The role of estrogen in hypothalamic regulation of hypothalamus-pituitary-adrenal axis activity, energy homeostasis and bone metabolism
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Intrahypothalamic estradiol regulates glucose metabolism via the sympathetic nervous system in female rats
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Intrahypothalamic estradiol regulates glucose metabolism via the sympathetic nervous system in female rats

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Abstract

Long-term reduced hypothalamic estrogen signalling leads to increased food intake, decreased locomotor activity and energy expenditure, which ultimately results in obesity and insulin resistance. In the current study we aimed to determine the acute obesity-independent effects of hypothalamic estrogen signaling on glucose metabolism. We studied endogenous glucose production (EGP) and insulin sensitivity during selective modulation of systemic or intrahypothalamic E2 signaling in rats one week after ovariectomy (OVX). OVX caused a 17% decrease in plasma glucose, which was completely restored by systemic E2. Likewise, the administration of E2 by microdialysis, either in the hypothalamic paraventricular nucleus (PVN) or in the ventromedial nucleus (VMH), restored plasma glucose. The infusion of an E2-antagonist via reverse microdialysis into the PVN or VMH attenuated the effect of systemic E2 on plasma glucose. Furthermore, E2 administration in the VMH, but not in the PVN, increased EGP and induced hepatic insulin resistance. E2 administration in both the PVN and the VMH resulted in peripheral insulin resistance. Finally, sympathetic, but not parasympathetic, hepatic denervation blunted the effect of E2 in the VMH on both EGP and hepatic insulin sensitivity. In conclusion, intrahypothalamic estrogen regulates peripheral and hepatic insulin sensitivity via sympathetic signalling to the liver.

Keywords: E2, hypothalamus, glucose, insulin sensitivity, sympathetic denervation
Estradiol (E2) plays a major role in the control of energy homeostasis (1, 2), as is exemplified by increased body weight after ovariectomy (OVX) in female rats, reversible with E2 replacement (3-6). E2’s effects on energy homeostasis are thought to be mediated primarily through the hypothalamus, as direct injections of E2 into the hypothalamic paraventricular nucleus (PVN), arcuate (Arc) or ventromedial nucleus (VMH) effectively reduce food intake and body weight after OVX in rodents (7-9). A link between hypothalamic E2 receptors and energy expenditure was elegantly shown by the obese phenotype induced by selective silencing of ERα in VMH (10, 11). Together with many more studies, these data have convincingly shown that reduced estrogen signaling in the hypothalamus increases body weight and is associated with impaired glucose tolerance and insulin resistance. At this stage it is less clear if estrogen affects glucose metabolism directly or whether it works indirectly - by inducing obesity. When ovariectomized rats are studied before the onset of obesity they exhibit higher glucose/insulin ratios (with decreased plasma insulin concentration) compared to intact rats, suggesting that ovariectomy increases insulin sensitivity (12). This surprising finding could represent a more direct obesity-independent effect of estrogen on glucose metabolism whose mechanisms have thus far been elucidated only partially. The hypothalamus, has emerged as a key player in the regulation of glucose production (13). The suppressive effects of peripheral hyperinsulinemia on endogenous glucose production (EGP) can be blocked by the central administration of NPY (14) or insulin antibodies (13) (15). Moreover, the suppressive effect of central insulin on EGP can be largely abolished by selective hepatic vagal denervation (16, 17), whereas the intrahypothalamic administration of various neurotransmitters stimulates EGP via the sympathetic efferent nerves to the liver (18-20). These hypothalamic neurotransmitter systems probably act as targets for circulating hormones such as insulin (14), thyroid hormone (21) and glucocorticoids (22). E2 receptors (ER) are abundantly expressed in the hypothalamic PVN and VMH (23), nuclei that are key players in the hypothalamic regulation of glucose metabolism via autonomic outflow towards the liver (24). Considering that the hypothalamus plays a key role in both the regulation of body weight by estrogen and in controlling glucose metabolism, we hypothesized that the direct, obesity-independent, effects of estrogen on glucose metabolism are, at least in part, mediated via the hypothalamus and the autonomic nervous system. To test our hypothesis we performed a series of experiments that involved the application of reverse microdialysis, selective hepatic autonomic denervations, euglycemic hyperinsulinemic clamps and stable isotope dilution. In order to prevent any effects of increased adiposity on glucose metabolism all experiments were performed 1 week after ovariectomy, i.e., before any increase in body weight or adiposity occurred.
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Research design and Methods

Animals

Female Wistar rats (Harlan, Horst), housed in a 12-h light–12-h dark schedule (lights on at 0700 h) were used for all experiments. Body weight was between 220 and 280 g. Food and drinking water were available ad libitum. All of the following experiments were conducted with the approval of the Animal Experimental Committee of the Academic Medical Center (AMC) in Amsterdam.

Surgery

Rats were anesthetized using a mixture of ketamine/xylazine (100:10 mg/kg IP). All animals underwent a bilateral ovariectomy (except for the control group in Experiment 1). Silicon catheters were inserted into the right jugular vein and left carotid artery for intravenous infusions (I.V.) and blood sampling, respectively. With a standard Kopf stereotaxic apparatus, bilateral microdialysis probes were placed adjacent to PVN or VMH. The coordinates for the PVN were AP: 1.6 mm, lateral: 1.8 mm (angled at 10°) and 9.1 mm ventral from the surface of the bone. The coordinates for the VMH were AP: 2.5 mm, lateral: 2.0 mm (angled at 10°) and 9.0 mm ventral from dura. We used dental cement to secure the microdialysis probes and the jugular and carotid outlets to 4 stainless steel screws inserted into the skull. Rats were allowed 1-week of post-operative recovery before the start of the actual experiment. The probe location was checked by thionin staining after sacrifice. Only the animals with correct probe placements were used for data analysis (supplemental data 1).

Hepatic sympathetic or parasympathetic branches were denervated according to our previously published methods (18). The effectiveness of the hepatic sympathetic denervation (SX) was checked by measuring the norepinephrine content in the liver (25). We have previously validated our method for selective hepatic parasympathectomy (PX) by using retrograde viral tracing (18).

During the experiments, animals were connected to blood-sampling and microdialysis lines, which were attached to a metal collar and kept out of reach from the rats by means of a counterbalanced beam. This allowed all manipulations to be performed outside the cages without handling the animals. The metal collars were attached at least 24 h before the actual experiment. Animals were handled and sham blood was sampled (i.e., blood was withdrawn and immediately returned) regularly in the week before the first experiment began to familiarize them with all the experimental procedures.

One mg β-E2 (Sigma, St. Louis, USA) was dissolved in 1 ml pure dimethyl sulfoxide (DMSO) and diluted 100 times with Ringer solution. The ER antagonist ICI 182,780 (TOCRIS, Bristol, UK) was dissolved at a final concentration of 10 μg/ml in Ringer solution containing 1% DMSO. With a measured estradiol recovery efficiency of 0.002% (in vitro), the drug
concentrations used in the present study (i.e., 10 μg/ml) are expected to result in tissue concentrations of around 1.0 nM, which is close to the tissue concentration of estradiol as measured in our previous study(26). The EC50 value of estradiol is around 0.15 nM for both ERα and ERβ. Thus, the doses used for infusion are expected to activate both ERα and ERβ. We cannot measure the recovery efficiency of the antagonist. The IC50 value of antagonist is 0.29nM. Thus if the recovery is similar to that of estradiol it will inhibit both ERα and ERβ.

**Plasma measurements**

Glucose enrichment was measured as described previously (27) (supplemental data2). Plasma insulin and corticosterone were measured by a commercially available ELISA. Plasma estradiol concentration was determined by ELISA kits (Biosource, Belgium).

**Statistics**

Data were analyzed by ANOVA with repeated measures, with Group (E2 or Veh) as the between-animal factor and Time as the within-animal factor. Post hoc tests (Tukey HSD) were performed if ANOVA revealed a significant effect. Significance was defined at P≤0.05.

**Experiment 1** was designed to investigate the difference in plasma glucose concentrations between intact and ovariectomized (OVX) animals, and the effect of an intravenous infusion (I.V.) of E2 in OVX animals on plasma glucose concentrations. Blood samples from both intact and OVX animals were collected at 10:30 a.m. for the measurement of basal plasma glucose concentrations. In OVX animals, E2 (3.5 ng/min) or vehicle (saline containing 1% DMSO) was continuously infused via the jugular vein catheter for 165 min (start from t=15 min). Blood samples were collected from the carotid artery at t=0 (just before infusion) and t=30, 45, 60, 90, 120, 150 and 180 min after infusion. In the sham animals blood samples were collected at the same time point.

**Experiment 2** was designed to investigate the changes in plasma glucose induced by the reverse microdialysis of the E2 receptor antagonist ICI 182,780 into the PVN and VMH combined with the I.V. administration of E2 in OVX animals. Ringer’s dialysis (3μl/min) in the PVN or VMH via the microdialysis probes was started at t= -60 min. E2 (3.5 ng/min) was infused I.V. starting at t=10 min, and E2 antagonist (10 μg/ml, 3μl /min) was infused via the microdialysis probes into the PVN or VMH starting at t=15 min. Blood samples were collected at t=0, 30, 45, 60, 90, 120, 150 and 180 min.

**Experiment 3** was designed to investigate the effects of reverse microdialysis of E2 into the PVN and VMH of OVX animals on glucose kinetics. To study glucose kinetics, [6,6-2H2] glucose (as a primed [8.0 umol in 5 min]-continuous [16.6 umol/h] infusion) was used as tracer (>99% enriched; Cambridge Isotopes, Andover, MA). Blood samples were taken at t = -95 min for measuring background enrichment of [6,6-2H2]glucose, at t =0, 5 and 10
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min for determining enrichment during the steady state, and at t =30, 45, 60, 90, 120, 150 and 180 min for determining enrichment during the retrodialysis of E2 (non-steady state). Ringer’s dialysis (3μl/min) in the PVN or VMH via the microdialysis probes was started at t=-60 min. At t=15 min E2 (10 μg/ml, 3 μl/min) or vehicle (Ringer with 1% DMSO, 3 μl/min) were infused by retrodialysis into PVN or VMH.

Experiment 4 was designed to investigate the effects of reverse microdialysis of E2 into the PVN and VMH of OVX animals on insulin sensitivity. Background blood samples and isotope tracer infusion were the same as for Exp.3. At t=15 min, insulin was administered in a primed I.V. infusion (3.6 mU/kg.min in 5 min for the “low” clamp#1, and 7.2 mU/kg.min in 5 min for the “high” clamp#2), followed by a continuous I.V. infusion (1.5 mU/kg.min and 3 mU/kg.min respectively). A variable infusion of a 25% glucose solution (containing 1% [6,6-2H2] glucose) was used to maintain euglycemia (5.5±0.5mmol/l) (supplemental data 3), as determined by carotid catheter blood sampling every 10 min. Thirty min after the start of the primary insulin infusion (t=45min), Ringer’s perfusion of the microdialysis probes was replaced by the E2 solution (10 μg/ml, 3 μl/min) or vehicle (Ringer’s containing 1%DMSO). At the end of the clamp, five blood samples were taken with a 5 min interval at t=120, 125, 130, 135 and 140 min (supplemental data 4).

Experiment 5 was designed to investigate the effect of selective hepatic autonomic nerve denervations on plasma glucose changes induced by the reverse microdialysis of E2 into the PVN and VMH. The experimental design is similar to that for Exp.3. Experiment 6 was designed to investigate the effects of a hepatic sympathetic nerve denervation combined with the reverse microdialysis of E2 into the VMH on hepatic insulin sensitivity. The experimental design was similar to that of Exp.4

Results

As expected, plasma E2 concentrations were lower in OVX than in intact animals (P=0.004) (Figure 4.1A). After 165 min of systemic E2 infusion, plasma E2 concentrations were higher in the E2 group (P=0.002), and comparable to those of the intact animals (P=0.357) (Figure 4.1A). OVX animals showed a 17% decrease of basal plasma glucose concentrations as compared to intact animals (P=0.035) (Figure 4.1B). During systemic infusion of E2, plasma glucose concentrations significantly increased as compared to the vehicle-infused group (Time effect: P<0.001; Group effect: P=0.028; Time×Group effect: P=0.001) (Figure 4.1B). The effect of systemic E2 on plasma glucose was blunted by intrahypothalamic administration of the E2 antagonist ICI 182,780 (ICI). Plasma glucose levels were significantly lower during systemic E2 infusion and simultaneous retrodialysis of ICI in the PVN than during vehicle retrodialysis (Time, P<0.001; Group, P=0.02; Time×Group, P=0.064) (Figure 4.1C). A similar effect was found after systemic E2 infusion and simultaneous retrodialysis of ICI in the VMH (Time, P<0.001; Group, P=0.009; Time×Group, P<0.001) (Figure 4.1D).
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During retrodialysis of E2 in the PVN of OVX animals, plasma glucose concentrations increased compared to vehicle retrodialysis (Group, P=0.015; Time×Group, P<0.001) (Figure 4.2A). Similarly, E2 treatment in the VMH also resulted in higher plasma glucose levels (Group, P=0.006; Time×Group, P<0.001) (Figure 4.2D). During the retrodialysis of vehicle in the PVN and VMH EGP showed a slow decline, probably due to the prolonged fasting(20). Retrodialysis of E2 in the PVN did not affect EGP (Figure 4.2B), but the same treatment in the VMH increased EGP as compared to the vehicle treatment, i.e., EGP showed no steady decrease, showing significant effects of Group (P<0.001) and Time×Group (P<0.001) (Figure 4.2E). Importantly, plasma E2 concentrations were not affected by the intrahypothalamic infusions of E2 in either the PVN or VMH (Figure 4.2C & 4.2F).

Figure 4.1 (A) Ovariectomy (OVX) reduced plasma E2 concentrations compared to intact animals. Intravenous E2 (E2 iv) administration restored plasma E2 concentrations (different letters indicate a significant difference, p<0.05). (B) After ovariectomy plasma glucose concentrations were lower compared to intact animals (p=0.035). Intravenous E2 administration acutely increased plasma glucose to concentrations observed in intact animals. The increase in plasma glucose concentrations during intravenous E2 administration was attenuated by simultaneous administration of the E2 receptor antagonist ICI 182,780 (ICI) in (C) the PVN (p=0.020 vs vehicle) and (D) VMH (p=0.009 vs vehicle). The hatched bars indicate the continuous infusion of vehicle, E2 and/or ICI.
We studied hepatic and peripheral insulin sensitivity using euglycemic hyperinsulinemic clamps at low and high insulin concentrations, respectively. Plasma insulin concentrations during the clamps were significantly higher than those during basal conditions in both the low- and high-dose clamp groups (time, $P<0.001$). No significant differences between the different infusion groups (i.e., Veh, PVN & VMH) were detected (group, $P>0.27$; time×group, $P>0.48$) (Figure 4.3C&F). The lower dose clamp experiment showed...
similar basal EGP levels between the vehicle and E2 infusion groups (P=0.687), but the intrahypothalamic administration of E2 differentially affected the insulin-induced decrease of EGP (P=0.003) (Figure 4.3A). In the vehicle and PVN E2 infusion groups, EGP was suppressed by 30-40%, while in the VMH E2 infusion group the expected decrease in EGP induced by hyperinsulinemia was completely blunted (P=0.017 vs vehicle) (Figure 4.3B).

**Figure 4.3 (A+B)** Insulin decreased endogenous glucose production (EGP) by 30%-40% during vehicle (VEH; n=6) and E2 infusion in the PVN E2 (n=6). Insulin-mediated suppression of EGP was completely blunted by simultaneous E2 infusion in the VMH (n=6). **(D+E)** Insulin infusion also stimulated glucose uptake (Rd), which was partly prevented by infusion of E2 in either the VMH or PVN. **(C+F)** Plasma insulin concentrations in the different groups during the “low” (1.5mU/kg.min) (C) and “high” (3.0mU/kg.min) (F) clamps. Different letters indicate a significant difference (p<0.05).

During the higher dose hyperinsulinemic clamp, the insulin-induced increase in glucose uptake was reduced by E2 treatment both in the PVN and VMH (both P=0.012) (Figure 4.3D). In the vehicle group, as expected, glucose uptake was increased by 130%, whereas in the PVN and VMH treatment group, the increase in glucose uptake by insulin was attenuated (Figure 4.3E).
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Figure 4.4 (A+B) The effect of E2 administration in the PVN on plasma glucose concentration and EGP was not affected by sympathetic (Sx) or parasympathetic (Px) denervation of the liver. (C+D) In contrast, sympathetic denervation reduced plasma glucose (p=0.002 SX vs sham) and EGP (p=0.006 SX vs sham) during E2 infusion in the VMH compared to sham denervation. The hatched bars indicate the continuous infusion of E2.

Figure 4.5 (A+B) During E2 administration in the VMH, insulin-mediated EGP suppression was restored after sympathetic denervation (Sx) of the liver. (C) Insulin concentrations under basal conditions and during intravenous insulin administration. n=6 for all groups. Different letters indicate a significant difference (p<0.05). (insulin administration rate: 1.5 mU/kg.min).
Liver noradrenalin levels were significantly lower (<10%) in all sympathectomy (SX) groups as compared to both the sham-denervated and parasympathectomy (PX) groups (Supplemental data 5). During E2 infusion in the PVN, the increase in plasma glucose did not differ between sham, hepatic Sx and Px groups (Group, $P=0.386$; $Time \times Group$, $P=0.163$) (Figure 4.4A). Likewise, there was no effect of hepatic denervations on EGP (Group, $P=0.528$; $Time \times Group$, $P=0.939$) either during steady state or non-steady state conditions (Figure 4.4B). During E2 infusion in the VMH, however, plasma glucose concentrations were lower in the Sx group than in the Px and sham groups (post hoc Group effect: $P=0.002$ and $P=0.001$, Sx versus Px and sham, respectively) (Figure 4.4C). The stimulatory effect of E2 infusion in the VMH on EGP (Figure 4.2E) was abolished by Sx, but not by Px or sham denervation (Group, $P=0.006$ and $Time \times Group$, $P<0.001$ for Sx versus sham, and Group, $P=0.604$ and $Time \times Group$, $P=0.487$ for Px versus sham) (Figure 4.4D). During E2 infusion in the VMH, insulin suppressed EGP by 35% in the Sx group, but only by 10% in the group with intact sympathetic signaling to the liver ($P=0.014$) (Figure 4.5A,B), indicating the necessity of the sympathetic hepatic innervation for the modulation of hepatic insulin sensitivity by E2 in the VMH. Plasma insulin levels during the clamp were significantly increased as compared to basal conditions before the clamp, but no significant differences between the 2 groups (Sham and Sx) were found ($p<0.001$ and $P=0.172$, respectively)(Figure 4.5C).

### E2 effects on glucoregulatory hormones

IV E2 infusions in Experiment#1 did not affect plasma insulin, corticosterone or glucagon levels when compared to the vehicle group (Table 1). Also E2 administration in the PVN or VMH did not affect plasma insulin or glucagon concentrations (Table 4.1). Both PVN and VMH administration of E2 resulted in increased plasma corticosterone values at t=180 min, but only the effect in the PVN reached significance (ANOVA basal values(t=0 min), $p=0.637$; t=180 min, $p=0.009$). The PVN-E2 corticosterone value differed significantly from

<table>
<thead>
<tr>
<th>Groups</th>
<th>Insulin (ng/ml)</th>
<th>Corticosterone (ng/ml)</th>
<th>Glucagon</th>
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<tbody>
<tr>
<td></td>
<td>Before infusion</td>
<td>After infusion</td>
<td>Before infusion</td>
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<tr>
<td></td>
<td>(t=0)</td>
<td>(t=180)</td>
<td>(t=0)</td>
</tr>
<tr>
<td>iv veh</td>
<td>1.25 ± 0.28</td>
<td>1.51±0.41</td>
<td>85.48±10.46</td>
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<tr>
<td>iv E2</td>
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<td>1.98±0.43</td>
<td>75.83±29.22</td>
</tr>
<tr>
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<td>0.88±0.13</td>
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</tr>
<tr>
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<td>81.00±18.80</td>
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<tr>
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<td>1.78±0.28</td>
<td>88.33±17.15</td>
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<tr>
<td>VMH E2</td>
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<td>1.65±0.35</td>
<td>55.40±12.30</td>
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$^a$: $p<0.05$ as compared to t=0 min values.
both vehicle groups at t=180 min (p<0.005), whereas the VMH-E2 t=180 value differed neither from the 2 vehicle groups nor from the PVN-E2 group.

Discussion

The key finding of the present study is that local changes in hypothalamic E2 availability have site-specific effects on peripheral glucose metabolism and insulin sensitivity. More specifically, in ovariectomized rats local supplementation of E2 in the PVN and VMH decreased peripheral insulin sensitivity and reduced glucose uptake, whereas local E2 supplementation in the VMH also reduced endogenous glucose production and caused hepatic insulin resistance. Finally, we demonstrated that the effect of E2 in the VMH on hepatic insulin sensitivity is mediated by the sympathetic innervation to the liver.

The effects of ovariectomy in animal models include increased food intake and decreased running activity, all of which are reversed upon E2 replacement (28, 29). A series of experiments by the group of Deborah Clegg nicely showed that local effects of E2 in the brain play an important role in these restorative effects of E2. Recent reports showed that a major part of the profound effects of E2 on energy metabolism are mediated via the hypothalamus. For instance, ICV infusion of E2 was sufficient to restore the normal pattern of body fat distribution in OVX females (6) and a local knock-out of ERα in the VMH was sufficient for animals to become obese (10). Thus E2 withdrawal and substitution have profound effects on adiposity and lipid metabolism (3, 6, 30). Direct effects of E2 on glucose metabolism via the brain, however, are not evident yet. On the other hand, previous studies by us and others revealed that the hypothalamus also plays a crucial role in the regulation of glucose production (19, 21). To further examine the possible neural mechanisms behind the modulation of peripheral E2 on glucose metabolism, we used reverse microdialysis technology in order to be able to administer E2 locally into PVN or VMH. In our first set of experiments, strikingly lower plasma glucose levels in OVX animals were found, which could be reversed by a systemic E2 replacement. Interestingly, the increase in plasma glucose concentrations induced by systemic E2 was blocked by the hypothalamic administration of the E2 antagonist ICI, both in PVN and VMH. The data indicated that, like glucocorticoid and thyroid hormone, hypothalamic E2, too, may play an important role in the regulation of peripheral glucose metabolism.

Next we infused E2 directly into the PVN and VMH by reverse microdialysis. The increased plasma glucose levels were consistent with the results of the antagonist experiment. The E2-induced changes could not be explained by the changed corticosterone or insulin levels, as during the VMH infusions neither plasma corticosterone nor insulin concentrations were affected. Infusions in the PVN did not affect plasma insulin concentrations either. In accordance with the well-known effects of estrogens on the hypothalamo-pituitary-adrenal axis, corticosterone concentrations were changed by E2 administration in the
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PVN. However, the E2-induced corticosterone changes occurred after t=150 min, which was much later than the E2-induced change in plasma glucose. Although both electrical stimulation of VMH neurons and direct insulin injections in the VMH have been reported to induce an increase of plasma glucagon concentrations (31-33), we did not find any change in plasma glucagon values after the different treatments in the present study. These data indicate that the increased EGP induced by VMH E2 is not mediated by an increased release of glucagon, and that different mechanisms maybe activated via the VMH. Although our denervation experiments suggest that the E2-induced increased EGP is caused by an increased sympathetic activity, at present we cannot exclude the involvement of other hormonal regulators such as the catecholamines.

Recently Yonezawa et al reported that during exposure to a high-fat diet both peripheral and central E2 receptors are involved in the regulation of glucose metabolism(34). However, central and peripheral E2 receptors seem to operate via different mechanisms. When treated with peripheral E2, fatty acid synthase was decreased in white adipose tissue. While treatment with central E2, changed both liver glucose production and peripheral tissue glucose uptake (34). Their results suggest that hypothalamic E2 may effect on EGP and glucose uptake by respectively increasing and decreasing insulin sensitivity. Estrogenic effects on peripheral organs were also addressed by several other studies. In the liver glucose homeostasis seems to be regulated mainly by estrogen acting via ER\textsubscript{α}, which was associated with a pronounced hepatic insulin resistance (35). Immunohistochemical analysis revealed that ER\textsubscript{α} and ER\textsubscript{β} are co-expressed in the nuclei of most muscle cells. These studies also showed that ER\textsubscript{α} is a positive regulator of GLUT4 expression, whereas ER\textsubscript{β} has a suppressive role (36).

In the current studies, E2 administration in the VMH but not PVN increased EGP, indicating that the increasing plasma glucose concentrations observed after PVN infusion were mainly due to a decreased glucose uptake, whereas the increased plasma glucose concentrations after E2 administration in the VMH were mainly due to an increased EGP. The two classic E2 receptors, ER\textsubscript{α} and ER\textsubscript{β}, show a distinct hypothalamic distribution, with the VMH mainly containing ER\textsubscript{α} and the PVN mainly containing ER\textsubscript{β} (23). At present it is not clear how this differential receptor distribution contributes to the different glucoregulatory effects of E2 in the PVN and VMH.

We used hyperinsulinemic-euglycemic clamps with 2 different insulin plasma levels. Consistent with the basal EGP results, PVN E2 treatment caused a peripheral insulin resistance. On the other hand, both peripheral and hepatic insulin resistance were found in the group treated with VMH E2. Previous studies in our group showed that the hypothalamus often increases hepatic glucose production by stimulating sympathetic efferent nerves (18, 19, 21). Also in the current experiments the stimulatory effect of E2 via the VMH on EGP (and plasma glucose concentrations) was abolished by a sympathetic, but not a parasympathetic, denervation of the liver. On the other hand, autonomic denervation of the liver (either sympathetic or parasympathetic) had no effect on the stimulatory effect
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of E2 on plasma glucose concentrations via the PVN. There is no evidence for a direct neural connection between the VMH and autonomic nuclei in the brainstem or spinal cord, contrary to the PVN. On the other hand, the VMH has pronounced projections to the PVN, which functions as the hypothalamic integration center for autonomic and endocrine information and serves as the final neuroendocrine and autonomic output nucleus from the hypothalamus (18, 37-40). ERα is expressed in the majority of glutamatergic neurons in VMH (41) and the PVN is known to receive a strong glutamatergic input from the VMH (42). Therefore, we propose that ERα-containing glutamatergic neurons in the VMH that project to the PVN are activated by local administration of E2, thereby exciting sympathetic pre-autonomic neurons in the PVN, that in turn stimulate the hepatic sympathetic tone. In line with our present findings, a number of previous experiments have provided evidence for hypothalamic effects on glucose uptake mediated via the autonomic nervous system (43, 44). From a physiological viewpoint the opposite effects of ERα stimulation in the VMH on hepatic glucose production and peripheral glucose uptake (stimulatory and inhibitory, respectively) are plausible, as in this way the two mechanisms will act in concert to increase plasma glucose concentration. In order to explain the opposite effect of the VMH on muscle-dedicated and liver-dedicated pre-autonomic neurons we propose that either the glutamatergic projection of the VMH to the muscle-dedicated pre-autonomic neurons involves a GABAergic interneuron in the subPVN or that the VMH contains GABAergic ERα-expressing neurons which contact and inhibit the muscle-dedicated pre-autonomic neurons directly. Indeed, E2 treatment has been shown to increase GABAergic activity in the VMH (45). Finally, the preferential effect of E2 in the PVN on peripheral glucose uptake indicates that in all likelihood the muscle-dedicated pre-autonomic neurons, but not the liver-dedicated pre-autonomic neurons, in the PVN express the ERβ. Although the expression of ERβ mRNA in pre-autonomic PVN neurons has been reported (46), the peripheral targets of these neurons are not known.

Together, our results show differential effects of intrahypothalamic E2 on hepatic and peripheral glucose metabolism that are, at least partly, mediated by the sympathetic branch of the autonomic nervous system. However, the current results seem contradictory with earlier studies that indicated increased plasma glucose levels in OVX animals. We think this apparent difference is induced by the different models used. Most studies thus far used a “chronic” model in which animals were studied at least one month after the OVX (47-49). Therefore, the increased levels of plasma glucose observed most likely are the results of hyperphagia and obesity. The current results indicate that the first effect of reduced plasma E2 concentrations is a lowering of plasma glucose levels. During the second stage, the OVX animals develop increased food intake and decreased energy expenditure and become obese. At the end of the second stage, the impaired energy homeostasis will overrule the glucose-lowering effect of E2 removal. How the short-term effects of intrahypothalamic E2 on glucose metabolism as observed in the present set
of experiments can be reconciled with the long-term effects of E2 deprivation on body weight and insulin sensitivity remains to be determined.

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Ji Liu is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. J.Liu contributed to the experiment and wrote the manuscript. P.Bisschop researched the data and reviewed/edited the manuscript. L.Eggels contributed to the experiment. E.Foppen contributed to the experiment. M. Ackermans contributed to the EGP measurement. JN Zhou reviewed/edited the manuscript. E.Fliers researched the data and reviewed/edited the manuscript. A.Kalsbeek researched data, reviewed/edited the manuscript and contributed to discussion.

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Chapter 4

Reference


Supplemental data 1: Probe location
At the end of the experiments all the animals were sacrificed for histological verification of the probe location. One hundred and forty-one animals were used in this part of the study. The data from 19 animals had to be discarded because of incorrect probe placements (n=11) or an incomplete data set due to catheter problems (n=8).

Supplemental Figure. Representative sections and schematic drawings demonstrating probe locations in the PVN (A) and VMH (B).

Supplemental data 2: Protocol for measurement of [6,6-2H2]glucose enrichment
The protocol was described previously (1). Briefly, the samples (25μl plasma) were deproteinized by mixing with 1 mL methanol. After centrifugation, the supernatant was evaporated to dryness under a stream of N2. The aldonitrile penta-acetate derivative of glucose was prepared with 100 μL hydroxylamine in methanol (5 mg hydroxylamine and 12.5 mg sodium acetate in 1 mL methanol), and the mixture was heated for 60 min at 60 C. After drying the sample under a stream of N2, 100 μL acetic anhydride were added, and the sample was heated for another 60 min at 120 C. The reaction mixture was cooled and partitioned between water (750 μL) and methylenechloride (750 μL). The lower methylenechloride layer was dried and reconstituted in ethylacetate, which was injected into the gas chromatograph (model 6890 gas chromatograph coupled to a model
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5973 mass selective detector, equipped with an electron impact ionization mode, Hewlett-Packard Co., Palo Alto, CA. The enrichment of \([6,6-\text{H}_2]\)glucose was determined by dividing the peak area at M+2 by the total peak area at M, and correction for the natural abundance was performed by subtracting the natural abundance from the measured M+2 enrichment.

The calculation was performed as described before (2). Briefly, when endogenous glucose production (Ra) and glucose disposal (Rd) are calculated, the added source of labeled glucose entering the system and the exogenous glucose infusate should be taken into account. Thus, Ra and Rd were calculated with a modified form of the Steele equations as described by Finegood et al (3):

\[
R_a(t) = \left[ \frac{I}{Pct_p(t)} - \frac{pVG(t)dPct_p(t)/dt}{Pct_p(t)} + \left( \frac{Pct_o}{Pct_p(t)} \times GInf(t) \right) \right] - pV \frac{dG(t)}{dt}
\]

and

\[
R_d(t) = \left[ \frac{I}{Pct_p(t)} - \frac{pVG(t)dPct_p(t)/dt}{Pct_p(t)} + \left( \frac{Pct_o}{Pct_p(t)} \times GInf(t) \right) \right] - GInf(t)
\]

where I is the constant tracer infusion rate (mg · kg\(^{-1}\)·min\(^{-1}\)), t is time, Pctp(t) is the percentage enrichment in plasma glucose taken as the average of 2 consecutive samples, p is the pool fraction, V is the distribution volume of glucose, G(t) is the plasma glucose concentration taken as the average of 2 consecutive samples, dPctp(t)/dt is the rate of change in the percentage enrichment in plasma (min\(^{-1}\)), GInf(t) is the rate of infusion of exogenous glucose, Pctg is the percentage enrichment of the glucose infusate, and dG(t)/dt is the rate of change in the plasma glucose concentration; V (pV) was set at 40 mL/kg.

The \([6,6-\text{H}_2]\)glucose enrichment (tracer/tracee ratio) total assay CV was 1%, the intra-assay CV 1%, and the detection limit 0.04%.

Supplemental data 3: time schedule for clamping experiment (Experiment 4&6)

Background blood samples and isotope tracer infusion were the same as for Exp.3. At t=15 min, insulin was administered in a primed I.V. infusion (3.6 mU/kg.min in 5 min for the “low” clamp, and 7.2 mU/kg.min in 5 min for the “high” clamp), followed by a continuous I.V. infusion (1.5 mU/kg.min for the “low” clamp and 3 mU/kg.min for the “high” clamp). A variable infusion of a 25% glucose solution (containing 1% \([6,6-\text{H}_2]\) glucose) was used to maintain euglycemia (5.5±0.5 mmol/l), as determined by carotid catheter blood sampling every 10 min. Thirty min after the start of the primary insulin infusion (t=45 min), Ringer’s perfusion of the microdialysis probes was replaced by the E2 solution (10 μg/ml, 3 μl/min) or vehicle (Ringer’s containing 1% DMSO). At the end of the clamp, five blood samples were taken with a 5 min interval at t=120, 125, 130, 135 and 140 min.
Supplemental data 4: Liver noradrenaline levels during denervation experiments.

After homogenizing the tissue samples, the noradrenaline was measured by an in-house HPLC method. Essentially norepinephrine and epinephrine were selectively isolated by liquid-liquid extraction (4) and derivatized with the fluorescent 1,2-diphenylethlenediamine (5). The fluorescent derivatives were separated by reversed phase liquid chromatography and detected by scanning fluorescence detection.

Supplemental Figure. The liver noradrenaline concentration is reduced to <10% of control values in the sympathetic denervation groups, but not in the parasympathetic denervation groups. (A) Experiment 5 (P<0.001) and (B) Experiment 6 (P<0.001).
Supplemental data 5: Plasma glucose level and variable glucose infusion rate during clamping experiment (Experiment 4 & 6)

**Supplemental table.** The table presents the mean of the plasma glucose concentration in the basal state before (t= 0, 5, 10 min) and at the end of the different clamp experiments. Plasma glucose levels were around 5.5 mmol/l during both the basal state and the clamp. No significant group differences were detected.

<table>
<thead>
<tr>
<th></th>
<th>basal</th>
<th>hyperinsulinemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;low&quot; clamp vehicle</td>
<td>5.32±0.27</td>
<td>5.36±0.45</td>
</tr>
<tr>
<td>PVN E2</td>
<td>5.50±0.36</td>
<td>5.54±0.34</td>
</tr>
<tr>
<td>VMH E2</td>
<td>5.56±0.33</td>
<td>6.02±0.38</td>
</tr>
<tr>
<td>&quot;high&quot; clamp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vehicle</td>
<td>5.23±0.34</td>
<td>5.49±0.56</td>
</tr>
<tr>
<td>PVN E2</td>
<td>5.00±0.68</td>
<td>5.13±0.53</td>
</tr>
<tr>
<td>VMH E2</td>
<td>5.50±0.57</td>
<td>5.46±0.39</td>
</tr>
<tr>
<td>Denervation plus &quot;low&quot; clamp</td>
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<tr>
<td>Sham</td>
<td>5.35±0.70</td>
<td>5.62±0.40</td>
</tr>
<tr>
<td>Sx</td>
<td>5.42±0.34</td>
<td>5.48±0.43</td>
</tr>
</tbody>
</table>

**Supplemental figure 5.** During the "low" dose insulin clamp, the variable glucose infusion rate was lower in both the PVN and VMH E2 treatment groups as compared to vehicle, but lowest in the VMH E2 group (Suppl. 5A). During the "high" clamp, the infusion rate was significantly lower in both the PVN and VMH E2 treatment groups as compared to vehicle (Suppl. 5B). After sympathetic denervation, the variable glucose infusion rate was highest in the SX group and comparable to the infusion rate in Vehicle treated animals of the "low" clamp group in suppl.5A.
References


