4

Sympathetic innervation, the major regulator that switches on and off

Sympathetic innervation is considered to be a crucial factor in melatonin production. This chapter describes a variety of experiments aimed to explore its role in great detail and thereby benefit from the opportunities that microdialysis gives in this kind of studies.

Melatonin levels could be greatly enhanced by systemic administration of the non-selective β-agonist isoprenaline. Also local infusion of isoproterenol or 8-Br-cAMP, an analogue of the second messenger cAMP, resulted in increased melatonin levels, demonstrating the presence of β-adrenergic receptors on the pineal gland, coupled to a cAMP based second messenger system. During night-time, the non-selective β-adrenergic receptor blocker propranolol appeared very effective in suppressing the melatonin production. Injection of the α₁-agonist phenylephrine had no effect on daytime levels. Only when administered during isoprenaline induced stimulation of melatonin release it enhanced this stimulated release. This proved the regulatory role of α₁-receptors on pinealocytes.

The sympathetic innervation of the rat pineal gland was further investigated, measuring the noradrenaline release directly. The high sensitivity of the analytical assay used, made it extremely useful to monitor the very low levels of noradrenaline in the dialysates. To increase noradrenaline levels,
the monoamine re-uptake inhibitor cocaine was added to Ringer's solution in concentrations of $10^{-6}$ M and $10^{-5}$ M. This resulted in increases of neurotransmitter output, but did not change the qualitative and/or quantitative outcome of other experiments. The characterization of the noradrenaline release was performed by inhibition of the release with the sodium channel blocker TTX and stimulation with the $\alpha_2$-receptor antagonist yohimbine. Perfusion with $10^{-6}$ M TTX for one hour resulted in a rapid decrease of the noradrenaline release, whereas perfusion with the $\alpha_2$-receptor antagonist yohimbine caused an increase. These results indicate that the noradrenaline release in the rat pineal is of neuronal origin and regulated by a negative feedback mechanism involving inhibitory presynaptic $\alpha_2$-receptors.

Long-term (i.e. 16 hours) measurements were performed, showing the circadian properties of noradrenaline release. A pronounced rhythm is reported, showing extremely sharp transitions between low daytime and high night-time values. Increases and decreases are reported to occur within the duration of collecting one sample (20 min). The on and off switches of the sympathetic input correlate well with the circadian rhythm of melatonin release and can thus be considered as the primary clock signal, inducing the nightly production of melatonin.

The presented method is of special interest for investigating the innervation of the pineal gland and the biochemical processes that regulate the biosynthesis of melatonin. Also for studies on the diurnal rhythms of melatonin release and factors that influence these rhythms in freely moving animals, this model can be of great value.

Data presented in this chapter are published in the following papers:


4.1 Introduction

One of the most interesting and probably most important functions of the pineal gland is the strong rhythmicity in which melatonin is produced and secreted. The resulting circadian rhythm of melatonin release with high levels during the night and low levels during the day are important in the photoperiodic regulation of reproduction and various other physiological functions.\textsuperscript{9,276} The circadian rhythm of pineal activity is driven by the suprachiasmatic nucleus (SCN).\textsuperscript{149,159,217} The connection between SCN and pineal consists of a multisynaptic pathway, finally resulting in sympathetic innervation of the pineal from the superior cervical ganglion. The release of noradrenaline from the sympathetic nerve terminals is considered as the primary stimulus for melatonin production.\textsuperscript{93,348}

The noradrenaline signal is mainly transduced by post-synaptic $\beta_1$- and $\alpha_1$-receptors. Activation of these receptors increases levels of intracellular cAMP, which enhance the activity of N-acetyltransferase, the rate-limiting enzyme in the biosynthesis of melatonin. Activation of N-acetyltransferase subsequently results in increased melatonin production and release. A wide range of other receptors on rat pinealocytes has been suggested, including $\alpha_2$-adrenergic,\textsuperscript{99,247,312} neuropeptide Y,\textsuperscript{326} vasointestinal peptide\textsuperscript{325,431} and $\gamma$-amino-butyric acid (GABA) receptors.\textsuperscript{298} Although the functionality of these receptors is partly known, their physiological importance remains mostly unclear. Some of these receptors show circadian variation in density and or sensitivity\textsuperscript{244,323} and are therefore believed to play a regulatory role in the rhythmic production of melatonin.

The effects of $\alpha$- and $\beta$-adrenergic drugs on melatonin production have been extensively studied. The interaction between $\alpha$- and $\beta$-adrenergic receptors, which may have a regulatory function, appeared to be interesting. In this chapter, the effects of $\alpha_2$- and $\beta$-adrenergic compounds and their interaction are investigated. The time dependency of the effects, a strong feature of microdialysis, is determined.

Although many studies describe the role of the sympathetic innervation indirectly by the effects of adrenergic drugs, data on the actual release of noradrenaline are scarce and only from in vitro studies. The possibility to measure noradrenaline with a very high sensitivity enabled us to examine in vivo the nocturnal release of noradrenaline. A comparison is made with the release of melatonin, in order to clarify to what extend noradrenaline release can regulate melatonin production and at what stages other regulatory mechanisms might act.

Experiments with the sodium channel blocker TTX, the monoamine re-uptake inhibitor cocaine, the $\alpha_2$-receptor antagonist yohimbine and a situation of acute stress were used to characterize the signal. Long-term experiments (16 h) were carried out to study the circadian properties of noradrenaline and melatonin release. Finally a comparison is made between the noradrenaline release and the melatonin production.

The fact that both melatonin and noradrenaline can be measured with the same kind of microdialysis technique, provide us with a unique situation in which we can measure the neuronal input of a gland, together with the hormonal output. In addition, to the best of our knowledge this is the first time that sympathetic innervation is sampled with in vivo microdialysis in freely moving rats.
4.2 Experimental setup

Animals were treated as described on page 58 and kept under a normal LD cycle (melatonin measurements) or a reversed LD cycle (noradrenaline measurements). They underwent surgery as described on page 59, one or two days before the experiments.

Melatonin production was measured (page 63) in the following experiments: injection with \((\pm)-\text{isoprenaline} \ (10 \text{ mg/kg i.p.})\), perfusion with \((\pm)-\text{isoprenaline} \ (10^{-6} \text{ M}, 1.5 \text{ h})\), perfusion with 8-Br-cAMP (5 mM, 1.5 h), injection with phenylephrine (5 mg/kg i.p.) and perfusion with \((\pm)-\text{isoprenaline} \ (10^{-6} \text{ M}, 6 \text{ h})\) followed by injection of phenylephrine (5 mg/kg i.p.) 3 h after start of the isoprenaline perfusion. A combination of melatonin, N-acetylsertotonin and serotonin (page 64), was measured during perfusion with propranolol (10^{-5} \text{ M}, 2 \text{ h}). The circadian profile of melatonin production was measured over a period of 16 h, from circadian time 10 (CT10) to CT2 in the absence of cocaine.

Noradrenaline release was measured (on-line, page 66) in the following experiments: perfusion with cocaine (10^{-6} \text{ and } 10^{-5} \text{ M}, 2 \text{ h}), perfusion with TTX (10^{-6} \text{ M}, 1 \text{ h}) in the absence and presence of cocaine (10^{-6} \text{ M } \text{ and } 10^{-5} \text{ M}), perfusion with yohimbine (10^{-5} \text{ M}, 1.5 h) in the absence and presence of cocaine (10^{-6} \text{ M } \text{ and } 10^{-5} \text{ M}) and handling for 10 min in the absence of cocaine. Furthermore the circadian profile of noradrenaline release was measured over a period of 16 h, from circadian time 10 (CT10) to CT2 in the absence and presence of cocaine (10^{-6} \text{ M } \text{ and } 10^{-5} \text{ M}).

![Figure 4.1](image-url) The effect of isoprenaline on melatonin production. Isoprenaline was injected in a concentration of 10 mg/kg i.p. at t= 0 min. Melatonin is expressed as percentage of average daytime levels and presented as the mean ± S.E.M. (n=3).
4.3 Results

- **Effect of isoprenaline injection and perfusion on melatonin levels**
  Since the main innervation of the pineal gland is β-adrenergic, we investigated the effects of the non-selective β-agonist isoprenaline on pineal melatonin content in the light period. Systemic injection of the non-selective β-agonist isoprenaline (10 mg/kg i.p.) resulted in an increase of melatonin production of approximately 2000%, being maximal after 1.5 h (Fig. 4.1). Melatonin levels did not reach basal levels within 5 h. Local perfusion with isoprenaline (1.5 h, 10⁻⁶ M) resulted in melatonin levels that were 1150 ± 130% higher than the basal levels (Fig. 4.2), returning back to about basal level rapidly within one hour, once the isoprenaline was removed from the perfusion fluid.

- **Effect of 8-Br-cAMP perfusion on melatonin levels**
  8-Br-cAMP, a cAMP analogue, infused at a concentration of 5 mM for 1.5 h increased the melatonin levels ~670% (Fig. 4.3). About 2 h after removing the 8-Br-cAMP from the perfusion fluid, melatonin levels returned to basal values.

- **Effect of propranolol on melatonin, N-acetylserotonin and serotonin levels**
  The β-adrenergic receptor blocker was applied during night-time. The results of a 2 h perfusion in a concentration of 10⁻⁵ M are shown in Fig. 4.4. Melatonin and N-acetylserotonin rapidly decreased upon perfusion with propranolol. Lowest levels reached were ~20% of normal night-time levels. Serotonin increased about twofold. The opposite response of N-acetylserotonin and serotonin indicate that the decrease in melatonin production was caused by inhibition of N-acetyltransferase.

![Figure 4.2](image-url) The effect of isoprenaline on melatonin production. Isoprenaline was perfused in a concentration of 10⁻⁶ M for 1.5 h, starting at t=0 min. Melatonin is expressed as percentage of average daytime levels and presented as the mean ± S.E.M. (n=4).

4. Sympathetic innervation, the major regulator that switches on and off
Figure 4.3 The effect of 8-Br-cAMP on melatonin production. 8-Br-cAMP was perfused in a concentration of 5 mM for 1.5 h, starting at t = 0 min. Melatonin is expressed as percentage of average daytime levels and presented as the mean ± S.E.M. (n = 3).

Figure 4.4 The effect of propranolol on melatonin, N-acetylserotonin and serotonin production. Propranolol was perfused in a concentration of 10⁻⁵ M for 2 h, starting at t = 0 min. Data are expressed as percentage of average night-time levels and presented as the mean ± S.E.M. (n = 4).

4. Sympathetic innervation, the major regulator that switches on and off
Figure 4.5 The effect of phenylephrine on melatonin production. Phenylephrine was injected in a concentration of 4 mg/kg i.p. at t = 0 min. Melatonin is expressed as percentage of average daytime levels and presented as the mean ± S.E.M. (n=4).

Figure 4.6 The effect of the combination of phenylephrine and isoprenaline on melatonin production. Isoprenaline was perfused in a concentration of 10^6 M starting from t = 0 min and throughout the rest of the experiment. Phenylephrine was injected in a concentration of 4 mg/kg i.p. at t = 180 min. Melatonin is expressed as percentage of average daytime levels and presented as the mean ± S.E.M. (n=3).

4. Sympathetic innervation, the major regulator that switches on and off
Effect of phenylephrine with or without isoprenaline on melatonin levels

In Fig. 4-5, the effect of systemic injection of the $\alpha_1$-agonist phenylephrine (4 mg/kg i.p.) is shown. No significant effect on the melatonin output was observed. Fig 4.6 shows the increase in melatonin levels during continuous isoprenaline infusion (10$^{-6}$ M), reaching a constant level of 1000%. At $t = 180$, a systemic injection of phenylephrine (4 mg/kg i.p.) yielded an enhancement of this increase to 1500%.

Basal levels of noradrenaline release

Basal levels during night-time were very low (12.5 ± 1.2 fmol/injection, $n = 39$) and were largely dependent on the placement of the cannula. However, during the light period, levels were even lower and often not detectable. In this delicate situation of changing noradrenaline release at the edge of the detection limit, good criteria for the right placement of the cannula were of the utmost importance. We used the very sharp transition from high to low levels and the presence of melatonin in the microdialysates as criteria for approval. Some animals did not show this transition, nor yielded detectable melatonin, indicating that the noradrenaline detected must have been from a different compartment. This led to exclusion of the animal from further experimentation. In some animals no noradrenaline could be detected, while melatonin was present. Also these animals were excluded. In cases of rhythmic noradrenaline release, melatonin was always present.

Figure 4.7 The effect of cocaine on noradrenaline release. Cocaine was perfused in concentrations of 10$^{-6}$ M ($\square$, $n = 3$) and 10$^{-5}$ M ($\bigtriangleup$, $n = 4$) starting from $t = 0$ min and throughout the rest of the experiment. Noradrenaline is expressed as percentage of average night-time levels and presented as the mean ± S.E.M.
Effect of cocaine on noradrenaline release

In order to increase the low basal levels, the monoamine re-uptake inhibitor cocaine was added to the perfusion medium. In Fig. 4.7 the effect is shown of two different concentrations. In a concentration of $10^{-6}$ M, cocaine increased basal output to $167 \pm 43 \%$, whereas in a concentration of $10^{-5}$ M, levels were increased to a maximum of $219 \pm 32 \%$. No behavioural changes of the animals were noticed.

Effect of TTX on noradrenaline release

The sodium channel blocker TTX was perfused in a concentration of $10^{-6}$ M (Fig. 4.8). During a perfusion of 1 h, noradrenaline levels decreased to a minimum of $11 \pm 2 \%$. As soon as the TTX was removed from the perfusion fluid, noradrenaline levels returned to their initial values within one sample. In some experiments even an overshoot was detected. The same experiments were also conducted in the presence of $10^{-6}$ M and $10^{-5}$ M cocaine respectively. The results of these experiments were essentially the same as the ones without cocaine. For reasons of comparison, also the effect of TTX $10^{-6}$ M on melatonin is shown (see page 77).

![Figure 4.8](image_url) The effect of TTX on noradrenaline release in the absence and presence of cocaine. TTX was perfused in a concentration of $10^{-6}$ M for 1 h, starting at $t = 0$ min. Cocaine was either absent (○, $n = 3$) or present in concentrations of $10^{-6}$ M (○, $n = 3$) and $10^{-5}$ M (□, $n = 3$) throughout the experiment. Noradrenaline is expressed as percentage of average night-time levels and presented as the mean ± S.E.M. The dotted line represents melatonin levels (without cocaine) during similar treatment with TTX, as shown previously (page 77).
Effect of yohimbine on noradrenaline release
The effect of the α<sub>2</sub>-receptor antagonist yohimbine was tested in a concentration of 10<sup>-5</sup> M (Fig. 4.9). During a perfusion of 1.5 h, noradrenaline levels increased substantially. The amount of increase varied among animals, with an average of 243± 42 %. After treatment, noradrenaline levels returned to their initial values. In the presence of 10<sup>-6</sup> M and 10<sup>-5</sup> M cocaine, the effects were comparable, although in the presence of 10<sup>-6</sup> M cocaine the increase was somewhat smaller (184± 14 %). This difference however was not significant.

Effect of handling on noradrenaline release
The animals were handled for a period of 10 min by taking them out of their cage and keep them rather immobilized. This handling is generally considered as an experimental model of acute stress. The resulting effects on pineal noradrenaline release are presented in Fig. 4.10. During the course of the experiment no significant changes in the levels of noradrenaline could be measured.

Circadian rhythm of noradrenaline release
In order to examine the circadian rhythm of noradrenaline and melatonin release, the output was measured for 16 h, from 2 h before until 2 h after the dark period. The results for melatonin are shown in Fig. 4.11. Levels started to increase 2 hours after lights off and reached a night-time level which was about 15 times higher than basal daytime levels.

Figure 4.9 The effect of yohimbine on noradrenaline release in the absence and presence of cocaine. Yohimbine was perfused in a concentration of 10<sup>-5</sup> M for 1.5 h, starting at t = 0 min. Cocaine was either absent (•, n= 3) or present in concentrations of 10<sup>-6</sup> M (○, n= 4) and 10<sup>-5</sup> M (□, n= 4) throughout the experiment. Noradrenaline is expressed as percentage of average night-time levels and presented as the mean ± S.E.M.
Melatonin started to decrease 1 h before lights on. In the case of noradrenaline (Fig. 4.12), daytime levels were extremely low and sometimes even below the detection limit. Therefore the data are presented as percentage of basal night-time values. The very low daytime levels started to increase rapidly after about 1.5 h in the dark period. In most individual experiments this increase occurred within one sample. Also a rapid decrease occurred about 1.5 h before lights on, often to levels below 20% of the night-time amounts. This sudden increase and decrease resulted in a circadian rhythm with an on/off character with two sharp phase markers that were fully symmetrical to the on- and offset of darkness. In between the two phase markers, levels were relatively stable. Small variations among animals in time of increase and decrease result in relatively large error bars at the phase markers and blurring of the sharp transitions seen in individual experiments (Fig. 4.13). In some animals the noradrenaline release was recorded during the remaining 8 h of the light period. However, no change whatsoever was seen in basal output, restricting the sympathetic activity in the pineal exclusively to the dark period.

When comparing the melatonin rhythm with the noradrenaline rhythm, there is a clear correlation between the two. Melatonin started to increase as soon noradrenaline levels were maximal and a decrease of the noradrenaline levels was immediately followed by a drop in melatonin contents.

Fig. 4.14 shows the circadian rhythm of noradrenaline release in the presence of two concentrations of cocaine ($10^{-6}$ M and $10^{-5}$ M). The same marked increases and decreases were seen around the times of lights on and off. The rhythm of noradrenaline release did not seem to be changed qualitatively by cocaine.

Figure 4.10 The effect of handling on noradrenaline release in the absence of cocaine. The animals were taken out of the cage and kept rather immobilized for a period of 10 min, starting at $t = 0$ min. Noradrenaline is expressed as percentage of average night-time levels and presented as the mean± S.E.M. ($n = 4$).
Figure 4.11. The circadian profile of melatonin production. Data were gathered from 2 h before until 2 h after the dark period. Melatonin is expressed as percentage of average daytime levels and presented as the mean ± S.E.M. (n = 5).

Figure 4.12. Circadian profile of noradrenaline release. Data were gathered from 2 h before until 2 h after the dark period. Noradrenaline is expressed as percentage of average night-time levels and presented as the mean ± SEM (n = 4). The dotted line represents the melatonin profile from the figure above, for reasons of comparison expressed as percentage of average night-time levels.
4.4 Discussion

- **Beta-receptors dominate melatonin production**
  Sympathetic nerves, originating from the superior cervical ganglion, innervate the pineal gland and can stimulate the biosynthesis of melatonin. This sympathetic innervation of the pineal gland is well established\(^{33,52,396}\) and is known to be the primary stimulus for the nightly increases in melatonin production.\(^{128,205}\) The suprachiasmatic nucleus (SCN), also known as the circadian pacemaker, plays a regulatory role in this sympathetic innervation.\(^{47,317}\)

  From *in vitro* and post-mortem studies, it is known, that \(\beta_1\) and \(\alpha_1\)-receptors are present on the pinealocytes.\(^{336,348}\) Stimulation of these receptors will increase the activity of \(\text{N-acyltransferase}\), which is the rate limiting step in the melatonin biosynthesis, and so increase melatonin levels. Our results clearly demonstrate this mechanism *in vivo*. Either systemic or local application of isoprenaline during daytime increased melatonin levels. After systemic injection, it takes several hours before melatonin levels reach their basal values. This is not surprising, since with systemic administration one has to deal with kinetic properties of the drug, such as distribution, metabolism etc. The possibility to apply compounds by local infusion is one of the advantages of microdialysis. Perfusion with isoprenaline clearly stimulated the release of melatonin; a sudden decline in melatonin levels was seen after withdrawal of the isoprenaline from the perfusion fluid. This confirms that \(\text{N-acyltransferase}\) is a labile enzyme when not induced.

  The \(\beta_1\)-receptors, that are activated by isoprenaline, are coupled to a G-protein. Activation of this receptor will cause the G-protein to activate adenylyl cyclase, which

![Figure 4.13](image)

**Figure 4.13** Typical example of a circadian profile of noradrenaline release. Data were gathered from 2 h before until 2 h after the dark period. Noradrenaline is expressed as percentage of average night-time levels.
can convert ATP into cAMP. High levels of cAMP in the pinealocytes increase the activity of N-acetyltransferase, resulting in enhanced production of melatonin. To demonstrate this biochemical event \textit{in vivo} we infused an analogue of cAMP, 8-Br-cAMP, in the pineal gland. The increase in melatonin production confirms that the concentration of the second messenger cAMP in the pinealocytes is an important factor in the stimulation of melatonin production.

The importance of \(\beta\)-adrenergic receptors in maintaining a high level of melatonin production was shown by the \(\beta\)-adrenergic antagonist propranolol. The steep decline in both melatonin and N-acetylserotonin and the low resulting level indicate that \(\beta\)-adrenergic receptors are at least for 80\% responsible for the endogenous melatonin production. Because it is not known whether \(10^5\) M is the maximal effective concentration, this percentage can even be higher. This marked effect of propranolol opens interesting possibilities. Although many receptor systems have been described to be present on the pinealocytes, their function remains largely unknown. Using a maximal effective concentration of propranolol, the remaining melatonin production will probably be induced by these other controlling mechanisms. Measuring melatonin at night during continuous propranolol perfusion might therefore be a model to investigate these mechanisms. Control mechanisms that act via an induction of \(\beta\)-adrenergic stimulated increased N-acetyltransferase activity would in this approach not be taken into account.

Figure 4.14 Circadian profile of noradrenaline release in the presence of cocaine. Dialysis occurred from 2 h before until 2 h after the dark period. Cocaine was present in concentrations of \(10^{-6}\) M (\(\bullet\), \(n = 3\)) and \(10^{-5}\) M (\(\circ\), \(n = 3\)) throughout the whole experiment. Noradrenaline is expressed as percentage of average night-time levels and presented as the mean\(\pm\) S.E.M.
Cross-talk between alpha- and beta-receptors

As mentioned before, also $\alpha_1$-receptors are present on the pinealocytes. These receptors are known to be involved in the regulation of the sympathetic innervation. The $\alpha_1$-receptors are coupled to a PI-system, which can increase protein kinase C (PKC) activity. This PKC activity does not have an effect on the melatonin production. It does have an effect, however, on stimulated adenyl cyclase activity. Stimulating the $\alpha_2$-receptors by the selective agonist phenylephrine alone had no effect on melatonin levels. However, when it was coadministered with isoprenaline, the increase in melatonin levels was enhanced, an example of cross-talk between receptor subtypes on the level of the second messenger system. This is another demonstration of the fact that cAMP is the main factor inside the pinealocytes that can enhance melatonin production. Regulatory mechanisms like the one described here are very efficient in fine tuning the melatonin production.

Noradrenaline release in the pineal gland

TTX has been widely used to discriminate between neuronal and non-neuronal release of neurotransmitters. The unambiguous decrease seen with TTX in our experiments clearly shows that all noradrenaline measured is of neuronal origin and not from other compartments, such as the blood. The remarkable rapid increase after withdrawal of the TTX is in contrast to similar experiments in several brain regions, where it takes several samples for the release to return to basal levels. This difference could be explained by the nature of the tissue. The pineal gland is the second best perfused organ in the body, with the primary function of releasing melatonin into the blood. Therefore it is likely that also drugs, added to the pineal, will be rapidly taken up into the blood. When the origin of sympathetic input remains unchanged during TTX perfusion, a rapid re-establishment of the original situation will be achieved. The close correlation between noradrenaline and melatonin during TTX treatment indicate a direct relationship between the two.

The presence of an $\alpha_2$-receptor in the rat pineal gland has been described in several papers. Most studies indicate its function as a presynaptic receptor, regulating noradrenaline release by negative feedback. Schaad et al. proposed the location of the $\alpha_2$-receptor as post-synaptic, concluding that the effects seen with $\alpha_2$-adrenergic drugs have to be interpreted with caution, especially in vivo. Our findings generated evidence in support of the $\alpha_2$-receptor as being presynaptic. It down regulates the noradrenaline release, since blockade of this receptor with yohimbine resulted in an increase of noradrenaline. The simultaneous presence of a postsynaptic $\alpha_2$-receptors, however, cannot be excluded.

A remarkable finding was that a situation of acute stress such as handling was not reflected in changes of noradrenaline release. Obviously the common increase in sympathetic activity normally seen under such circumstances is not homogenous in all tissues. Apparently the innervation of the pineal behaves differently from sympathetic innervation of systems directly involved in the "fight-or-flight" reaction such as the cardiovascular system. This is a clear demonstration of the robust character of pineal activity, which is not readily disturbed by environmental conditions, including stress. In addition this finding supports the conclusion that the noradrenaline measured is of neuronal origin.
and does not reflect blood concentrations, because these would certainly increase during stress situations.

A major problem in studying noradrenaline release from the rat pineal gland is the extremely low level of noradrenaline, both in vivo (this study) and in vitro. Often the levels are in the range of the detection limit. The use of an uptake inhibitor in such cases can be helpful, as long as it does not change the pharmacology of the system. Experiments that proof this lack of effect on the responsiveness of the system have to be carried out. In this study cocaine was used to increase the basal levels, resulting in a twofold increase of the night-time values. Although this effect on basal levels is modest, it can mean the difference between measurable and unmeasurable noradrenaline release. As far as the pharmacology is concerned, the effect of TTX was not influenced by cocaine. Since TTX blocks the electrical conductivity of the neuron, a process ahead of noradrenaline release and re-uptake, this seems reasonable. Also the effect of yohimbine was not affected. Since both drugs increase noradrenaline by different mechanisms, a synergistic effect could be expected. The absence of such synergism indicates that under basal conditions the presynaptic receptors are fully occupied. Increase of noradrenaline release by re-uptake inhibition under such circumstances does not influence the negative feedback. Melatonin levels seem to be maximal during night-time. Attempts made in our laboratory to increase night-time levels by infusion of isoprenaline or cocaine have been unsuccessful. Furthermore, the day/night rhythm of noradrenaline release seems not to be affected by cocaine. Taken together, apart from the effect on basal levels being modest, there seems to be no reason not to use a re-uptake inhibitor in pineal and circadian studies.

Concerning basal levels at daytime, variability in the placement of the cannula makes the situation even more complicated. When levels in the light period were relatively high, there was never rhythmicity, indicating extra-pineal sources of noradrenaline caused by improper implantation of the cannula. This could always be confirmed by post mortem localization. When levels were below the detection limit during the light period, this did not exclude the night-time rise. Many times normal night-time values were measured under such circumstances and also melatonin levels were normal. These experiments were considered to be the best. Often the situation was in between these two extremes, so low levels were measured in the light period, which increased during the dark period. An important conclusion from this is that little importance should be attached to the level of noradrenaline during the light period, since it will mainly not be originating from the pineal.

Once in a while, levels were undetectable at night as well as during daytime. Mostly improper implantation was responsible for it, but occasionally implantation seemed correct and also normal melatonin was released. These findings might indicate a rather specific anatomical region of innervation. Since the localization technique used was based on visual inspection of the dissected brain, an exact determination of this region was not possible. A detailed histological study could address this point further.
Sympathetic clock input switches on and off

The circadian rhythm of rat pineal activity has been described using several parameters. Melatonin production, N-acetyltransferase activity, β-adrenergic receptor density, and noradrenaline content of the pineal all show circadian variation. Most of these studies have been done with relatively poor time-resolution. The large amount of animals needed to increase time resolution is a serious drawback of conventional techniques. With microdialysis one animal can generate data during the complete LD cycle with a time resolution of minutes.

The noradrenaline release measured in the present study showed a remarkable rhythmicity. Being switched on about 1.5 h after lights off, it was switched off again about 1.5 h before lights on, fully symmetrical to the LD cycle. The delay of 1.5 h is too long to make it likely that the dark onset is the direct trigger for the increase in noradrenaline release. Since no other external triggers could be responsible for the changes seen, an endogenous time keeping system must be responsible for the fast transitions in noradrenaline release. Such a time keeping system is thought to be located in the SCN. Originating from the SCN, a multisynaptic pathway finally innervates the pineal gland by postganglionic sympathetic nerve fibers. The SCN neurons show gradually changing electrical activity both in vivo and in vitro, with peak activity during daytime. Based on the present data it seems likely that somewhere along the way from the SCN to the pineal, this rhythmicity is reversed and transformed to an on/off pattern of noradrenaline release. The sharp transitions can be best explained by a system in which a threshold has to be passed before signals can pass through. The physiological nature and anatomical localization of such a threshold remains still unclear and demands further research.

The correlation between the rhythms of noradrenaline and melatonin is remarkable. Noradrenaline starts to increase 1.5 h after the dark onset, shortly followed by an increase in melatonin production. While noradrenaline levels reach maximal values within the duration of one sample, maximum melatonin levels are not reached until about four hours after the dark onset. This discrepancy is caused by the production and induction of N-acetyltransferase, known to be necessary for the production of large amounts of melatonin. The fact that noradrenaline and melatonin decrease simultaneously indicates a very short half-life of N-acetyltransferase activity under non stimulated conditions.

Since noradrenaline release is considered as the primary driving force behind N-acetyltransferase activity and melatonin synthesis, it seems likely that its strong rhythmicity is the primary timing signal for N-acetyltransferase and melatonin rhythmicity. This makes it questionable what the function is of circadian variations in adrenergic receptor density. Some speculations have been made on their role in the circadian control of the N-acetyltransferase activity, but based on these data it seems more likely that varying receptor densities only play a regulatory role in fine tuning the response of the pinealocytes to their innervation.

Various families of receptors have been demonstrated to be present on pinealocytes. The prominent role of noradrenaline in the regulation of melatonin production evokes questions about the function of possible other neurotransmitters. Do they act directly on melatonin production, or do they influence noradrenaline release, and what is the physiological function of these regulatory mechanisms? Also effects on other pineal functions, such as serotonin production should not be excluded.
The present study describes for the first time the use of microdialysis in freely moving rats in measuring the characteristics of sympathetic innervation of a peripheral gland. The results indicate that this approach can answer important questions about the mechanisms that are involved in the regulation of pineal metabolism. The application of this technique in other peripheral tissues seems to be a logical next step.