Biological clock control of daily glucose metabolism: hormonal and autonomic pathways

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CHAPTER III

The diurnal modulation of hormonal responses in the rat varies with different stimuli

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Abstract
The circadian clock, located in the suprachiasmatic nuclei (SCN) of the hypothalamus, does not only control the basal daily temporal organization of many neuroendocrine functions, but also its responsiveness. We studied the time-of-day influence on plasma changes in ACTH, corticosterone, glucagon and leptin concentrations elicited by an insulin-induced hypoglycemic event. Male Wistar rats were exposed to an insulin challenge at 6 different times during the light/dark-cycle. The time of day of exposure markedly affected the responses of all 4 hormones studied. Generally, the magnitude of the different hormone responses correlated with their basal daily release pattern, i.e. the responses of ACTH and corticosterone were largest around lights off, and glucagon and leptin responses were most pronounced during the dark period. With regard to the hormones of the hypothalamo-pituitary-adrenal axis, the presently reported time-of-day dependent modulation is completely opposite to that previously reported for novelty or restraint. Therefore, these findings provide further support for the existence of at least two different neural pathways that are able to activate the hypothalamo-pituitary-adrenal axis, and provide different substrates for modulation by the biological clock. This observation warrants a thorough examination of possible functional explanations for the observed differences.

Introduction
The plasma concentrations of many hormones demonstrate marked fluctuations along a 24-h light/dark cycle. Most of these rhythms are generally entrained to the sleep-wake or activity cycle of the animal, but nevertheless show a large variety in the timing of their peaks and troughs. Daily rhythms in hormone release, as most other daily rhythms, are ultimately controlled by the outputs from the master clock contained in the suprachiasmatic nuclei (SCN) in the anterior hypothalamus. The SCN receives signals from the environment and provides, via its outputs, the
principal timing cues for synchronizing the daily oscillations in peripheral tissues. Apart from timing outputs in the form of diffusible signals\textsuperscript{253,255}, at least part of this timing information is transmitted to other areas of the brain via physical connections. As clearly indicated by the results from transplantation studies, especially in the case of hormonal rhythms, the neural connections of the SCN are of the utmost importance for transmitting its endogenous rhythm\textsuperscript{256,257}. The rhythmic message of the SCN is forced onto the hormone release patterns by a combination of synaptic inputs to both neuroendocrine and pre-autonomic neurons located in the hypothalamic target areas of the SCN\textsuperscript{258,259,260,261}.

Several reports have shown that next to controlling the secretion of hormones in basal conditions, the biological clock also clearly affects the responsiveness of a hormonal system to stimuli from the environment. Best studied in this regard is the time-dependent activation of the hypothalamo-pituitary-adrenal (HPA) axis\textsuperscript{262-267}. At present, the general agreement is that early morning exposure to psychological stress, such as a novel cage, results in a pronounced activation of the HPA-axis, whereas exposure to the same stress in the early evening causes a less pronounced increase of plasma corticosterone concentrations, and hardly affects plasma ACTH concentrations\textsuperscript{265,267,268}. Combining the neuro-anatomical information on SCN connections with CRH neurons with the data on the diurnal variation of the stress-evoked activation of the HPA-axis proved to be a valuable tool for obtaining additional insights into the organization of the SCN control of the HPA-axis. From the time-dependent and differential modulation of the ACTH and corticosterone response we concluded that the SCN is able to control separately the release of ACTH and the sensitivity of the adrenal cortex for ACTH\textsuperscript{278}. Indeed, subsequently, direct projections from the SCN to the CRF-containing neurons in the paraventricular hypothalamic nucleus (PVN) were demonstrated\textsuperscript{258}, as well as direct projections to the pre-autonomic PVN neurons that are at the origin of the sympathetic input to the adrenal cortex\textsuperscript{14}. Furthermore, an indirect projection to the CRF neurons was proposed, involving SCN projections to the dorsomedial hypothalamus (DMH) and the sub-paraventricular zone of the PVN (subPVN)\textsuperscript{277,278}. Limbic circuits involved in arousal also project heavily to these peri-PVN areas\textsuperscript{281-283}, thus enabling the SCN to modulate the HPA-activating stimuli already before they reach the CRH neuron.

Stress can be defined as the imposition or perception of environmental or physical stimuli, either negatively (i.e. threatening) or positively (i.e. rewarding), that elicit physiological and behavioral changes adaptive to the organism\textsuperscript{281}. Stimuli that act as stressors can elicit changes in a variety of physiological and neuroendocrine systems. Depending on the stimulus, apart from the HPA-axis often also the secretion of pituitary hormones such as GH and prolactin, and peripheral hormones such as glucagon and leptin are affected. Although the potential interaction of time-of-day and
the hormonal release subsequent to stress exposure is generally acknowledged, few studies, even in rats, have systematically assessed the response of various hormones and the time of day. Recently the daily fluctuations in the basal concentrations of plasma leptin and plasma glucagon have been described in the rat. In order to get further insight into the SCN control of these two hormone rhythms we decided to study the time dependency of their stress-induced activation. Insulin-induced hypoglycemia is a well-known and widely used physiological stimulus that, apart from the hormones from the adrenal cortex and the adrenal medulla, also elicits the release of glucagon from the endocrine pancreas and leptin release from the adipose tissue. Therefore, rats were provided with permanent jugular vein cannulas and treated with an intravenous insulin bolus at 6 different time points along the light/dark-cycle. Subsequently, blood samples were taken and the responses of corticosterone, ACTH, glucagon and leptin analyzed.

Materials and Methods

Animals
Male Wistar rats (Harlan, The Netherlands) were housed at a room temperature of 20 °C with a 12-h light/dark schedule, with lights-off being defined as Zeitgeber Time 12 (ZT12). The rats were allowed to adapt to the new laboratory environment for several weeks before the first experiments. The rats were kept with 4 - 6 animals per cage until one week before surgery, at which time they were transferred to individual cages (25x25x35 cm). Food and water were available ad libitum, except during experiments, when only water was available. For all experiments the “Principles of laboratory animal care” (NIH publication no. 85-23, revised 1985) were followed and approval of the Local Animal Care Committee was obtained.

Surgery
An intra-atrial silicone catheter was implanted through the right jugular vein, according to the method of Steffens when the body weight had reached 300 g. After surgery, rats were allowed to recover for at least 10 days. During experiments, the rats were permanently connected to the blood-sampling catheter, which was attached to a metal collar and kept out of reach of the rats by means of a counterbalanced beam. This allowed all manipulations to be performed outside the cages, without handling the rats. The metal collars were attached at least 48 h before the actual experiment. The rats were handled and sham blood sampled (i.e. blood was withdrawn and immediately returned) regularly in the week before the first experiment in order to adapt them to all experimental procedures.
**Experiment 1**

A total of 31 rats (out of the 32 operated upon) were subjected to an intravenous injection of an insulin bolus (at one of 6 different time points along the light/dark cycle. Insulin injections were performed through the silicone catheter at ZT2, ZT8, ZT11, ZT14, ZT18 and ZT22. Most rats participated in 2 experiments at different time points (3 rats underwent only 1 experimental session and 6 rats participated in 3 experiments), i.e. a total of 65 insulin injections. Rats were randomly assigned to testing at the different time points. On the day of an experiment rats were deprived of food 2 h before the start of the insulin injection by placing them in a new, but similar cage, without food. Sham blood samples were taken at t=-120, t=-90, t=-60 and t=-30. At t=0 the first blood sample was withdrawn (0.3 ml) immediately followed by a bolus injection of the insulin solution (approximately 0.5 ml of a 0.012 mg/ml solution, i.e. 0.5 IU/kg body weight). Subsequent blood samples were collected at t=5, 10, 20, 30 and 60 min. The insulin solution (bovine insulin from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was prepared freshly shortly before every experiment from a concentrated (frozen) stock solution. All experiments were performed with the same stock. After an experiment, the total volume of blood removed (i.e. 1.8 ml) was returned as Ringer and rats were allowed to rest for at least 1 week before the next experiment was started.

**Experiment 2**

Due to the large number of experiments in Exp.1 we performed no control or sham-infusions. Therefore, in Exp.2 we tested for the existence of a time-of-day effect on the corticosterone response due to the experimental procedure itself, i.e. an intravenous injection and blood sampling. In order to further test the specificity of the insulin-induced responses of the HPA-axis in Exp.1 we administered an intravenous glucose bolus to elicit the release of insulin. Six rats were subjected to an intravenous injection of a glucose bolus at 3 different times along the light/dark-cycle (i.e. ZT2, ZT8 and ZT14). Food was removed 30 min before t=0. At t=0 the first blood sample was withdrawn (0.2 ml) immediately followed by a bolus injection of the glucose solution through the silicone catheter (approximately 0.6 ml of a 250 mg/ml solution, i.e. 500 mg/kg body weight). Subsequent blood samples were collected at t=5, 10, 20, 30 and 60 min. Rats were randomly assigned to testing at the 3 different time points. After an experiment, the total volume of blood removed (i.e. 1.2 ml) was returned as Ringer and rats were allowed to rest for at least 1 week before the next experiment was started.

**Experiment 3**

In order to be sure that the non-responsiveness of the HPA-axis at ZT2, which was
Counterregulation completely in contrast to our previous "novel environment" experiments, was not due to some unrecognized difference in the experimental setup, we submitted a new group of rats to both an insulin challenge and a novel environment. Eight rats were subjected to an intravenous injection of insulin, exposure to a novel environment and a control experiment at ZT2 on 3 separate occasions. Insulin injections were performed as described in Exp. 1. Exposure to a novel cage was performed as described previously. In short, at t=0 the animal was picked up, removed from its home cage and transferred to an identical clean experimental cage, the only difference being that there was no sawdust on the floor. A blood sample was taken before the animal had been taken from its home cage (t=0), as well as 5, 10, 20, 30 and 60 min after. During the control experiment access to food was denied and blood was sampled. During all 3 experimental sessions, access to food was prevented, starting 2-h before t=0, by a sliding door in front of the food hopper. The rats were accustomed to the noise of door closure on several non-experimental days by door closure for 5 min at ZT0. After each experiment, the total volume of blood removed (i.e. 1.8 ml) was returned as Ringer and rats were allowed to rest for at least 1 week before the next experiment was started.

**Analytical methods**

Blood samples were immediately chilled on ice in tubes containing a 10 μl solution of 2.5% EDTA + 10% BDH and centrifuged at 4 °C. Plasma was then stored at -80 °C until further analysis. Plasma glucose concentrations were determined using a Glucose/GOD-Perid method (Boehringer Mannheim, Mannheim, Germany). This method actually measures the amount of H₂O₂ and the subsequent amount of dye formed by peroxidase, produced by the oxidation of glucose through glucose oxidase (GOD). Plasma immunoreactive glucagon, leptin, corticosterone and ACTH concentrations were measured using radioimmunoassay kits (LINCO Research Inc., Missouri, USA and ICN Biomedicals, Costa Mesa, CA, respectively), and all samples were assayed in duplicate. Inter- and intra-assay coefficients (measured at 50% binding of the standard curve) for all hormones were 10% or less.

**Statistical analysis**

We evaluated the kinetics of the plasma glucose and hormone concentrations by using 2 physiological parameters: 1) the increments of their plasma concentrations as compared to the t=0 value, and 2) the areas under the curve (AUC) as a reflection of the overall change in the 60-min experimental period. The AUC was calculated from the incremental data. All results are expressed as the mean ± SEM. Statistical analysis was conducted using a repeated-measures analysis of variance (ANOVA) to test for effects of treatment, time of day and interaction. ANOVA was followed by post-hoc
testing if significant effects of interaction or time of day were detected. For Experiment 1 all injections at a certain ZT were considered as a different (independent) group. Statistical significance was set at p < 0.05 for a two-tailed test.

Results

Experiment 1

Injection of the insulin bolus resulted in an immediate decrease of plasma glucose concentrations in all but 4 experiments (1 at ZT2, 2 at ZT8 and 1 at ZT18), which were removed from the final analysis since they were considered unsuccessful injections. Also, in a number of experiments the basal corticosterone values (i.e. before injection of the insulin bolus) were clearly above the normal range, probably due to an arousing event during the preparation for the experiment. As the perception of a stressful event may affect multiple hormonal systems in addition to the adrenocortical system, we excluded the data from rats that had t=0 corticosterone values well above the normal daily range (i.e. >200 ng/ml; 1 at ZT2, 4 at ZT8, 4 at ZT11, 3 at ZT14, 3 at ZT18 and 1 at ZT22; total n=16). The mean corticosterone concentration at t=0 in these 16 rats was 360±97 ng/ml vs 60±48 ng/ml in the remaining animals. These 2 selection criteria resulted in a total of 45 remaining experiments and the following number of injections at the different time points: ZT2 (n=8), ZT8 (n=5), ZT11 (n=6), ZT14 (n=7), ZT18 (n=6) and ZT22 (n=13).

Glucose

Absolute plasma glucose concentrations before the insulin bolus are shown in Table I and exhibited no daily variations. Minimal glucose concentrations were reached 10 min after the injection of the insulin bolus (Fig.1), with the maximum decreases at t=10 not differing between the different ZT-times (F(5,39)=2.1, p=0.083). ANOVA indicated significant effects of injection, time of day and interaction on the total 60 min glucose response (Table II). Analysis of the AUC's revealed that the total amount of glucose uptake during the 60 min post-injection period was significantly higher at ZT14 than the glucose uptake at ZT2 and ZT8 (Fig.2).

Corticosterone

The mean t=0 corticosterone values at the different ZT-times showed a significant effect of time of day (Table I), with the highest and lowest concentrations being found at ZT18 and ZT2, respectively. Injection of the insulin bolus resulted in a significant increase of plasma corticosterone at all ZT times, except ZT18 (p=0.592), but the most pronounced increases were seen at ZT11 and ZT14 (Figs.1, 2). ANOVA revealed significant effects of injection, time of day and interaction for the corticosterone
responses. Also a comparison of the AUC of the different corticosterone responses showed a pronounced effect of time of day (Table II), with most corticosterone being released at ZT14 (Fig.2).

Table I. Basal levels (mean ± S.E.M.) of plasma glucose and the different hormones studied before injection of the insulin bolus in Exp 1.

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Corticosterone</th>
<th>ACTH</th>
<th>Glucagon</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZT2</td>
<td>5.9 ± 0.2</td>
<td>21.1 ± 11.2</td>
<td>44.2 ± 10.2</td>
<td>74.0 ± 2.5</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>ZT8</td>
<td>5.9 ± 0.2</td>
<td>53.6 ± 15.3</td>
<td>60.3 ± 16.2</td>
<td>60.3 ± 4.2</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>ZT11</td>
<td>5.8 ± 0.2</td>
<td>73.5 ± 9.1</td>
<td>60.6 ± 4.1</td>
<td>59.6 ± 4.1</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>ZT14</td>
<td>5.6 ± 0.1</td>
<td>62.9 ± 11.3</td>
<td>55.6 ± 3.4</td>
<td>63.7 ± 5.3</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>ZT18</td>
<td>5.6 ± 0.2</td>
<td>109.0 ± 27.6</td>
<td>94.5 ± 6.9</td>
<td>72.1 ± 7.5</td>
<td>x</td>
</tr>
<tr>
<td>ZT22</td>
<td>5.5 ± 0.2</td>
<td>55.4 ± 14.8</td>
<td>62.2 ± 6.1</td>
<td>72.5 ± 7.5</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

Common subscripts indicate daytime showing a significant difference in basal hormone levels. The last column shows the ANOVA results. Glucose (mmol/l), Corticosterone (ng/ml), ACTH (pg/ml), Glucagon (pg/ml) and Leptin (ng/ml).

Table II. Statistical analysis of the hypoglycemia-induced glucose and hormone responses in Exp 1.

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Corticosterone</th>
<th>ACTH</th>
<th>Glucagon</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time of day</td>
<td>0.002</td>
<td>0.007</td>
<td>0.195</td>
<td>0.002</td>
<td>0.264</td>
</tr>
<tr>
<td>I x T</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.293</td>
<td>&lt;0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>AUC</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.25</td>
<td>0.002</td>
<td>0.315</td>
</tr>
<tr>
<td>n</td>
<td>45</td>
<td>45</td>
<td>43</td>
<td>42</td>
<td>45</td>
</tr>
</tbody>
</table>

Indicated are the p values according to ANOVA for the effects of injection, time of day, interaction (I x T) and AUC. The AUC data of ACTH are based on the first 30 min of the response.
Fig. 1 Plasma glucose, corticosterone, ACTH, glucagon and leptin responses after injection of an insulin bolus at different times during the L/D-cycle. Responses are expressed as the difference from the respective t=0 values. Absolute values at t=0 are displayed in Table I. The bars on top of the figure indicate the light period (open bars) and the dark period (solid bars).
Fig. 2  Daily changes in the total amount of glucose removed from the general circulation or corticosterone, ACTH, glucagon and leptin released in the general circulation as a result of the injection of an insulin bolus at different times of the L/D-cycle. The area under the curve (AUC) for each individual glucose or hormone response was calculated and expressed as percentage of the daily mean. Bars indicate the mean response at the different ZT-times. For ACTH and leptin only the first 30 min of the response were used for calculating the AUC. Bars sharing a common superscript (a - e) are significantly different according to post-hoc testing.
ACTH
Plasma ACTH concentrations could be measured in plasma samples of all experiments except 2 (i.e. #2 at ZT2 and #46 at ZT18). Basal plasma ACTH concentrations at the different ZT-times showed a significant effect of time of day (Table I), with the highest concentrations found at ZT18. ACTH responses induced by the insulin challenge at the different time points are shown in Fig.1. The most pronounced ACTH responses were observed at ZT11 and ZT14. ANOVA, however, only indicated a significant effect of injection and not of time of day or interaction on the ACTH responses (Table II). Also, analysis of the AUC of the ACTH response did not reveal a significant effect of time of day ($F(5,37)=1.7$, $p=0.170$). Only when the calculation of the AUC was restricted to the first 30 min of the response a significant effect of time of day appeared, with the highest values being attained at ZT14.

Glucagon
Plasma glucagon concentrations could be determined in 42 out of the 45 experiments (i.e. #2 at ZT2, #46 at ZT18 and #28 at ZT22 are lacking). Basal plasma glucagon concentrations showed a small diurnal variation, with the highest and lowest concentrations found at ZT18 and ZT11, respectively (Table I). ANOVA for the separate ZT-times showed a significant effect of injection after every injection of insulin (all $p<0.001$). In addition, the overall ANOVA showed significant effects of injection, time of day and interaction for the 6 glucagon responses (Table II). Post-hoc analysis of the AUC data revealed that significantly more glucagon was released at ZT22 than at ZT18 and ZT2 (Fig.2).

Leptin
Plasma leptin concentrations were measured in the samples of all 45 experiments. Before injection of the insulin bolus, plasma leptin concentrations showed no significant daily variation (Table I). Although injection of the insulin bolus induced a clear leptin response, no significant effects of time of day or interaction were observed (Table II). Also analysis of the AUC of the leptin response showed no significant time of day effect (Table II). A significant diurnal variation could only be detected when the leptin responses in respectively the light period and the dark period were combined ($F(1,43)=6.07$, $p=0.018$).

Comparing the presently found corticosterone and ACTH responses, as a consequence of the hypoglycemic stressor, with our previously published corticosterone and ACTH responses as a consequence of a novel cage stressor revealed completely opposite results of the two stressors at ZT2 and ZT14, whereas at the end of the dark period both stressors evoked similar (i.e. small or non-significant) responses (Fig.3).
Fig. 3 Changes in plasma corticosterone and plasma ACTH concentrations as a consequence of exposure to novelty (closed circles) or an insulin challenge (open circles) at different times of the light/dark-cycle. Data from the novelty exposure are derived from our previously published experiment\textsuperscript{278}. Since no insulin challenge was performed at ZT20, the results from both the ZT18 and ZT22 insulin challenge are shown together with the results from the novelty exposure at ZT20.

In order to ensure that these differences were indeed due to the nature of the stressor and not to a critical difference in the experimental setup, two additional control experiments (as described in the Materials & Methods section) were performed.

Experiment 2
Basal plasma corticosterone (i.e. t=0) values showed a significant effect of time, with the highest basal values being found at ZT14 (p<0.001). The injection of the glucose bolus caused a significant increase in plasma glucose and plasma insulin concentrations at all 3 ZT points investigated (data not shown), but no significant effects of the glucose injection on plasma corticosterone responses were detected for the individual ZT-times (all p>0.1; Fig.4). The overall ANOVA did not reveal any significant effects
Fig. 4 Changes in plasma corticosterone concentrations as a consequence of either an iv bolus of insulin (0.5 IU/kg body weight; open circles) or glucose (500 mg/kg body weight; closed circles) at 3 different times during the light/dark-cycle. Basal plasma corticosterone concentrations of the glucose treated rats are: 6.8±4.1 (n=6, ZT2), 37.7±13.8 (n=6, ZT8) and 129.0±24.7 ng/ml (n=5, ZT14), with ZT14 values being significantly higher than those at ZT2 and ZT8 (p<0.01).

of injection (p=0.279), time of day (p=0.289) or interaction (p=0.213) either. On the other hand, analysis of the corticosterone data after administration of the insulin bolus at the same 3 ZT points showed very significant effects for injection, time of day and interaction (all p<0.001).

Experiment 3
The 3 different treatments showed no differences as to basal concentrations of corticosterone, glucose and glucagon; only plasma leptin concentrations were significantly higher at the start of the control experiments (Table III). Corticosterone release patterns at ZT2 as a consequence of the insulin challenge, a novel environment, and a control experiment are displayed in Fig.5A. As indicated in Table IV, ANOVA revealed significant effects of time (p<0.001), treatment (p=0.003) and treatment x time (p<0.001), clearly indicating the stressor specific activation of the HPA-axis. Post-hoc analysis showed that corticosterone concentrations after exposure to a novel cage significantly differed from those during an insulin challenge and control exposure at t=10, 20 and 30 (effects of treatment p=0.005 and p=0.020, respectively). Despite the pronounced differences in glucose and glucagon responses between the insulin and control treatment, post-hoc analysis revealed no significant differences between the corticosterone response during the insulin-induced hypoglycemia and the control experiment, i.e. effects of treatment (p=0.338) and injection x treatment (p=0.123).
Fig. 5 Changes in plasma corticosterone (A), leptin (B), glucose (C) and glucagon (D) concentrations as a consequence of exposure to either novelty (closed circles) or an insulin challenge (closed triangles). The same 8 rats were exposed at random, at ZT2, to either novelty, an insulin challenge or a control experiment, i.e. only blood sampling (open circles). One week of recovery was allowed in between the different experiments. Superscripts indicate time points at which treatments at significantly different effects on plasma glucose and hormone levels. * Novelty v Control; ^ Novelty v Insulin; + Insulin v Control. *,^,+ p<0.05; **,^^,++ p<0.01; ^^^,+++ p<0.001.

Discussion
The present results once again clearly show that the magnitude of a hormonal response strongly depends on the time of day that such a response is elicited. The daily variation in the responsiveness of the 4 hormonal systems investigated in the present study mainly depended on the diurnal setting of their basal release activity, at least in case of insulin-induced hypoglycemia. But, in addition, the present results clearly show that the time-of-day dependent modulation of the HPA response varies consid-
Table III. Basal levels (mean ± SEM) of plasma glucose and the different hormones studied before the start of the 3 different treatments in Experiment 3.

<table>
<thead>
<tr>
<th></th>
<th>Hypoglycemia</th>
<th>Novelty</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>6.2 ± 0.1</td>
<td>6.6 ± 0.1</td>
<td>6.4 ± 0.3</td>
<td>0.224</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>6.8 ± 1.0</td>
<td>10.9 ± 4.5</td>
<td>6.7 ± 0.9</td>
<td>0.451</td>
</tr>
<tr>
<td>Glucagon</td>
<td>86.7 ± 2.0</td>
<td>88.0 ± 3.1</td>
<td>86.5 ± 6.3</td>
<td>0.936</td>
</tr>
<tr>
<td>Leptin</td>
<td>2.9 ± 0.3</td>
<td>3.2 ± 0.4</td>
<td>3.9 ± 0.3</td>
<td>0.002</td>
</tr>
</tbody>
</table>

P-values indicate the result of the statistical analysis (ANOVA). Glucose (mmol/l), corticosterone (ng/ml), ACTH (pg/ml), glucagon (pg/ml) and leptin (ng/ml).

Table VI. Statistical analysis of novelty- and hypoglycemia-induced glucose and hormone responses during experiment 3

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Glucose</th>
<th>Corticosterone</th>
<th>Glucagon</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.030</td>
</tr>
<tr>
<td>S x T</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.003</td>
<td>0.263</td>
</tr>
<tr>
<td>NOV x HYP</td>
<td>&lt;0.001/&lt;0.001</td>
<td>0.005/&lt;0.001</td>
<td>0.002/&lt;0.001</td>
<td>0.401/0.057/0.114</td>
</tr>
<tr>
<td>NOV x CTR</td>
<td>0.162/0.407</td>
<td>0.014/&lt;0.001</td>
<td>0.474/0.363</td>
<td>0.198/0.233</td>
</tr>
<tr>
<td>HYP x CTR</td>
<td>0.001/&lt;0.001</td>
<td>0.338/0.123</td>
<td>&lt;0.001/&lt;0.001</td>
<td>0.863/0.556</td>
</tr>
</tbody>
</table>

P-values indicate the results of the ANOVA for the effects of sampling, treatment and interaction (S x T) considering all three treatments: novelty (NOV), hypoglycemia (HYP) and control (CTR). The lower three lines show the ANOVA results (treatment and interaction, respectively) for the different combinations of two treatments.

erably with various stimuli. Therefore, these data also enforce the idea that stimuli can access the HPA-axis by different neural pathways, depending on the type of stressor. Moreover, the opposite reaction pattern of corticosterone (and ACTH), with respect to the time of day, during exposure to novelty or an insulin challenge, clearly shows that the diurnal variation in the responsiveness of the HPA-axis cannot be solely due to the diurnal variation in basal, unstressed plasma corticosterone values.

As outlined in the introduction, a time-of-day dependent modulation of the ACTH and corticosterone response evoked by insulin-induced hypoglycemia was not unexpected, since a diurnal modulation of stress-evoked ACTH and corticosterone responses had been reported previously many times\(^\text{262-265,267}\). Unexpected, however, was
the completely opposite reaction pattern of the corticosterone and ACTH responses, i.e. no response at ZT2 and high responses at ZT14 (and ZT11). The most surprising fact was the apparent unresponsiveness of the HPA-axis in the early morning (i.e. ZT2), because most, if not all, previous reports had shown clear ACTH and corticosterone responses at this time of day, including our own results. The most straightforward explanation seems to be the somewhat smaller hypoglycemia induced at ZT2. Although the maximum decrease in plasma glucose concentrations at t=10 did not differ at the different ZT-times, the total amount of glucose removed from the circulation did, and for individual rats correlated significantly (p=0.026) with the magnitude of the corticosterone response. As we reported previously, this diurnal difference in glucose removal is due to an increased insulin sensitivity and glucose tolerance at the light/dark transition. However, it is also clear that the hypoglycemia evoked at ZT2 did not remain unnoticed, since a significant glucagon response was evoked at every ZT point, including ZT2.

The glucagon responses also showed a significant diurnal variation, but contrary to the corticosterone responses, no significant correlation was found between the amount of glucose disappearing from the circulation and the glucagon response (p=0.121). In addition, the results of EXP.3 clearly show that the glucagon response at ZT2 cannot be attributed to the psychological stress of the experiment itself, since, if anything, the novel cage exposure and the blood sampling procedure only have a small inhibitory effect (Fig.4c). Finally, the results of EXP.3 provide clear evidence that the lack of an HPA response at ZT2 in EXP.1 is not due to a typical experimental condition (such as the 2-h food deprivation or the cage change), the time of year, or an aberrant group of rats, but specific to the type of stressor used. Therefore, although there is a small daily variation in the severity of the hypoglycemia induced, the glucagon data indicate that the insulin challenge itself is equally effective in eliciting a counterregulatory hormone response at all time points.

The present results clearly show that the absence (or presence) of an HPA response at a certain time of day is specific for the type of stimulus encountered by an organism. In addition, the pronounced response of the HPA-axis following the insulin challenge at ZT11 and ZT14, once again demonstrates that the diminished responsiveness of the HPA-axis to a psychological stressor at about the time of lights off cannot be due to the elevated basal concentrations of plasma corticosterone at this time of the day. Indeed, it is difficult to compare the amount of stress evoked between different stressors, but both our currently applied stressors were able to elicit a significant (and comparable) activation of the HPA-axis, be it at different times of the light/dark-cycle. Therefore, it seems reasonable to assume that the refractoriness of the HPA-axis to insulin-induced hypoglycemia at ZT2 (or to novelty at ZT14) is not due to an insufficient intensity of the stressor.
Provided that the lack of an HPA response at a certain time of day is not due to an insufficient intensity of the stressor, this means that the initiation of an HPA response is blocked at a brain level somewhere between the sensory input and the CRH neuron. Several authors have raised the hypothesis that at least two types of stress pathways exist in the central nervous system, especially with regard to the activation of the HPA-axis\textsuperscript{281,292-294}. Stressors involving an immediate physiological threat (such as hypoglycemia) are called 'systemic' or 'physical' stressors and are expected to be relayed to the PVN mainly via brainstem catecholaminergic projections, whereas stressors that require interpretation by higher brain structures (such as novelty) are called 'processive' or 'psychological' stressors and appear to be relayed to the PVN mainly via the limbic system in the forebrain.

In order to explain the time-of-day effects of insulin on the corticosterone response it could of course be argued that insulin (or glucose levels, or some other consequence of insulin administration) interacts with ACTH at the level of the adrenal, or with CRH at the level of the pituitary. On the other hand, insulin release elicited by the glucose bolus did not evoke any corticosterone response. Therefore, it is likely that the temporally different response pattern of the HPA-axis in case of an insulin challenge and novelty is the consequence of the different neural pathways used to reach the PVN.

The outflow of the limbic circuits activated by 'processive' or 'psychological' stimuli is mainly aimed at the area immediately surrounding the PVN. The stress-responsiveness of cell populations in peri-PVN structures such as subPVN, DMH, MPOA and BNST is evidenced by the c-fos induction following stress exposure\textsuperscript{27,292,295-298}. Indeed, many of these peri-PVN brain areas are also contacted by the SCN. Moreover, it has been shown that stress-activated neurons in the subPVN and DMH receive a synaptic input from the SCN\textsuperscript{27}. Thus it is very likely that the final effectiveness of the limbic input to the PVN is modulated by the SCN input to the interneurons in the peri-PVN areas.

A similar circadian modulation of the pathway employed by 'systemic' or 'physical' stimuli is less likely because SCN projections to the catecholaminergic cell groups in the brainstem are unlikely to exist; i.e. until now the rostral mesencephalon is the most caudal level at which efferent projections of the SCN have been observed\textsuperscript{12,299-303} and depletion of the catecholaminergic innervation of the hypothalamus does not disturb the circadian rhythm of plasma corticosterone concentrations\textsuperscript{304,305}. Therefore, in case of novelty, the SCN control involves direct projections to endocrine and autonomic neurons in the PVN as well as SCN inputs to intermediate neurons (receiving stress inputs) around the PVN, whereas in case of the insulin challenge only the direct SCN inputs to the PVN are involved\textsuperscript{278}. Not only the responses of the HPA-axis, but also the hypoglycemia-induced excur-
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sions of glucagon and leptin release demonstrated a clear variation along the light/dark cycle, although for leptin the effect only became significant when the respective data for the light period and the dark period were combined. In contrast to the increased responsiveness of the HPA-axis around lights off, the leptin and glucagon responses were most pronounced during the dark period. The different diurnal reaction patterns of ACTH and corticosterone on the one hand, and glucagon and leptin on the other are probably due to the different motorneurons in the PVN responsible for these responses, i.e. endocrine CRH neurons versus pre-autonomic sympathetic neurons, and the different SCN projections to these neurons. As we and others have previously shown, pre-autonomic neurons in the PVN receive a pronounced GABA-ergic input from the SCN, which is mainly active during the light period. It is well known that the control of both glucagon and leptin release heavily depends on the sympathetic nervous system. Therefore, it is likely that the GABA-ergic inhibition of the sympathetic pre-autonomic neurons in the PVN is responsible for the smaller glucagon and leptin responses during the light period. The more pronounced responses of glucagon and leptin during the dark period coincide with the higher basal concentrations of these 2 hormones during the dark period. Also for corticosterone and ACTH the magnitude of the responses varied along with the basal plasma hormone concentrations, since the highest excursions were found at the time of the daily peak, i.e. ZT11 and ZT14. Although in the present study no significant correlations were found between basal hormone concentrations and the subsequent hormone response of individual rats, the basal hormone rhythms as published previously are clearly recognizable in the response data as presented in Fig.2.

Presently, it is still not known exactly how and at which site (decreasing) glucose concentrations are sensed. It is likely that both peripheral glucose sensors at the level of the pancreas, liver, portal vein and carotid bodies and central glucose sensing areas in the hypothalamus and brainstem contribute to the final response. Information from peripheral as well as brainstem glucose sensors will be relayed directly to the CRH neurons via ascending noradrenergic and adrenergic fibres from the A1/C1 and A2/C2 cell groups in the brainstem. Removal of the noradrenergic input to the PVN, however, does not prevent the glucagon response induced by hypoglycemia. Therefore, the major part of the hypoglycemia-induced release of glucagon appears to be induced by direct effects of hypoglycemia (and insulin) on the pancreas.

The same may hold true for the release of leptin. In a previous study using human subjects, an inhibitory effect of insulin-induced hypoglycemia on plasma leptin concentrations was found. These authors suggested that the decrease in plasma leptin concentrations was caused by the hypoglycemia-induced activation of the sympathetic nervous system. Surprisingly, however, we observed a stimulation of plasma leptin
concentrations, despite the fact that hypoglycemia induces a clear activation of the sympathetic nervous system in rats as well\textsuperscript{318}.

Clearly the increased plasma leptin concentrations are not caused by a stimulatory effect of corticosterone\textsuperscript{291,319}, since the plasma corticosterone concentrations only started to increase when the leptin response was already at its maximum (i.e. at t=10). A direct effect of the insulin bolus, however, able to override the inhibitory effect of the autonomic activation, is very well possible\textsuperscript{291,319}. But apparently the release of NA in the PVN or the direct peripheral effects of hypoglycemia and insulin on the pancreas and adipose tissue are not able to overrule completely the inhibitory effect of the SCN on the endocrine and autonomic neurons in the PVN. Therefore, the daily variation in the magnitude of the hormone responses as a consequence of ‘systemic’ stimuli is determined for a large part by the basal settings of the hormone system by the SCN. During ‘psychological’ stimuli, on the other hand, the direct catecholaminergic projections to the PVN seem to be of less importance\textsuperscript{292}. Now the indirect pathway involving the GABA-ergic interneurons in the peri-PVN comes into play, and apparently the limbic input is able to overrule the SCN input to these neurons\textsuperscript{27}. Indeed, lesions of the entorhinal cortex do affect the response of the HPA-axis to the stress of immobilization, but do not change its response to hypoglycemia\textsuperscript{320}.

In conclusion, the present data once again confirm the time-of-day dependent modulation of hormone responses, but, more importantly, they also show that the time-of-day dependent modulation differs for different hormones and different stimuli. All our experiments were performed under L/D=12/12 conditions; therefore the persistence of these temporal variations under free-running conditions (and thus their circadian nature) have not yet been established. However, for the time-dependent modulation of the novelty response the involvement of the SCN has been clearly determined\textsuperscript{231,278,280,321,322}. The different time-of-day dependent modulation of the novelty and hypoglycemia-induced responses of the HPA-axis is probably due to the use of different neural pathways. However, a more functional explanation is also possible, since the time-of-day dependent modulation could also depend on the fact that the severity of the homeostatic disturbance may vary with time-of-day. ‘Psychological’ stimuli like novelty, involving behavioral activation, are more disturbing at the beginning of the light period, when rats are asleep, than at the end of the light period, when the rat has to wake up anyway. On the other hand, hypoglycemia is certainly more threatening at a time when the rats have not been feeding for a long time and glycogen stores in the liver are almost depleted (i.e. at the end of the light period), than when glycogen stores are plenty after a night of feeding. Additional experiments using a time-of-day dependent exposure to other ‘physiological’ and ‘psychological’ stimuli, such as cold exposure, haemorrhage, immune challenge, noise, or forced swim, will be necessary to validate either of the two explanations discussed.