlingame, CA), the reaction product was visualized by incubation in 1% diaminobenzidine (DAB) (0.05% nickel ammonium sulphate was added to the DAB solution to darken the reaction product, DAB/Ni) with 0.01% hydrogen peroxide for 5-7 min. After Fos immunostaining, sections were rinsed in 0.1M TBS, incubated 1 hr in biotinylated goat anti-rabbit IgG, and then 1 h in ABC, the reaction product was visualized by DAB staining only. Sections were mounted on gelatine-coated glass slides, dried, run through ethanol and xylene and covered for observation by light microscopy.

Supplemental data.3

Table 2, RT-PCR primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Pepck</td>
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<td>CGCTTCCGAAGGAGATGATCT</td>
</tr>
<tr>
<td>G6Pase</td>
<td>CCCATCTGTGTTCCACATTCAA</td>
<td>GGCCTGGTCCAAAAAAGAATC</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>TCCTCTCAATTGGACCAAGG</td>
<td>TGCCACCATCCATCTCAA</td>
</tr>
<tr>
<td>IL-6</td>
<td>TGCCCTCCAGGAACAGCTATG</td>
<td>TGTCACAACATCAGTCCCAAGAA</td>
</tr>
<tr>
<td>TNF-a</td>
<td>CTAACTCCACAAAAAGCAAGCAA</td>
<td>CCTCGGGCCAGTTATGAGA</td>
</tr>
<tr>
<td>SOCS3</td>
<td>CCTCCACGTCTTTGGTCCGAAGAC</td>
<td>TACTGGTCCAGGAATCTCAGGCC</td>
</tr>
<tr>
<td>Ubi*</td>
<td>CTCCCAACGGACCTGTGAAG</td>
<td>CTGAAGAGAATTCACCAAGGAATTGA</td>
</tr>
</tbody>
</table>

* Ubi: ubiquitin conjugate enzyme, as reference gene.
Supplemental data.4

Supplemental Figure 1  EGP response to Ringer’s retrodialysis into hypothalamus or purified water i.c.v. infusion. Food was removed from the rat’s home cage at the beginning of the light period. Experiments started five hours later, after the baseline samples for calculating the equilibration of isotope tracer [6.6-^2H_2] glucose were taken between 90-100 min. Rats received either Ringer’s retrodialysis into the hypothalamus (3μl/min) or purified water i.c.v. (5μl/hr), blood samples were taken continuously during 2 hrs with a 50 min intervals. With similar basal EGP, both groups show similar decline of EGP along the 2 hrs sampling period and also ended up with similar EGP’s at the last time point (t=250 min, 54.9±0.74 vs 54.8 ± 2.52 μmol/kg.min, p=0.65). Another group of rats that went through a similar fasting protocol but without any brain infusion showed a similar EGP on each time point. Under ad libitum conditions, without any brain infusion, EGP does not show a declining trend. This indicates that the decline of EGP is an endogenous process resulting from the removal of food from rats’ home cage at the beginning of the light period, and not from the brain infusion.

Supplemental data.5

Supplemental Figure 2  Orexin neurons (dark brown) and Fos positive orexin neurons were counted in the rectangle box outlined in a Nissl staining (blue) section (1.5 mm x 1 mm). III, third ventricle, f: fornix. Scale bar: 500 μm.