Control of the daily melatonin rhythm: A model of time distribution by the biological clock mediated through the autonomic nervous system

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CHAPTER 3

Glutamatergic clock output stimulates melatonin synthesis at night

Stéphanie Perreau-Lenz, Andries Kalsbeek, Paul Pévet & Ruud M. Buijs


Abstract

The rhythm of melatonin synthesis in the rat pineal gland is under the control of the biological clock, which is located in the suprachiasmatic nucleus of the hypothalamus (SCN). Previous studies demonstrated a daytime inhibitory influence of the SCN on melatonin synthesis, by using γ-aminobutyric acid (GABA) input to the paraventricular nucleus of the hypothalamus (PVN). Nevertheless, a recent lesion study suggested the presence of a stimulatory clock output in the control of the melatonin rhythm as well. In order to investigate further this output in acute in vivo conditions, we first measured the release of melatonin in the pineal gland before, during and after a temporary shutdown of either SCN or PVN neuronal activity, using multiple microdialysis. For both targets, SCN and PVN, the application of Tetrodotoxin (TTX) by reverse dialysis in the middle of the night decreased melatonin levels. Because of recent evidence of the existence of glutamatergic clock output, we then studied the effect on melatonin release of glutamate antagonist application within the PVN in the middle of the night. Blockade of the glutamatergic input to the PVN significantly decreased melatonin release. These results demonstrate that (1) neuronal activity of both PVN and SCN is necessary to stimulate melatonin synthesis during the dark period, and that (2) glutamatergic signalling within the PVN plays an important role in melatonin synthesis.

Introduction

In mammals, the endogenous circadian rhythms of physiological functions (such as hormone release, metabolism, and body temperature) are controlled by a central biological clock, located in the suprachiasmatic nucleus of the hypothalamus (SCN) (Buijs & Kalsbeek, 2001). The daily synthesis of melatonin in the pineal gland, which exclusively occurs during the dark phase of the light-dark cycle, represents a stable and reliable output of the biological clock. The SCN is connected to the pineal gland...
by a multisynaptic pathway, including, successively, neurons of the paraventricular nucleus of the hypothalamus (PVN), sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord, and noradrenergic sympathetic neurons of the superior cervical ganglion (SCG) (Moore & Klein, 1974; Klein et al., 1983a; Lehman et al., 1984; Bittman et al., 1989; Tessonneaud et al., 1995; Larsen et al., 1998; Teclemariam-Mesbaah et al., 1999; Garidou et al., 2001). Recently, we suggested that, in rat, the SCN uses a combination of inhibitory and stimulatory signals towards the PVN for that purpose (Perreau-Lenz et al., 2003). In the present study, we tested this concept for the first time in acute in vivo conditions. In view of the fact that a stimulatory role of the SCN at night was suggested, and that the main neuronal activity of the SCN is reported during daytime (Schwartz & Gainer, 1977; Inouye & Kawamura, 1979; Shibata et al., 1982; Bos & Mirmiran, 1990), we measured the effect of a temporary shutdown of the neuronal activity of either the SCN or the PVN, using a local tetrodotoxin (TTX) application by reverse dialysis, on melatonin release. Both interventions resulted in a diminished melatonin secretion, as measured by microdialysis in the pineal gland. We previously showed that blocking GABA-ergic transmission to the PVN lifts the inhibition on melatonin synthesis in the pineal gland (Kalsbeek et al., 1999; Kalsbeek et al., 2000c), which suggested that GABA represents the inhibitory signal of the SCN to the PVN during daytime. On the other hand, the melatonin decrease observed after the shutdown of the SCN neuronal activity demonstrates the presence of a stimulatory signal during nighttime. Interestingly, recent studies indicate that both GABA and glutamate may be used, respectively, as inhibitory and stimulatory SCN inputs to regions of the preoptic area involved in the control of sleep/wake rhythm (Sun et al., 2000; Sun et al., 2001). In addition, evidence of glutamate immunoreactivity within presynaptic boutons in the PVN (van den Pol, 1991), as well as evidence of a specific glutamate release from the SCN onto (preautonomic) PVN neurons (Hermes et al., 1996; Csaki et al., 2000; Cui et al., 2001) shore up the idea of a glutamatergic SCN input to the PVN as well. On the basis of this evidence, we proposed that SCN-derived glutamate release within the PVN is responsible for stimulation of melatonin synthesis. Hence, we investigated the effect on melatonin release of nightly glutamate signalling blockage within the PVN.

Material and methods

Animals
Male Wistar rats (Harlan, Zeist, The Netherlands) were kept in a temperature-controlled environment under 12-h light/12-h dark conditions, and fed ad libitum. Time is expressed in Zeitgeber Time (ZT) with ZT12 corresponding to the beginning of the dark period. For the experiments requiring sampling during the dark phase of
the animals, the rats were kept in reverse light/dark conditions during 4 weeks before surgery, in order to assure a total adaptation to the new light/dark regime. Rats weighed 125-150 g and were housed at five animals per cage upon their arrival. For the experiments realized during the light period, the animals (250-275 g) arrived one week before their operation. After surgery, the rats were housed individually. All experiments were conducted under the approval of the Animal Care Committee of the Royal Netherlands Academy of Sciences.

Experimental set-up

**IN VIVO ELECTRICAL ACTIVITY SHUTDOWN IN THE SCN OR THE PVN**

The first part of the study aimed at measuring the effect on melatonin release of a temporary shutdown of the nighttime electrical activity of either the SCN or the PVN. We used a multiple microdialysis technique in order to follow the secretion of melatonin, by transspineal microdialysis, as described previously by Drijfhout et al. (1993), before, during, and after a 2 hours bilateral application, by reverse microdialysis, of the Na\(^+\) channel blocker Tetrodotoxin (TTX) within either the PVN (experiment A) or the SCN (experiment B) in the middle of the dark-period. For both experiments, a Ringer solution (NPBI BV, Emmer-Compascum, The Netherlands) was perfused during 3 consecutive days (Ctr1, ExpTTX, Ctr2) from the beginning to the end of the dark period (from ZT12 to ZT24) through the microdialysis probe implanted in the pineal gland and the ones implanted in either the PVN or the SCN. Besides, during the second dark period (ExpTTX), TTX was added to the Ringer solution (concentration of 10\(^{-6}\)M) and perfused through the hypothalamic probes from ZT17 to ZT19. In addition, in order to measure the effect of the hypothalamic Ringer perfusion itself on melatonin release, Ringer was perfused only through the pineal probes on an extra-control-day (CTR).

In addition, we measured the effect of a temporary shutdown of SCN neuronal activity during the light period (experiment C). For this purpose we followed the secretion of melatonin during two daytime periods. Therefore, Ringer solution was perfused within the pineal gland and the SCN from ZT1 to ZT18 for the first day (Ctr), and from ZT1 to ZT11 for the second light period (ExpTTX). We followed the melatonin release during one dark period in order to confirm the correct placement of the microdialysis probe. In addition, during the second light period (ExpTTX), TTX was added to the Ringer solution (concentration of 10\(^{-6}\)M) and perfused from ZT4-ZT8 within the SCN through the hypothalamic probes.

**BLOCKAGE OF GLUTAMATE SIGNALLING WITHIN THE PVN AND MELATONIN RELEASE**

The second part of this study aimed at investigating the role of the glutamatergic input to the PVN with regard to the rhythm of melatonin synthesis. Therefore, we also used
the multiple microdialysis technique to measure the secretion of melatonin before, during and after the application of an N-methyl-D-aspartate (NMDA) receptor-specific glutamate antagonist in the middle of the night. Ringer was perfused through the pineal gland and the bilateral PVN for three consecutive days, from the beginning to the end of the dark period. During the second dark period (ExpMK801), the NMDA antagonist (+/-) MK-801 was added to the Ringer (concentration of 10^-4 M) and applied by reverse microdialysis from ZT17 to ZT19 within the PVN.

**Microdialysis Probes and Chemicals**

Two kinds of microdialysis probes were used in this study. The microdialysis probes implanted in the pineal gland, or transversal probes, were made as previously described (Drijfhout et al., 1993), using the microdialysis membrane Hospal (AN69; cut-off: 35-40 kDa). The hypothalamic probes, or U-shaped probes, used for reverse microdialysis in the hypothalamus, were made as previously described (Engelmann et al., 1992; Barassin et al., 2002) using the microdialysis membrane C-DAK™ Artifical Kidney cut-off: 6 kDa (135 SCE catalogue nr. 201-8000, CD Medical Inc., Miami Lakes, Florida, USA).

Different chemicals were applied by reverse dialysis in the SCN or PVN. TTX (Tocris Cookson Ltd, Avonmouth, Bristol, UK) was used at a concentration of 10^-6 M, and (+/-) MK-801 (Research Biomedical International, Natrick, MA, USA) was used at a concentration of 10^-4 M.

**Surgeries**

Surgeries were performed in rats of 300-350 g. The animals were anaesthetized with a neuroleptanalgesic mix (fentanyl citrate, 0.315 mg/ml, and fluanisone, 10 mg/ml; Hypnorm*, Jansen Pharmaceutical Ltd., Oxford, England; 0.06 ml/100 g i.m.) and a benzodiazepine (midazolam hydrochloride, 5mg/ml; Dormicum*, Roche Nederland B.V, Mijdrecht, The Netherlands; 0.04ml/100 g s.c.) and placed in a David Kopf stereotaxic apparatus. The transpineal microdialysis probes were implanted transversally in the pineal gland as described previously by Drijfhout et al. (1993). In addition, U-shaped hypothalamic microdialysis probes were implanted bilaterally within either the SCN (tooth bar set at +5.0 mm; angle of -6°; coordinates: 1.4 mm rostral to bregma; 1.3 mm to midline; 8.5 mm below the brain surface) or the PVN (tooth bar set at -3.3 mm; angle of -10°; coordinates: 2 mm caudal to bregma; 2 mm to midline; 7.8 mm below the brain surface). In order to reduce post-operative pain, all rats were subcutaneously injected with depressant analgesic opiate (buprenorphine hydrochloride, 0.324 mg in 50 mg dextrose; Temgesic*, Reckitt & Colman Products Ltd., Kingston-Upon-Hull, UK; 0.03 ml/100 g) after post-anaesthesia arousal.
Microdialysis
After a recovery period of 6-8 days, the microdialysis probes were connected to the system of microinjection pumps as described previously (Bothorel et al., 2002). Ringer was perfused through the dialysis probes at a flow rate of 3 μl/min during experimental periods and 1 μl/min during resting periods. Dialysate samples were collected every hour or every half-hour (during the application of TTX and glutamate antagonist) from the pineal probe, and stored at -20°C until melatonin and corticosterone assay. Chemical solutions (such as Ringer, TTX or glutamate antagonist) were perfused through the hypothalamic probes at a flow rate of 3 μl/min.

Radio Immunoassays
Melatonin concentration of dialysates was measured in duplicate by radioimmunoassay using [125I] melatonin (Amersham, Bucks, UK; specific activity 2000 Ci/mmol) and rabbit antiserum (AB/R/03, Stockgrand Ltd, Guildford, UK) with a final dilution of 1:200,000. Stock melatonin (Sigma Chemicals) was stored at a concentration of 1 mg/ml. The standard range of dilutions extended from 3.75 pg/ml to 4 ng/ml, and the minimum detection limit level was 4 pg/ml. The intra-assay coefficients of variation were 19, 13 and 13 % for standards containing 60, 300 and 1,500 pg/ml, respectively, and the inter-assay coefficient of variation between seven assays was 22, 13 and 3%, for the 3 concentrations mentioned before (Barassin et al., 1999). Corticosterone concentrations in the dialysates were measured in duplicates by radioimmunoassay using a commercially available [125I] corticosterone radioimmunoassay kit (ICN Biomedical Division, Carson, CA, USA). The intraassay and interassay (at 50% binding of the standard curve) coefficients of variation were 7.4% and 6.7%, respectively. The lower limit of sensitivity using this method is 12.5 pg/ml.

Immunocytochemistry
In order to check the placement of the probes, the animals were perfused with fixative (paraformaldehyde 4%) at the end of each experiment, and immunocytochemical staining was performed on the brain sections obtained with a vibratome apparatus. Alternating hypothalamic sections were processed for a Nissl counterstaining or to detect the presence of vasopressin or vasoactive intestinal peptide containing neurons and fibers.

Data analysis
Data from all experiments are expressed as mean ± SEM. In order to avoid the factor of microdialysis probe placement, we only analysed the data of animals that had a mean ≥ 200 pg/ml/hr of melatonin release during the control dark periods. The data were analysed using two-way MANOVA with both treatment (i.e. 3 levels, Ctrl,
ExpTTX or MK-801, and Ctr2) and time of day (depending on the experiment 10 or 14 levels) as repeated measures within-subject factors. MANOVA tests were followed, if F-values were appropriate, by a Student-Newman-Keuls post-hoc test. For all analyses realized in this study P <0.05 was considered significant.

Results
The MANOVA analysis applied for the comparison of the different control days (CTR, Ctr1 and Ctr2) measured in 5 animals did not reveal any significant effect of the Ringer perfusion within the hypothalamic probes on melatonin release (Treatment, F(2,6)=2.65, P = 0.15; Time, F(13, 39)=3.56, P <0.001; Interaction, F(26,78)=0.89, P = 0.62).

In vivo shutdown of electrical activity of PVN/SCN and melatonin release
In the first place, we tested in vivo the effect of the shutdown of neuronal activity of the PVN in the middle of the night on pineal melatonin release (experiment A). For this experiment 18 rats were operated. Due to a too strong decrease of their body weight (more than 10%) one week after the operation, or to an obstruction of the pineal probe, 5 animals were not connected to the perfusion system of dialysis. Of the 13 rats perfused only 11 were perfused for the entire experiment. Of these 11 rats, 3 more animals had to be excluded due to the low level of melatonin measured, suggesting misplacement or damage of the pineal microdialysis probe. All hypothalamic probes of the 8 remaining animals were located in or directly adjacent to the PVN as observed after histological analysis of the brain sections (for an example see Fig.1).

As shown in Figure 2A, melatonin release quickly, strongly and significantly (Table 1A) decreased following the application of TTX within the PVN (from ZT17 to ZT19). Levels started to decrease 30 minutes after the start of the TTX perfusion and reached daytime basal levels already after 1h. This effect was reversible, and melatonin levels rose to control levels 2 hours after the end of the TTX perfusion.

Next, we tested the in vivo effect of shutting-down neuronal activity within the SCN in the middle of the night, on melatonin release in the pineal gland (experiment B). For this experiment 20 rats were operated and only 13 animals were connected to the perfusion system of dialysis for the same reasons explained above. Of the 13 rats perfused, 11 were perfused for the entire experiment. Due to too low levels of melatonin concentration, 2 additional animals were excluded. Of the 9 remaining animals, 6 showed a significant drop of melatonin after TTX application (>80%) and 3 did not. Post-hoc analysis of the brain sections revealed a placement clearly anterior of the SCN for the probes of these 3 animals. As shown in Figure 2B for the 6 remaining animals, TTX application in the middle of the night within the SCN also strongly,
quickly and significantly (Table 1A) decreased melatonin levels, as observed after TTX application at the level of the PVN. Levels started to decrease 30 minutes after the start of the TTX perfusion. Like in the PVN, the effect of TTX application in the SCN was quickly reversible after the end of the perfusion. Indeed, melatonin levels reached control levels 2 hours after the end of the TTX perfusion.

In order to test the in vivo effect of shutting-down the neuronal activity of the SCN on melatonin release in the pineal gland during the light period, we performed a third experiment (experiment C). For this experiment 14 animals were operated. Of these 14 rats 3 were not connected to the perfusion system of dialysis for the same reasons explained above, and 8 of the 11 rats remaining were perfused for the entire experiment. Of these 8 rats, 3 were excluded, due to either the misplacement of the hypothalamic microdialysis probe (n=1) or due to too low levels of melatonin release.

Table 1  Statistical effects of neuronal activity shutdown of either the PVN (experiment A) or the SCN (experiment B and experiment C) on melatonin release in the pineal gland (A) or on corticosterone release (B). Data result from the MANOVA analyses (See Material and Methods). “F” indicates the degree of freedom and “p” the probability for each factor (Time, Treatment, and the interaction between Time and Treatment). The significance threshold is p<0.05.

<table>
<thead>
<tr>
<th>A. Melatonin</th>
<th>Factors</th>
<th>F</th>
<th>p</th>
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<tbody>
<tr>
<td><strong>Experiment A</strong></td>
<td>Time</td>
<td>F(13,91) = 4.45</td>
<td>p&lt;0.001</td>
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<td>Treatment</td>
<td>F (2,14) = 5.86</td>
<td>p&lt;0.05</td>
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<td></td>
<td>Interaction</td>
<td>F(28,182)= 3.75</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td>Time</td>
<td>F(13,52) =10.23</td>
<td>p&lt;0.001</td>
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<td></td>
<td>Treatment</td>
<td>F (2,8) = 0.93</td>
<td>p=0.434</td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>F(26,104)= 2.24</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td><strong>Experiment C</strong></td>
<td>Time</td>
<td>F(9,36) = 2.08</td>
<td>p=0.058</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>F(1,4) = 0.04</td>
<td>p=0.851</td>
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<tr>
<td></td>
<td>Interaction</td>
<td>F(9,36) = 1.66</td>
<td>p=0.136</td>
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<th>B. Corticosterone</th>
<th>Factors</th>
<th>F</th>
<th>p</th>
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</thead>
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<tr>
<td><strong>Experiment A</strong></td>
<td>Time</td>
<td>F(13,78) = 3.08</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>F (1,6) = 0.99</td>
<td>p=0.358</td>
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<tr>
<td></td>
<td>Interaction</td>
<td>F(13,78) = 0.74</td>
<td>p=0.72</td>
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<tr>
<td><strong>Experiment B</strong></td>
<td>Time</td>
<td>F(13,65) = 2.39</td>
<td>p&lt;0.05</td>
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<td></td>
<td>Treatment</td>
<td>F (1,5) =27.88</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>F(13,65) = 2.80</td>
<td>p&lt;0.005</td>
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<tr>
<td><strong>Experiment C</strong></td>
<td>Time</td>
<td>F (9,9) = 1.47</td>
<td>p=0.286</td>
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<td></td>
<td>Treatment</td>
<td>F (1,1) =90.48</td>
<td>p=0.07</td>
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<tr>
<td></td>
<td>Interaction</td>
<td>F (9,9) = 2.43</td>
<td>p=0.101</td>
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The melatonin data of the remaining 5 rats are shown in Figure 2C. Interestingly, daytime TTX application within the SCN had no effect on melatonin release. Indeed, melatonin levels were not significantly different (Table 1A) from the daytime basal levels measured during the control day.

In order to assess the local specificity and efficiency of the TTX administration, we also measured the corticosterone levels in the same dialysate samples collected for these 3 experiments. TTX application in either the PVN or the SCN caused very different effects regarding corticosterone release (Fig.3).

TTX in the PVN did not cause a change in corticosterone release as compared to Ctrl, but TTX in the SCN caused a clear and significant increase of corticosterone release during the dark period (Table 1B). During the light period, the TTX application increased the levels of corticosterone, but due to the small number of samples (the samples of 3 animals were lost) the statistical analysis did not reach significance (Table 1B).
Figure 2 Effect of a temporary shutdown of the neuronal electrical activity of the PVN or the SCN on melatonin release in the pineal gland. A local 2h perfusion of TTX in the middle of the dark period within either the PVN (A) or in the SCN (B) decreased the release of melatonin when applied between ZT17-ZT19. But, a 4h perfusion of TTX within the SCN during the middle of the light period, from ZT4 to ZT8, has no effect on melatonin synthesis (C). Data are expressed in mean levels of melatonin concentration for each time point of 5 to 8 rats, error bars represent SEM. Student-Newman-Keuls post-hoc tests realized after a MANOVA indicate the time-points that for the day of TTX perfusion were significantly different compared to the first control day. Significant differences P <0.05 and P <0.001 are respectively indicated by * and **.
Figure 3 Effect of a temporary shutdown of the neuronal electrical activity of the PVN or the SCN on Corticosterone release. A local 2h perfusion of TTX during the middle of the dark period, from ZT17 to ZT19, did not affect corticosterone release when applied within the PVN (A) but did strongly increase it when applied within the SCN (B). A 4h perfusion of TTX within the SCN during the middle of the light period, from ZT4 to ZT8, also strongly increased the release of corticosterone (C). Data are expressed in mean levels of corticosterone concentration for each time point of 2 to 8 rats, error bars represent SEM. Student-Newman-Keuls post-hoc tests realized after a MANOVA indicate the time-points that for the day of TTX perfusion were significantly different compared to the first control day. Significant differences P<0.001 are indicated by **.
Blockage of glutamate signalling within the PVN and melatonin release

In a second series of experiments, we tested the role of endogenous glutamate release within the PVN on melatonin release. For this purpose, we measured the effect of a glutamate transmission blockage (application of a glutamatergic antagonist) in the PVN on the nocturnal release of melatonin in pineal gland. For this experiment 12 rats were implanted with two bilateral hypothalamic and one transpineal microdialysis probe. Due to a too strong decrease of their body weight (more than 10%) one

![Graph A](image1)

![Graph B](image2)

**Figure 4** Effect of a temporary blockade of the glutamatergic signalling within the PVN on the nocturnal melatonin release in the pineal gland (A) and on corticosterone release (B). A local 2h perfusion of glutamate antagonist, +/-MK-801, within the PVN during the middle of the dark period (ZT17-ZT19) decreased the release of melatonin in the pineal gland (A), but did not affect the corticosterone secretion (B). For each time point, data of melatonin and corticosterone concentration are expressed in mean levels of 6 rats, error bars representing the SEM. Student-Newman-Keuls post-hoc tests realized after a MANOVA indicate the time-points that were significantly different for the day of TTX perfusion as compared to the first control day. Significant differences P <0.05 are indicated by *. 

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Table 2  Statistical effects of glutamatergic signalling blockade on nocturnal melatonin release in the pineal gland (A) and on corticosterone release (B). Data result from the MANOVA analyses (See Material and Methods). “F” indicates the degree of freedom and “p” the probability for each factor (Time, Treatment, and the interaction between Time and Treatment). The significance threshold is p<0.05.

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<th>A. Melatonin</th>
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<td>Time</td>
<td>F(13,65) = 1.97</td>
<td>p&lt;0.05</td>
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<td>Treatment</td>
<td>F (2,10) = 3.27</td>
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<td>Interaction</td>
<td>F(26,130) = 2.46</td>
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<td></td>
<td>Time</td>
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<td>p&lt;0.001</td>
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<td>Treatment</td>
<td>F (1,5) = 0.15</td>
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<td></td>
<td>Interaction</td>
<td>F(13,65) = 1.08</td>
<td>p=0.389</td>
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week after the operation, or to an obstruction of the pineal probe, 2 animals were not connected to the dialysis system of perfusion. Of the 10 rats perfused, 4 animals were excluded due to either an instability between the different control days, suggesting damage of the pineal microdialysis probe (n=2), or due to an incomplete sampling pattern (n=2). The melatonin release patterns of the 6 remaining animals are shown in Figure 4A.

The glutamate antagonist infusion within the PVN significantly decreased melatonin release (Table 2A). The effect of MK-801 on melatonin is not as strong and as rapid as the effect observed after TTX application. But just as with TTX, this effect is also reversible, and the levels of melatonin increased again and reached the level of the control days 3 hours after the end of the application. As shown in Figure 4B and Table 2B, the perfusion of the glutamate antagonist did not have any significant effect on the release of corticosterone.

Discussion
The present in vivo experiments (1) demonstrate for the first time that nocturnal neuronal activity of both the SCN and the PVN is crucial to stimulate melatonin synthesis in the rat pineal gland, and (2) clearly reveal that glutamate is an essential neurotransmitter used by the SCN to stimulate the PVN→pineal pathway and consequently the melatonin synthesis.

Acute in vivo shutdown of the neuronal activity of both SCN and PVN during the dark period caused, indeed, an immediate diminution of melatonin release in the pineal gland. The effect of silencing nocturnal neuronal activity in the PVN could be
expected from previous lesions studies (Klein et al., 1983a; Lehman et al., 1984; Hastings & Herbert, 1986; Bittman et al., 1989; Larsen et al., 1998). However, the present results for the first time establish the importance of PVN output for a proficient melatonin production in an in vivo experimental setting and after an acute and reversible shutdown of its neuronal activity. Nocturnal melatonin release was also decreased in animals with permanent thermal lesions of the SCN. But, despite its strong decrease nocturnal levels of mRNA expression of the limiting enzyme for melatonin synthesis, arylalkylamine N-acetyltransferase, and melatonin release in SCN-lesioned animals remained significantly elevated as compared to basal daytime levels in control animals (Bittman et al., 1989; Tessonneaud et al., 1995; Kalsbeek et al., 2000c; Perreau-Lenz et al., 2003). The intermediate levels of melatonin release in SCN-lesioned animals and the complete arrest of melatonin release after a temporal shutdown of nocturnal SCN neuronal activity, confirm the dual role of the SCN (i.e. inhibitory and stimulatory) in the control of melatonin synthesis.

It could be argued that due to the very similar effects of TTX administration in the PVN and the SCN on melatonin release, TTX diffusion from one target affects the other target. However, the analysis of corticosterone release, in the same samples, showed a clear differentiation of PVN and SCN infusions. Indeed, silencing PVN neuronal activity in the middle of the night did not affect the corticosterone levels, as expected, while silencing SCN neuronal activity in the middle of the night, clearly increased the corticosterone levels. Apparently, SCN neuronal activity is already necessary in the middle of the night to inhibit corticosterone suggesting that the decrease of corticosterone secretion at this time is not a simple passive mechanism, as previously suggested (Kalsbeek et al., 1996c). Consequently, a shutdown of the SCN removed its inhibitory influence on the corticotropin-releasing-hormone neurons of the PVN and then on corticosterone secretion. On the other hand, in control conditions corticotropin-releasing-hormone neurons in the PVN apparently show little activity during the dark period, and an additional shutdown of its activity by TTX administration in the PVN does not affect corticosterone levels. Thus, the diminution of melatonin release observed after TTX application within the SCN is site specific and cannot be explained by a possible diffusion of the TTX to the PVN. The present in vivo results, therefore, show a clear functional evidence of nocturnal neuronal activity of the SCN, which is not only crucial for melatonin synthesis in the pineal gland, but also for the inhibition of corticosterone secretion.

It may seem surprising to propose a significant output of the SCN during the dark period since, as reported so far, the SCN is mainly active during (subjective) daytime (Schwartz & Gainer, 1977; Inouye & Kawamura, 1979; Shibata et al., 1982; Bos & Mirmiran, 1990). However, more recent in vitro studies measuring the activity of individual SCN neurons have shown evidence for SCN neurons with an opposite-
phase of activity (Herzog et al., 1997; Nakamura et al., 2001). In addition, Saeb-Parsy & Dyball (2003a) have shown recently that defined cell groups in the rat SCN have different day/night rhythms of single-unit activity in vivo, suggesting that for different subpopulations of SCN cells the peak time of their activity differs. Therefore, it is likely that, although the general neuronal activity recorded within the SCN at night is lower than during the day, the activity of selected populations of SCN neurons will be responsible for the stimulation of melatonin secretion in the pineal gland and for the inhibition of corticosterone secretion.

In addition, the present study, further confirms our previous data (Perreau-Lenz et al., 2003) showing that, during the light period, the SCN sustains both an inhibitory and a stimulatory input to the PVN at the same time. We have previously shown that blocking GABA input to the PVN during 4 hours in the (subjective) day allows the stimulation of melatonin synthesis and release in the pineal gland (Kalsbeek et al., 2000c). On the other hand, the present results revealed that silencing all SCN neuronal activity during daytime has no such effect on melatonin release, although the TTX solution was applied during a period long enough (i.e. 4 hours) for a neo-synthesis of melatonin. Moreover, although no effect could be observed on melatonin release, the strong increase of corticosterone following TTX application in the SCN attests for an effective infusion. Thus, we can conclude that during daytime the effect of a total SCN neuronal activity shutdown is different from the effect of only a blockade of its inhibitory (GABA-ergic) output to the PVN. These results show that the stimulatory SCN input to the PVN→pineal pathway is also present and functional during the light period, and is silenced in case of a TTX application in the SCN but not by a blockade of GABA signalling in the PVN. In addition, as already shown by previous studies (Buijs et al., 1993a; Kalsbeek & Buijs, 1996; Kalsbeek et al., 1996c), the present results confirm that the SCN control of corticosterone release at this time of the day (middle of light-period) is mainly inhibitory.

The present evidence of glutamate as a SCN neurotransmitter responsible for the stimulation of melatonin secretion fits very well with previous studies showing glutamate as a dominant central excitatory neurotransmitter with an important role within the hypothalamus (van den Pol et al., 1990; van den Pol, 1991). Moreover, Sun et al. (2000; 2001) recently proposed that SCN projections to the ventromedial and ventrolateral regions of the preoptic area use both GABA and glutamate as inhibitory and stimulatory inputs, respectively, for the control of the sleep/wake rhythm. In addition, several recent studies revealed specific glutamate release from the SCN onto (preautonomic) PVN neurons (Hermes et al., 1996; Csaki et al., 2000; Cui et al., 2001). In the present study, we clearly demonstrated that glutamatergic signalling in the PVN is involved in the stimulation of melatonin synthesis in the pineal gland. Indeed, the blockade of glutamatergic transmission (at least via NMDA receptors)
within the PVN selectively inhibits melatonin synthesis. At present we cannot exclude the involvement of either other glutamatergic receptors or other stimulating neurotransmitters or neuropeptides in the nocturnal stimulation of melatonin release. Accordingly, further studies will be necessary to complete our knowledge on the stimulating SCN input towards the PVN. However, we can already clearly conclude from the present study that release of glutamate within the PVN at night is necessary to stimulate melatonin synthesis. The present results, obtained with a non-competitive NMDA receptor antagonist, MK-801, infused specifically in the PVN, confirm and can explain previous results obtained in hamsters after an intra-peritoneal injection of MK-801, at ZT19, showing that MK-801 induces a dose-dependent inhibition of melatonin production (Vuillez et al., 1998). It is likely that in this experiment the glutamate antagonist reached the PVN.

In conclusion, the present study demonstrates that even if the general neuronal activity of the SCN is rather weak at night, it is sufficient, and, even more, necessary, to stimulate melatonin synthesis and to inhibit corticosterone at the same time. Furthermore, the present study clearly demonstrates that glutamate release within the PVN is a major component for the stimulation of melatonin synthesis at night, suggesting a clear functionality for direct glutamatergic SCN inputs to the preautonomic neurons of the PVN. These results consequently bring new insight on the way the biological clock can control diverse physiological functions. In future experiments it will be interesting to investigate further the pattern of release of this SCN stimulatory signal and to identify the subpopulation of SCN neurons that sustains a stimulatory output to the pineal gland.

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