The suprachiasmatic nucleus generated rhythm in blood glucose. A role for the automatic nervous system

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CHAPTER 6

Role for the pineal and melatonin in glucose homeostasis: pinealectomy increases night-time concentrations

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Submitted

Abstract

The effects of melatonin on glucose metabolism are far from understood. In rats, the biological clock generates a 24h-rhythm in plasma glucose concentrations, with declining concentrations in the dark period. We hypothesized that, in the rat melatonin enhances the dark signal of the biological clock, decreasing glucose concentrations in the dark period. We measured 24h-rhythms of plasma concentrations of glucose and insulin in pinealectomized rats fed ad libitum with and without melatonin-treatment and compared them with data of intact rats fed ad libitum. Pinealectomy abolished the nocturnal decline in plasma glucose concentrations, resulting in increased 24h-mean plasma glucose concentrations, while plasma insulin concentrations did not change. These findings suggest a decreased insulin sensitivity. Melatonin replacement restored 24h-mean plasma glucose concentrations in pinealectomized rats, but interestingly it did not restore the 24h-rhythm. Melatonin treatment also resulted in higher meal-induced insulin responses, probably mediated via an increased sensitivity of the β-cells. Taken together, our data demonstrate that the pineal hormone melatonin, influences both glucose metabolism and insulin secretion from the pancreatic β-cell. The present paper also demonstrates that removal of the pineal gland cannot be compensated by mimicking plasma melatonin concentrations only.
Introduction

In mammals, circadian rhythms are generated by the biological clock, located in the suprachiasmatic nucleus of the hypothalamus (SCN)\(^\text{176}\). Recently, we have shown that the SCN generates a 24-h rhythm in plasma glucose concentrations in the rat, which is independent from the SCN generated rhythm in feeding activity. Plasma glucose concentrations rise towards the end of the light period just before awakening and the onset of the rats' main activity period and then decrease again during the dark period\(^\text{106}\). Time information from the SCN is transmitted to the rest of the organism by way of SCN projections to neuronal, neuroendocrine and autonomic hypothalamic output centers. One of the best known SCN output signals is the pineal hormone melatonin. Effects of melatonin on glucose metabolism have been suggested for both humans\(^\text{199}\) and rodents\(^\text{4,51}\), but the exact mechanism of its actions has not yet been identified. It is thought that melatonin acts directly on its target cells, e.g. hepatocytes and pancreatic \(\beta\)-cells\(^\text{1145}\), which are known to contain melatonin-binding elements. It is also possible that melatonin influences glucose metabolism via its modulatory action on SCN activity\(^\text{125}\). The data concerning the effects of melatonin on glucose metabolism in rodents are rather contradictory. Removal of the endogenous source of melatonin in rodents, i.e. pinealectomy, causes a decrease in hepatic and muscular glycogenesis\(^\text{128}\), it increases plasma concentrations of glucose and glucagon, and decreases insulin concentrations\(^\text{52}\), it exacerbates hyperglycemia to an alloxan treatment\(^\text{42}\) and recently, it has been suggested that pinealectomy causes glucose intolerance and impairs insulin secretion and action\(^\text{119}\). However, the effects of melatonin administration are less clear, i.e. some studies have suggested that it has no effect on insulin release and glucose metabolism\(^\text{10,60}\), whereas others have described either an inhibitory\(^\text{4}\) or a stimulatory\(^\text{74}\) effect. A possible reason for these different results is that experiments were executed at different time-points of the day/night-cycle. Many investigators focus on one time point in the light/dark-cycle or execute their experiments during the light phase only\(^\text{10,42,52,119,128}\), while melatonin is released during the dark period.

In our view the main (circadian) function of melatonin in rats is to enhance the dark signal of the SCN, through a combination of inhibition of the firing rate of SCN neurons\(^\text{125}\) and peripheral effects. As one function of the SCN is to lower night-time glucose concentrations in the rat\(^\text{106}\), we hypothesized that removing the pineal gland, and thus the source of melatonin, results in higher plasma glucose concentrations during the dark period. Therefore, we measured 24h-profiles of plasma concentrations of glucose and insulin in pinealectomized (PNx) rats. As melatonin modulates the SCN, which in turn also generates a daily rhythm in feeding behavior, changes in the 24h-rhythm in plasma glucose concentrations may result from indirect effects of melatonin on the daily rhythm in feeding behavior. To suppress the influence of the SCN on feeding behavior, we examined 24h-profiles of plasma concentrations of glucose and insulin in PNx rats and in
melatonin-treated PNx rats that were subjected to a scheduled feeding regimen with six identical meals equally distributed over the light/dark-cycle.

Materials and methods

Animals and Food Intake
Male Wistar rats (Harlan) were used in all experiments. Rats were housed in separate cages (35 x 35 x 40 cm) at a room temperature of 20 °C, on a 12h/12h light-dark regimen (lights on at 5.00 PM). For experiment 2&3, rats were entrained to a feeding schedule. Food pellets were available in metal food hoppers. A rat could gnaw off pieces of food through vertical stainless steel bars situated at the front of the food hopper. Access to the food could be prevented by a sliding door situated in front of the food hopper. Door opening and door closing were activated by an electrical motor and controlled by a clock. During all experiments, water was available ad libitum. All experiments were performed in the rat's home cage. All experiments were conducted under the approval of the Local Animal Care Committee.

Pinealectomy
Eighteen rats (175-190 gr) were anesthetized with Hypnorm® (0.04ml/100 gr, i.m.; Janssen Pharmaceutical Ltd., Oxford, England) and Dormicum® (0.08 ml/100 gr, s.c.; Roche Nederland B.V., Mijdrecht, The Netherlands). A square section of bone covering the sinus confluence was removed. With a sharp needle, an incision was made in the dura mater next to the sinus confluence. The tip of a suction tube was inserted under the sinus and the pineal gland was ablated by suction. The square section of bone was placed back in its original position and fixed with tissue glue. Buprenorphine hydrochloride (Temgesic®) (0.03 ml/100 gr, s.c.; Schering-Plow, Amstelveen, The Netherlands) was given for pain relief. When their body weight had reached 250 gr, blood was withdrawn from the tail vein at ZT 17 (when melatonin levels are normally high) and plasma melatonin concentrations were measured. Those rats that showed plasma melatonin concentrations > 25 pg/ml were considered to be not totally pinealectomized. Three rats that showed normal concentrations of plasma melatonin were excluded from the experiment.

First experiment
An intra-atrial silicone catheter was implanted through the right jugular vein according to the method of Steffens in six PNx rats that were fed ad libitum when their body weight had reached 300gr. After surgery, the animals were given two weeks to recover. During the experiments, the animals were connected permanently 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 carried out out-
side the cages without the need to handle the animal. The complete circadian profile of plasma concentrations of glucose and insulin were determined by taking 0.2 ml of blood once every hour for 12 consecutive hours on 2 different occasions within a period of 2 weeks. The 2 runs started at ZT6.5 and ZT18.5.

Second experiment
Six PNx rats were entrained to a feeding schedule of six, 10-min-meals spaced equally over the light/dark-cycle. Food became available at ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22. Rats had two weeks to adapt to this feeding schedule. Adaptation was considered complete when rats had learned to consume ± 3.5 gr during every meal. Rats adapted readily to this feeding schedule and resumed growth (2.5 gr/day). A jugular vein catheter was implanted and after two weeks of recovery, the complete circadian profile of plasma concentrations of glucose, insulin and corticosterone was determined by taking 0.2 ml of blood once every hour for 12 consecutive hours on 2 different occasions within a period of 2 weeks. The 2 runs started at ZT6.5 and ZT18.5.

Third experiment
Five PNx rats were entrained to the same feeding schedule as described above. After one week of adapting to the feeding schedule, PNx rats received melatonin (100 ng/ml) through their drinking water from ZT15.5 until ZT23.5 (during the remaining hours normal water was available) every day until the end of the experiment. One week later a jugular vein catheter was implanted and the rats were allowed to recover for two weeks. The complete circadian profiles of plasma concentrations of glucose, insulin and corticosterone were determined as described for the first two experiments. To verify whether drinking a melatonin solution resulted in high plasma melatonin concentrations 1 ml blood was taken at ZT 1, ZT 14.5, ZT 16 and at ZT 21.5 one week after the final experiment.

Melatonin
Crystalline melatonin (Sigma, St. Louis, MO) was dissolved in absolute alcohol (and stored at -20°C). This solution was subsequently diluted with water to a final concentration of 100 ng/ml melatonin in 0.1% alcohol solution. Melatonin solutions were freshly prepared every morning.

Analytical methods
Blood samples were immediately chilled at 0°C and centrifuged at 4°C, and the plasma was stored at -20°C until analysis. The plasma glucose concentrations were determined using a Glucose/GOD-Perid method (Boehringer Mannheim, GmBH, Germany). Plasma immunoreactive insulin concentrations in experiment 1 were determined with a radio
immuno-assay kit (ICN Biochemicals, Costa Mesa, CA, USA) and in experiment 2 and 3 with a different radio immuno-assay kit (Linco Research, St. Charles, MO). Since the plasma controls used in these radio-immuno assays were not the same, insulin concentrations of experiment 1 may not be compared with those of experiment 2 and 3. The samples were measured in duplicate. The lower limit of the assay was 10 μU/ml and the coefficient of variation of the immunoassay was < 8%. Plasma corticosterone concentrations were determined with a radio immuno-assay kit (ICN Biomedicals, Costa Mesa, CA); samples were measured in duplicate. From the samples, 10 μl was taken and diluted in 4 ml of assay buffer. The lower limit of the assay was 1 ng/ml and the coefficient of variation of the immunoassay was < 4%. Plasma melatonin concentrations were measured in duplicate by radio immunoassay (RIA) using (125I) melatonin (Amersham Bucks., UK; specific activity 2,000 Ci/mmol) and a rabbit antiserum (AB/R/03, Stockgrand Ltd., Guilford, UK) at a final dilution of 1:160,000. Stock melatonin (Sigma Chemicals) was stored at a concentration of 1 mg/ml. Melatonin was extracted from plasma samples (250 μl) in 5 ml dichloromethane and dried by vacuum evaporation. Next, samples were reconstituted in 150 μl assay buffer and two 50 μl aliquots were taken for assay. Standards were diluted in assay buffer to give a range of dilutions from 0.5 to 200 pg/ml. The minimum detection level for the assay was 10-15 pg/ml plasma. 

Comparison with previous data

We were able to compare plasma concentrations of glucose, insulin and corticosterone in PNX rats with those in intact rats that we have published previously106, because the experiments in PNX rats were performed with similar experimental procedures and under similar conditions as for those in intact rats (i.e. same source of animals and food, same experimental room, same experimenter). Plasma concentrations of glucose, insulin and corticosterone in PNX rats and in intact rats were measured with similar analysis methods; the inter-assay variations were respectively < 2.5%, <8% and < 5%. The plasma insulin concentrations in PNX rats subjected to a scheduled feeding regimen were determined with an analysis method from a different company (Linco Research, St. Charles, MO instead of ICN Biochemicals, Costa Mesa, CA). However, the data from both PNX rats and intact rats may be compared because the inter-assay variation (comparing methods of the two companies) was <7%.

Recent measurements (unpublished results from 2000) and measurements from 1998106 illustrate the remarkable stability of the plasma concentrations of glucose and insulin in intact rats subjected to a scheduled feeding regimen, i.e. no significant differences were detected106. Furthermore, for the daily rhythm in plasma corticosterone concentrations in intact rats we demonstrated a similar stability comparing data from 1992 with those from 199593. Thus, over the years the control values remain stable as measured in our experiments and we, therefore, believe we are justified in using historical data. Concen-
trations of glucose, insulin and corticosterone of intact rats from the previous study\textsuperscript{106} are shown as gray background in the figures, and these data sets were used for statistical analysis.

Data analysis
Plasma concentrations of glucose, insulin and corticosterone were expressed as mean ± SEM. Statistical analysis was conducted using a repeated-measures analysis of variance (ANOVA) to test for an effect of time. If ANOVA detected a significant effect of time, paired t-tests were used to detect differences between peak and trough values. In addition, a single cosinor analysis was performed, using the fundamental period (24 h) for the individual sets of data points. The statistical significance of the cosine fit is expressed as F statistic. The fitted function is defined by its mesor (rhythm-adjusted mean), amplitude (50% of the difference between the maximum and the minimum), and acrophase (time of the maximum). The software package Table-Curve (Jandel Scientific GmbH, Erkrath, Germany) was used for fitting cosine functions to the data. ANOVA and the Student’s t-test were used to detect significant differences between intact rats and PNx rats. For the ANOVA and (paired) t-tests, p<0.05 was considered to be a significant difference.

Results

Growth profiles, food and water intake in PNx rats
The growth patterns of PNx rats in all three experiments were comparable to those of intact rats. In between the pinealectomy and the jugular vein implantation, the average daily growth rate was 3.7 ± 0.3 gr; this rate was not significantly different from that of intact rats (3.8 ± 0.4 gr; p>0.05). The body weights of PNx rats and intact rats at the beginning of the experiments were not significantly different (PNx: 334 gr ± 3; intact 338 gr ± 4). PNx rats had no difficulties adapting to the scheduled feeding regimen (Experiment 2&3), but PNx rats (with and without melatonin treatment) consumed smaller amounts of food as compared to intact rats in our previous study, respectively 2.7 ± 0.2 gr/meal and 3.3 ± 0.1 gr/meal (p<0.01), however their growth rates were not significantly different (PNx: 2.5 gr/day ± 0.2; intact: 2.7 gr/day ± 0.2, p>0.06)). Under \textit{ad libitum} feeding conditions food intake of PNx rats (experiment 1) was similar to that of intact rats (respectively 21.2 ± 1.1 gr/24h and 20.1 ± 1.0 gr/24h (p>0.05)).

Plasma concentrations of glucose and insulin in PNx rats on \textit{ad libitum} feeding
Fig. 1 shows the daily variation in plasma glucose concentrations in PNx rats as well as in intact rats (in grey\textsuperscript{106}). Plasma glucose concentrations in PNx rats fed \textit{ad libitum} showed
Fig. 1 Plasma glucose concentrations (A) and plasma insulin concentrations (B) across the light/dark-cycle in PNx rats (n = 6) fed ad libitum. The black bars indicate the night-time. The gray areas indicate the plasma glucose concentrations (A) and plasma insulin concentrations (B) in intact rats as measured in our previous study.106

Significant fluctuations over the light/dark-cycle (ANOVA, F(23,93)= 3.4, p<0.001). Sets of data points of all rats could be fitted with the single cosinor analysis so that the fitted curve was not significantly different from the curve of the individual rat (Table 1). The amplitude of the rhythm in PNx rats was significantly smaller as compared to that in intact rats and the peak of the rhythm occurred later than the peak in intact rats (Table 1). Statistical analysis of 24h-profiles of plasma glucose concentrations indicated that those of PNx rats and intact rats differed significantly and that plasma glucose concentrations in PNx rats were significantly higher than those in intact rats (7.1 mmol/l ± 0.1 in PNx rats vs. 6.3 mmol/l ± 0.1 in intact rats). ANOVA detected a significant effect of time (F(22, 264)= 8.2, p<0.001), of 'group x time' (F(22, 253)= 0.7, p<0.001) and of group (F(1,11)= 47, p< 0.001).
Table 1 Rhythm parameters of plasma glucose concentrations in PNx rats as compared to those in intact rats.

<table>
<thead>
<tr>
<th></th>
<th>Ad libitum fed</th>
<th>Scheduled fed</th>
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<tbody>
<tr>
<td></td>
<td>PNx</td>
<td>Intact</td>
</tr>
<tr>
<td>R^2</td>
<td>0.35 ± 0.02</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0.47 ± 0.05</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>M (mmol/l)</td>
<td>PNx</td>
<td>Intact</td>
</tr>
<tr>
<td></td>
<td>7.1 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>6.3 ± 0.1</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>Am (%)</td>
<td>PNx</td>
<td>Intact</td>
</tr>
<tr>
<td></td>
<td>7.0 ± 0.4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10.6 ± 1.4</td>
<td>12.9 ± 1.1</td>
</tr>
<tr>
<td>A (ZT)</td>
<td>PNx</td>
<td>Intact</td>
</tr>
<tr>
<td></td>
<td>12.9 ± 0.5</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>11.5 ± 0.6</td>
<td>11.3 ± 0.4</td>
</tr>
</tbody>
</table>

R^2 = Goodness of fit; M = absolute 24 h mean (mmol/l); Am = Amplitude (%); A = Acrophase (ZT); PNx = pinealectomized rats. Values are means ± SEM.

There was no diurnal variation in plasma insulin concentrations in PNx rats over the light/dark-cycle (Fig 1B) (ANOVA, F (23,93)= 1.0, p= 0.5). Moreover, 24h-profiles of plasma insulin concentrations in PNx rats were very similar to those in intact rats (ANOVA: time (F(22,264)=1.6, p<0.05); ‘group x time” (F(22,253)=0.9, p=0.6) and group (F(1,11)= 0.1, p=0.9)). There was also no significant difference in 24h-means of plasma insulin concentrations between PNx and intact rats (56.1 µIU/ml ± 4.2 in PNx rats vs. 57.0 µIU/ml ± 7.5 in intact rats).

**Plasma concentrations of glucose and insulin in PNx rats on a scheduled feeding regimen**

There was no diurnal variation over the light/dark-cycle in plasma glucose concentrations in PNx rats on a scheduled feeding regimen (Fig 2A) (ANOVA, F(22,110)=1.2, p=0.2). Comparisons revealed that the profiles for PNx rats differed significantly from those of intact rats, and that glucose concentrations in PNx rats were significant higher over the 24h than those in intact rats (Table 2), because ANOVA detected an effect of group (F(1,12)= 5.3, p<0.04), of time (F(22, 164)= 3.3, p<0.001), and of ‘group x time’ (F(22,164)= 1.8, p<0.02).

Plasma insulin concentrations in PNx rats on a scheduled feeding regimen showed clear fluctuations over the light/dark-cycle (ANOVA, F(22,110)= 8.5, p<0.001) and they were similar to those in intact rats (Fig. 3). ANOVA only detected an effect of time (F(22,164)=8.6, p<0.001), but not of group (F(1,12)= 0.5, p= 0.5) or ‘group x time’ (F(22,
Fig. 2  Plasma glucose concentrations across the light/dark-cycle under scheduled feeding conditions in (A) PNx rats (●, n = 6) and in (B) melatonin-treated PNx rats (○, n=5). The black bars indicate the night-time. The gray areas indicate the plasma glucose concentrations in intact rats as measured in our previous study. The straight line (B) indicates the plasma glucose concentrations in PNx rats, the same as depicted in (A).

Table 2  24h-mean plasma concentrations of glucose and insulin in intact rats and PNx rats on a scheduled feeding regimen with and without melatonin

<table>
<thead>
<tr>
<th></th>
<th>Intact</th>
<th>PNx</th>
<th>PNx + mel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.5 ± 0.1</td>
<td>7.1 ± 0.1*†</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>Insulin (μIU/ml)</td>
<td>111 ± 6</td>
<td>106 ± 4</td>
<td>152 ± 16*‡</td>
</tr>
</tbody>
</table>

+ mel = melatonin treated, * P < 0.01 for comparison with intact rats, † P < 0.02 for comparison with “PNx rats + mel”, ‡ P < 0.001 for comparison with intact rats.
Fig. 3  Plasma insulin concentrations across the light/dark-cycle under scheduled feeding conditions in (A) PNx rats (●, n = 6) and in (B) melatonin-treated PNx rats (○, n=5). The black bars indicate the night-time. The gray areas indicate the plasma insulin concentrations in intact rats as measured in our previous study\textsuperscript{106}. The straight line (B) indicates the plasma insulin concentrations in PNx rats, the same as depicted in (A).

164) = 1.3, p = 0.15). Insulin concentrations rose after most meals in PNx rats. This increase in insulin (expressed as the difference with the last sample point before a meal) reached significance for ZT 2, 10 and 22 (p<0.05). Mean insulin increments in PNx rats were significantly lower as compared to those in intact rats (p<0.01) (Fig 4).

Melatonin treatment in PNx rats on scheduled feeding regimen
We wanted to mimic normal levels of plasma melatonin during the dark period. We, therefore, chose to provide melatonin in the drinking water in a 100 ng/ml concentrations, since experiments showed that this concentration resulted in physiological levels
of urinary melatonin metabolites in Wistar rats subjected to light-mediated melatonin reduction and in physiological levels of plasma melatonin (unpublished observation).

Addition of melatonin to the drinking water had no effect on the water intake of the PNx rats (32.1 ± 2.0 ml/24h vs 33.2 ± 1.3 ml/24h, respectively, for PNx and intact rats drinking normal water (P>0.05)). Plasma melatonin concentrations in PNx rats provided with melatonin are shown in Fig. 5. The plasma melatonin concentrations were significantly elevated at ZT 16 and ZT 21.5 (p<0.001) and were very similar to plasma melatonin concentrations in intact rats as observed previously.

Melatonin treatment in PNx rats did not restore the 24h-rhythm in plasma glucose concentrations. Although plasma glucose concentrations showed significant fluctuations over the light/dark-cycle (ANOVA; F(22,88)=2.0, p<0.02), the sets of data points of the rats could not be fitted with the single cosinor analysis. The 24h-mean plasma glucose concentration decreased with 0.5mmol/l after treatment with melatonin (average glucose in PNx vs. melatonin treated PNx rats: p<0.01) (Fig 2B; Table 2). Mean 24-h glucose concentrations in melatonin-treated PNx rats were similar to those in intact rats (p=0.6).

Treatment of PNx rats with melatonin resulted in significantly increased overall insulin concentrations. ANOVA detected an effect of time (F(22,198)=7.2; p<0.001), an effect of group x time: (F (22,198)= 6.2; p<0.001), and an effect of treatment (F(1,9)= 8.8; p<0.016). Their insulin concentrations were now even higher than those in intact rats (ANOVA: effect of time (F(22,242)=9.2; p<0.001), an effect of group x time: F(22,242)=5.6; p<0.001), and an effect of treatment (F1,11)=7.7; p<0.018)).
Fig. 5  Plasma melatonin concentrations in PNx rats during the first experiment (○) and second experiment (□) and in melatonin-treated PNx rats (●). The gray area indicates the plasma melatonin concentrations in intact rats as measured in a previous study\textsuperscript{46}.

For melatonin-treated PNx rats, insulin increments after a meal reached significance for ZT 2, 14, 18 and 22 (p<0.05). Insulin increments after a meal in melatonin treated PNx rats were significantly higher than those in PNx rats (p<0.02) and in intact rats (p<0.01).

Plasma corticosterone concentrations in PNx rats subjected to a scheduled feeding regimen
As changes in plasma corticosterone concentrations influence glucose metabolism, changes in the 24h-rhythm in plasma glucose concentrations may result from indirect effects of pinealectomy on the 24h-rhythm in plasma corticosterone concentrations. Therefore, plasma corticosterone concentrations were measured in PNx rats subjected to a scheduled feeding regimen. They showed a clear 24h-rhythm in plasma corticosterone concentrations (Fig. 6). This rhythm did not differ from the one observed previously in intact rats\textsuperscript{106}.

Discussion
The present data provide evidence that removal of the pineal gland dampens the amplitude of the 24h-rhythm in plasma glucose concentrations in rats fed \textit{ad libitum}, but abolished it completely in rats subjected to a scheduled feeding regimen. Most importantly, pinealectomy increased plasma glucose concentrations at the end of the dark period irrespective of whether rats were fed \textit{ad libitum} or subjected to a scheduled feeding regimen. The higher glucose concentrations confirm previous observations\textsuperscript{52,128} and appeared
Fig. 6 Plasma corticosterone concentrations across the light/dark-cycle under scheduled feeding conditions in PNx rats (n = 6). The black bars indicate the night-time. The gray area indicates the plasma corticosterone concentrations in intact rats as measured in our previous study.\textsuperscript{106}

not to have resulted from a higher food intake, because we did not find effects of pinealectomy on the total amount of food consumed when rats were fed ad libitum. These findings are consistent with the results of previous studies reporting no effects of pinealectomy on the rhythm of food intake or the amount of food intake.\textsuperscript{155,186} In addition, we observed that PNx rats subjected to the scheduled feeding regimen consumed smaller meals than intact rats. The increased plasma glucose concentrations, especially during the dark period and the early light period, led to a strong dampening of the 24h-rhythm. Removal of a second time cue, i.e. the nocturnal feeding rhythm, completely abolished the endogenous rhythm in plasma glucose concentrations. This appeared not to be the result of a general disturbed rhythm in PNx rats, as a clear 24h-rhythm in corticosterone concentrations was observed in PNx rats (Fig 6) and there was also no disturbance in their rhythm in locomotor activity or food intake.\textsuperscript{155,186}

Measuring the glucose concentrations over a period of 24-hours enabled us to provide a more detailed description of the effects of pinealectomy on plasma glucose concentrations than previous studies.\textsuperscript{42,52} An increase of the average glucose concentrations may have resulted from decreased insulin concentrations after pinealectomy. However, we did not find an effect of pinealectomy on insulin concentrations under both ad libitum feeding conditions and scheduled feeding conditions. Therefore, the higher glucose concentrations together with the unchanged insulin concentrations suggests a decreased glucose tolerance after pinealectomy as a result of an increased insulin insensitivity. This is supported by the study of Lima and colleagues, who showed a reduced insulin action after pinealectomy and observed a down regulation of the GLUT-4 transporter, which is
an insulin-dependent glucose transporter. In addition, in PNx rats subjected to a scheduled feeding regimen we also observed a decrease in insulin increments after a meal in the dark period as compared to those in intact rats in our previous study, indicating that pinealectomy also decreases the ability of the pancreas to respond to a meal. This observation is in agreement with the data of Lima and colleagues.

To examine whether the observed changes in glucose metabolism were solely due to the lack of melatonin, we administered melatonin via the drinking water during the hours that the pineal normally releases melatonin. This resulted in plasma melatonin concentrations in PNx rats very much comparable to those described previously for intact rats. Furthermore, a different study showed that replacing melatonin via drinking water restored urinary 6-sulphatoxymelatonin in Wistar rats subjected to a light-mediated melatonin reduction. In the present study, a long-term replacement strategy was chosen to enable a restoration of the down-regulated melatonin receptor, because melatonin receptors in the SCN appear to be down regulated after pinealectomy.

Three weeks of melatonin replacement did not restore the 24h-rhythm in plasma glucose concentrations. However, it did lower the 24h-mean concentrations to a level comparable to those in intact rats. This suggests that the underlying mechanisms responsible for these two effects may be different. The reduction in glucose concentrations may be explained by the higher meal-induced insulin increments observed after melatonin replacement, suggesting a much higher responsiveness of the pancreas. Indeed, meal-induced insulin increments were significantly higher in melatonin-treated PNx rats. However, the elevation of meal-induced insulin was so pronounced that with a normal tissue sensitivity to insulin we would have expected a pronounced hypoglycemia. Apparently, melatonin replacement in PNx rats did not abolish the insulin insensitivity that resulted from the pinealectomy.

Whether the process of maintaining homeostasis, under influence of the exogenous melatonin in PNx rats, is regulated at the level of the central nervous system, for example at the level of the SCN as was suggested by Shima and colleagues, or at a peripheral level is as yet unclear. A peripheral regulation alone, i.e. melatonin binding to peripheral organs including the liver and the β-cells in the pancreas, is not likely since the effects of pinealectomy, and melatonin replacement are not restricted to dark period, i.e. the time window in which melatonin is normally secreted. In addition, administration of melatonin to PNx rats did not result in restoration of the 24h-rhythm in plasma glucose concentrations. These effects may be due to changes in the central nervous system as a consequence of the relatively long period between the pinealectomy and the melatonin administration. Indeed, Peschke observed changes in the ventromedial hypothalamic nucleus (VMH) after pinealectomy. It is not known whether these changes are transient or permanent even after melatonin replacement. The VMH is known to be involved in glucose regulation by influencing the autonomic nervous system. Also other central
sites that influence the autonomic nervous system, for example in the hypothalamus and brainstem, may be changed as a consequence of pinealectomy.

The observation that the rhythm in plasma glucose concentrations and the insulin insensitivity in PNx rats were not restored following melatonin replacement, suggests that pinealectomy has more severe effects on the organism than only removing the source of melatonin. The pineal is richly innervated by the autonomic nervous system, and removing the pineal may result in degeneration of neurons in the superior cervical ganglia that innervate the pineal gland. This may result in changes in the autonomic nervous system innervating other organs, and may modify the level of activity of the autonomic nervous system. The assumption that pinealectomy changes the autonomic nervous system is supported by the observation that melatonin treatment only partly reversed the increments in heart rate observed in PNx rats. In addition, the high insulin responses in melatonin-treated PNx rats may be explained by a change in the reactivity of the parasympathetic nervous system that innervates the β-cells of the pancreas. We observed elevated meal-induced insulin responses after all 6 meals, and not only during the period melatonin was provided. This may only be explained assuming that melatonin acts indirectly on meal-induced insulin responses and that the autonomic nervous system is affected over the total 24 hours. In addition, in pancreatic islets maintained in vitro melatonin reduces the insulin response to a glucose application, suggesting that our finding of elevated insulin responses after melatonin treatment results from changes outside the pancreas. Further experiments are needed to clarify this issue.

In conclusion, the present study showed that pinealectomy prevents the nocturnal decline in plasma glucose concentrations resulting in increased 24h-mean glucose concentrations. In addition, melatonin replacement in PNx rats restored 24h-mean glucose concentrations, but did not restore the 24h-rhythm. As the 24h-mean insulin concentrations did not change after pinealectomy our finding suggests an increased insensitivity to insulin. Melatonin treatment of PNx rats resulted in higher meal-induced insulin responses, suggesting an increased sensitivity of the β-cells. These data suggest that the pineal hormone melatonin influences both glucose metabolism and the pancreatic β-cell. However, other mechanisms also changed due to pinealectomy are probably responsible for the disappearance of the 24h-rhythm in plasma glucose concentrations.