Hypothalamic neural networks in control of glucose homeostasis
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Glucocorticoid signaling in the arcuate nucleus is involved in the control of hepatic insulin sensitivity

In preparation
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Abstract

Objective  Glucocorticoid receptors are, amongst others, highly expressed in the hypothalamic paraventricular (PVN) and arcuate nucleus (ARC). As glucocorticoids have pronounced effects on neuropeptide Y (NPY) expression, and NPY neurons projecting from the ARC to the PVN are pivotal in feeding behaviour and glucose metabolism, we investigated the effect of glucocorticoid signaling in the ARC and PVN on endogenous glucose production (EGP) and hepatic insulin sensitivity.

Research Design and Methods  We investigated the acute effects of local administration of the glucocorticoid receptor agonist dexamethasone (Dex) in the PVN or ARC, by retrodialysis, on EGP and hepatic insulin sensitivity. EGP was measured by the stable isotope dilution method, and hepatic insulin sensitivity was studied during a hyperinsulinemic-euglycemic clamp.

Results  Retrodialysis of dexamethasone for 90 min into the PVN, the ARC, or the brain area surrounding the PVN and ARC (misplacement control, MC) had no significant effects on basal plasma glucose concentration. EGP showed a decline over time in all groups (i.e., dexamethasone and vehicle), but only in the Dex-ARC and Dex-MC group this decline reached significance. No significant differences were found between the six treatment groups, nor when each dexamethasone group was compared to its own vehicle control group. During the hyperinsulinemic-euglycemic clamp, retrodialysis of dexamethasone into the ARC, but not into the PVN or MC, largely prevented the suppressive effect of peripheral hyperinsulinemia on EGP. Circulating plasma corticosterone levels were inhibited in all dexamethasone, but not vehicle, treated groups.

Conclusions  Dexamethasone administration in the ARC, but not in the PVN, induces hepatic insulin resistance. Whereas glucocorticoids profoundly affect glucose metabolism through their signalling in specific hypothalamic nuclei, their inhibitory effect on the hypothalamus-pituitary-adrenal (HPA) axis is much more widespread in the hypothalamus.
Introduction
Clinical conditions with glucocorticoid excess such as Cushing’s syndrome are accompanied by a disturbed glucose metabolism, including hepatic insulin resistance, which is reversible after treatment. Several hypothalamic nuclei including the arcuate nucleus (ARC) and the paraventricular nucleus (PVN) have recently been proven to be involved in regulating hepatic glucose production and insulin sensitivity. Thus in addition to their well known direct peripheral effects on glucose metabolism, glucocorticoids may also affect glucose metabolism through their binding in the central nervous system (CNS).

Glucocorticoid signaling involves two receptor systems, i.e., the mineralocorticoid receptor (MR), which in the CNS is mainly restricted to the septum, hippocampus and amygdala, and the glucocorticoid receptor (GR), which is localized throughout the brain including the hypothalamus. In the hypothalamus, GRs are expressed abundantly in the PVN and in the ARC as demonstrated by immunohistochemistry, in situ hybridization and receptor autoradiography studies, while MRs show weak immunoreactivity in the PVN and its mRNA expression is not detectable by in situ hybridization methods. The strong expression of GRs in the PVN, especially in the parvocellular subdivision, is thought to be mainly involved in the negative feedback of corticosterone on the hypothalamus-pituitary-adrenal (HPA) axis. However, the PVN is not only responsible for neuroendocrine regulation, but it is also an important hypothalamic centre for the control of the autonomic nervous system (ANS) by virtue of the local abundance of pre-autonomic neurons. Recently the ANS has been strongly implicated in the regulation of glucose metabolism and hepatic insulin sensitivity, therefore glucocorticoids might affect glucose metabolism through their action on pre-autonomic neurons in the PVN.

Interestingly, i.c.v. infusion of dexamethasone (i.e., a glucocorticoid receptor agonist) stimulates food intake and body weight gain, while it decreases glucose uptake. The specific neuronal target area(s) for these i.c.v. administered dexamethasone effects have not been clearly identified yet. One possibility is that it involves glucocorticoid signaling in the ARC, thereby antagonizing the effects of insulin on neuropeptide Y (NPY) containing neurons in the ARC. This is supported by the observation that peripheral administration of dexamethasone or corticosterone increases NPY mRNA expression in the ARC.

In the present study we investigated whether modulation of hypothalamic glucocorticoid signaling specifically in the ARC or the PVN would influence peripheral glucose metabolism. By using the retrodialysis technique we delivered dexamethasone locally into the ARC and PVN under stress-free experimental conditions. EGP was measured by the stable isotope dilution method and hepatic insulin sensitivity was determined by combining it with a hyperinsulinemic-euglycemic clamp.
Materials 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, with a 12/12-h light-dark schedule (lights on at 07.00 h, as Zeitgeber time 0 (ZT0)). 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 i.m. (Janssen, High Wycombe, Buckinghamshire, UK), and 0.4 ml/kg Dormicum s.c. (Roche, Almere, The Netherlands).

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 were placed into the ARC and PVN. Coordinates were adapted from the atlas of Paxinos and Watson (see supplemental data.1; table.1). All catheters and probes were fixed on top of the skull and secured with dental cement. 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 restricted at 20g on the night prior to experiments. The start of the experiment (i.e., t=0) was at ZT3.

Glucose kinetics
To study glucose kinetics, [6.6-2H2] glucose as a primed (8.0 μmol in 5 min) followed by a continuous (16.6 μmol/h) infusion was used as a tracer (>99% enriched; Cambridge Isotopes, Andover, USA). A first blood sample was taken at t=-5 min for measuring background enrichment of [6.6-2H2] glucose. The [6.6-2H2] glucose infusion started at t=0 min. After an equilibration time of 90 minutes, blood samples were taken at t=90, t=95 and t=100 min for determining glucose enrichment and plasma glucose and corticosterone concentrations. Thereafter the [6.6-3H2] glucose infusion was continued for another 110 min where after blood samples were taken at t=200, t=205 and t=210 min for determining glucose enrichment during the retrodialysis of dexamethasone and vehicle (Ringer’s solution). Thereafter, the insulin infusion started (prime 7.2mU/kg-min for 4 min followed by a continuous infusion (3mU/kg-min) from T= 210 min until T=370 min (vide infra). At t=210 min an additional and variable infusion of a 25% glucose solution (enriched 1% with [6,6-2H2] glucose) was started to maintain euglycemia (5.5±0.2mmol/L), as determined by 10 min carotid catheter blood sampling. At the end of the clamp, five blood samples were taken with a 10 min interval from t=330-370 min.
Retrodialysis

Retrodialysis probes were aimed to be placed into PVN and ARC. We included animals in which the probes appeared to be misplaced upon histological examination as a separate post-hoc misplacement control (MC) group, thus animals were divided into six groups: vehicle-PVN, dexamethasone-PVN (Dex-PVN), vehicle-ARC, Dex-ARC, vehicle-MC and Dex-MC. From t=0-100 min, all six groups received retrodialysis of vehicle (Ringer’s, 3μl/min). After taking the blood samples for the calculation of basal EGP, i.e., from t=100 min onwards, in the dexamethasone groups (Dex-PVN, Dex-ARC and Dex-MC) the retrodialysis of Ringer’s was replaced by dexamethasone retrodialysis (200 μM, 3μl/min). In the vehicle group the retrodialysis of Ringer’s was continued until the end of the study. At the end of the experiments, brains were removed for Nissl staining to validate the probe placements.

Analytical methods

Plasma glucose concentration was measured by a handheld glucometer (Freestyle Flash, Abbott), plasma corticosterone concentrations were measured using radioimmunoassay kits [LINCO Research (St. Charles, MO, USA). Plasma [6,6-2H2] glucose enrichment was measured by gas chromatography-mass spectrometry (GCMS) 605.

Statistics and calculations

Results are expressed by averaging values from three plasma samples at the end of the equilibration state (t=90-95-100 min), the end of the dexamethasone infusion state (t=200-205-210 min), and five plasma samples at the end of the clamp experimental period (t=330-340-350-360-370 min). ANOVA and post-hoc Student’s t-tests were performed to compare the group differences. EGP was calculated using the modified forms of the Steele Equations (ref Finegood) 500.

Figure 1 Schematic drawing illustrating the correct placement zones for of retrodialysis probes. If probes were inside the PVN and ARC nuclei or within the boundaries of the gray dotted lines (=200μm around the nuclei), rats were categorized as vehicle/Dex-PVN (Dex-PVN is illustrated by gray triangles) or vehicle/Dex-ARC (Dex-ARC is illustrated by gray circles) groups. Rats with probes outside of the gray dotted lines were grouped as misplacement control (Vehicle/Dex-MC).
Results

Glucose metabolism in the basal state

Probe placements were examined in Nissl stained sections. For the ARC and PVN groups, rats were included if bilateral probes were placed either inside the nuclei or within the direct vicinity of the nucleus, i.e., not beyond 200μm distance from the borders of the nuclei. Rats with probes exceeding these ranges were categorized as misplacement control. The anatomical criteria are illustrated in Fig. 1.

Basal plasma glucose levels did not differ between the six experimental groups (F(5,28)=0.74, p=0.60) and glucose concentration did not change within any of the groups during the retrodialysis of vehicle or dexamethasone; also no difference in plasma glucose level was found between the six groups after retrodialysis (F(5,28)=0.80, p=0.56) (Fig. 2).

Basal EGP showed no significant difference between all groups (F(5,28)=0.42, p=0.83) (Fig. 3). Also after 90 min of dexamethasone or vehicle treatment, EGP was not different between the 6 treatment groups (F(5,28)=1.03, p=0.42). In all groups mean EGP decreased (average 27%) during the retrodialysis of vehicle or dexamethasone. As we demonstrated previously (6), this decline of EGP is caused by the removal of food from the rat home cage before the start of the experiment and fasting along the experimental period. Only in the Dex-ARC and Dex-MC groups, this decrease reached significance (p=0.03 for both groups).

Figure 2 Plasma glucose concentrations at the end of the equilibration state (basal) and at the end of the 90-min dexamethasone treatment (before the start of the clamp).
Hepatic insulin sensitivity

Insulin significantly suppressed EGP in the three vehicle groups, as well as in the Dex-PVN and the Dex-MC groups (P<0.01 for all five groups, compared to their own basal state, and P<0.05 for five all groups, compared to their own Dex state) (Fig. 3). Mean suppression of EGP in those groups was 64%. However, in the Dex-ARC group, severe hepatic insulin resistance was found with no significant suppression of EGP by insulin infusion (P= 0.43 vs. Basal state, and P=0.54 vs. Dex state). Indeed ANOVA detected a significant effect of Group (F(5,28)=5.66, p=0.002) during the clamp condition. Post-hoc testing showed that EGP during hyperinsulinemia was significantly higher and suppression significantly less in the Dex-ARC group than in the other five groups (P<0.01 for all groups).

The rate of glucose disappearance (Rd) was not significantly different between the six groups (F(5,28)=1.41, p=0.26), however, Rd in Dex-PVN was significantly higher than in vehicle-PVN (p=0.02) as well as in Dex-ARC group (p=0.01) (Fig. 5).

The present study used a freely moving, non-stressed animal model, and during the basal state (i.e., before dexamethasone administration) plasma corticosterone concentrations of all animals were in the normal daytime range, i.e., 0 – 25 ng/ml (Fig. 6), without significant differences between any of the groups (F(5,28)=0.78, p=0.80). After dexamethasone or vehicle treatment in the basal state no significant difference

**Figure 3** Endogenous glucose production (EGP) at the end of equilibration state (basal vehicle state), at the end of the 90-min dexamethasone or vehicle treatment and at the end of the hyperinsulinemic-euglycemic clamp. # p<0.05 vs. basal vehicle state; ^ p<0.05 vs. Dex state; * p<0.05 vs. other five groups during clamp.
was found between the 6 groups (F(5,28)=2.63, p=0.17). A clear rise in the plasma corticosterone concentration was observed in the three vehicle groups at the end of the hyperinsulinemic clamp (ZT9-10) compared to their basal state (p=0.01 for vehicle-PVN; p=0.04 for vehicle-ARC; p=0.006 for vehicle-MC); no difference was found between the end of the dexamethasone period and the clamp period (p=0.064 for vehicle-PVN; p=0.26 for vehicle-ARC); p=0.007 for vehicle-mc), i.e., nicely in line with the daily rhythm in plasma corticosterone concentrations that show a pronounced circadian peak before the onset of the dark period (i.e., ZT12). This rise in corticosterone at the end of the hyperinsulinemic period was completely absent in

![Figure 4](image_url)

**Figure 4** Percentage suppression of EGP by hyperinsulinemia at the end of the clamp.

# p<0.05 vs. Vehicle-ARC;

* p<0.05 vs. Dex-PVN and Dex-MC.

![Figure 5](image_url)

**Figure 5** Rd at the end of the euglycemic hyperinsulinemic clamp.

# p<0.05 vs. Vehicle-PVN,

* p<0.05 vs. Dex-ARC.
all three dexamethasone treated groups (p=0.12 for Dex-PVN; p=0.87 for Dex-ARC; p=0.48 for Dex-mc). ANOVA detected a significant effect of Group (F(5,28)=12.31, p<0.001), (p<0.01, for each vehicle group vs. dexamethasone group). No differences were found between the three vehicle groups (F(2,11)=3.35, p=0.99), nor between the three dexamethasone-treated groups (F(2,16)=3.35, p=0.07).

**Discussion**

To investigate whether glucocorticoids are able to affect glucose metabolism via a central mechanism and whether this modulation is mediated within the hypothalamus by the ARC or the PVN, we delivered the glucocorticoid receptor agonist dexamethasone or vehicle locally into the ARC as well as in the PVN. After 90 min of treatment, dexamethasone appeared to have no effects on either basal plasma glucose concentrations or basal EGP in comparison with the vehicle control groups. However, dexamethasone treatment into the ARC during hyperinsulinemia induced severe hepatic insulin resistance. Peripheral corticosterone concentrations were similar in all groups, but the endogenous rise in plasma corticosterone before the onset of the dark period was absent in all dexamethasone treated animals. These results show that glucocorticoids acting specifically in the ARC reduce hepatic insulin sensitivity, whereas
their inhibitory effect on the HPA-axis is not different between ARC and PVN or the areas around these two nuclei. Therefore, contrary to its feedback action on the HPA-axis, the antagonistic effect of glucocorticoids on the insulin-induced inhibition of EGP appears to be localized specifically in the ARC. Moreover, the insulin-resistance promoting effect of hypothalamic glucocorticoids is independent from its effects on circulating glucocorticoids.

Due to a locally leaky blood brain barrier, the ARC neurons represent a neuron population in the brain with an easy access to many blood born signals, including hormones such as insulin, leptin, thyroid hormone, ghrelin and glucocorticoids as well as other circulating metabolic factors. Moreover, the receptors for these hormones are abundantly expressed in this nucleus. The clearest evidence for glucocorticoids to act on the ARC are the changes induced by glucocorticoids in NPY expression and release, in conjunction with the presence of glucocorticoid binding elements in the NPY gene. In addition, the effects of ghrelin, which is released by the stomach and also acts within the ARC to alter food intake depend on glucocorticoids because none of these actions of ghrelin can be induced after adrenalectomy (ADX). In addition, we found that during fasting, which increases circulating plasma corticosterone levels, Fos-ir is induced in the ARC neurons that express the GR (see supplemental data 2 and supplemental Fig. 1). Together these data indicate that glucocorticoids are able to interact with ARC signaling and that this interference may act as an important integrative element for ARC neurons in the control of glucose metabolism. In our previous study, i.c.v. infusion of NPY induced hepatic insulin resistance via the hepatic sympathetic innervation. Therefore, it is tempting to speculate that in the present study the main action of dexamethasone in the ARC was to increase NPY activity and release, thereby inducing hepatic insulin resistance via an increased sympathetic input to the liver.

Chronic (2 days) i.c.v. infusion of dexamethasone increases food intake and decreases muscle tissue glucose uptake, and both effects need an intact subdiaphragmatic vagus nerve in order to occur. Interestingly, some of the metabolic effects of chronic i.c.v. infusions of NPY depend on the presence of circulating corticosterone because bilateral adrenalectomy prevents the effects on muscle glucose uptake and insulin sensitivity of adipose tissue. Moreover these effects also dependent upon an intact subdiaphragmatic vagus nerve. Taken together, these data support a synergy between NPY and corticosteroids in the ARC in the control of metabolism.

To further clarify the effects of central dexamethasone on glucose disappearance, plasma insulin concentrations representing ~6 times physiological levels are needed. Moreover, such studies will also enable us to separate possible direct effects of dexamethasone on glucose metabolism from indirect effects via changes in food intake and other aspects of energy balance.
Interestingly, although it has been suggested that dexamethasone poorly penetrates the brain \(^{648}\), by using radioautographic studies in ADX rats, it has been shown that “small amounts” of dexamethasone do selectively reach the ventral part of the ARC \(^{649}\). Whether the observed specificity of dexamethasone in the ARC on glucoregulation in the present study has a clinical implication and thus whether peripherally administered dexamethasone can act via the ARC to regulate hepatic insulin sensitivity needs further investigation.

In summary, by delivering dexamethasone via retrodialysis into specific hypothalamic nuclei, we showed that increasing glucocorticoid signaling in the ARC induces severe hepatic insulin resistance while it had no effect on basal endogenous glucose production. This centrally mediated effect of dexamethasone cannot be attributed to increased circulating plasma corticosterone concentrations. Moreover, the hypothalamic system responsible for the glucocorticoid feedback on the HPA-axis seems much more widespread than the one responsible for the inhibitory effect on EGP.

**Supplemental data.1**

**Table 1** Stereotactic coordinates for placements of microdialysis probes.

<table>
<thead>
<tr>
<th>(mm)</th>
<th>Antero-posterior</th>
<th>Lateral</th>
<th>Ventral</th>
<th>Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVN</td>
<td>-1.88</td>
<td>2.0</td>
<td>-7.9</td>
<td>10</td>
</tr>
<tr>
<td>ARC</td>
<td>-3.30</td>
<td>2.0</td>
<td>-9.7</td>
<td>8</td>
</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

Methods

To examine whether ARC neurons that express GR can be activated by long-term fasting, food (but not water) was removed at ZT1 from three intact rats for 48 h. Rats were then deeply anesthetized with a lethal dose of sodium pentobarbital and perfused with saline, followed by a solution of 4% paraformaldehyde in 0.1M PBS (pH 7.4) at 4°C. The brains were removed and kept in fixative at 4°C for overnight post-fixation, equilibrated 48h with 30% sucrose in 0.1M Tris-buffered saline (TBS; pH 7.2). Brains were coronally cut in a cryostat into 30 μm sections; sections were rinsed in 0.1M TBS and incubated overnight at 4°C in goat anti c-Fos antibody (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-GR antiserum (1:2000). Sections were then further incubated with horse anti-goat IgG for 1h, thereafter, donkey anti-rabbit-Cy2 and streptavidin-Cy3 were co-incubated for 1h to check colocalization of c-Fos and GR by confocal laser scanning microscopy.

Supplemental Figure 1  c-Fos expression (red), induced by 48 hours fasting, in the ventromedial part of the ARC is co-localized with glucocorticoid-receptor staining (green) in the same neurons (yellow) (arrow). III: third cerebral ventricle.