This study describes the use of the microdialysis technique to elucidate specific properties of the circadian pacemaking system in the hypothalamus, by measurement of melatonin production in the pineal gland. Melatonin has appeared to be a reliable marker of the pacemaker activity, which is influenced by the light/dark (LD) cycle. A phase shift in the LD cycle was applied to perturb the rhythm generating system. An 8 h phase advance resulted in the disappearance of melatonin production over 2 days, with basal levels comparable to normal daytime levels. In the subsequent return of rhythmic melatonin production, new clock characteristics could be revealed, due to the high time-resolution measurements of microdialysis. While half of the animals still did not show any rhythmicity, the other half of the animals regained rhythmicity with entrained onset of melatonin production, while the offset was variable and not entrained to lights on. Ten days after the shift, the system had completely recovered and all animals regained normal rhythmicity, in phase with the new LD cycle. The results are interpreted in terms of the two-oscillator model, with one oscillator reacting with a phase advance and the other with a phase delay to adapt to the phase shift.
Data presented in this chapter are published in the following paper:

8.1 Introduction

The complex system by which animals keep track with the daily changes in their environment, in terms of light, temperature, food availability etc., is generally referred to as "the biological clock". In mammals, the two suprachiasmatic nuclei (SCN) in the hypothalamus are the major components of this clock. The SCN are oscillating continuously and have the capability of maintaining these oscillations without any stimuli from outside. They are therefore considered as having a pacemaker function for circadian rhythms. Light information from the environment, which is transferred from the eyes to the SCN through the retinohypothalamic tract, synchronizes this pacemaker to the 24 h of the earth’s rotation. Efferent projections from this area result in the generation of a wealth of behavioral, physiological and endocrine rhythms.

From all these circadian rhythms, rhythmic production of melatonin in the pineal may be one of the most robust. Pineal metabolism shows a marked day/night variation with high levels during night-time and low levels during daytime. Although it is generally unaffected by masking conditions, it is extremely sensitive towards light and changes in the light/dark cycle.

Parameters of pineal metabolism used in circadian rhythm studies include activity of the rate-limiting enzyme in melatonin biosynthesis, N-acetyltransferase, plasma levels of melatonin and urinary excretion of the liver metabolite 6-sulphatoxymelatonin. As shown in the previous chapter, the use of microdialysis to monitor the melatonin production directly in the pineal gland is a useful new tool in circadian research. Most importantly, it provides detailed information on the phase markers of the biological clock and simultaneously information on the actual level of melatonin production.

Since melatonin rhythms are robust and closely coupled to pacemaker activity, the aim of the present study was to investigate characteristics of this pacemaker by studying the response of the melatonin profile after perturbing the pacemaker. Therefore, an 8 h phase advance shift in the light/dark (LD) cycle was applied to rats. From previous studies, it is known that such a condition of artificial jet-lag perturbs many circadian rhythms, including N-acetyltransferase activity and 6-sulphatoxy-melatonin secretion. The time dependence of the effects was investigated by recording the melatonin profile 2, 5 and 10 days after the phase shift. The high time-resolution of the microdialysis technique used may reveal specific characteristics of the pacemaking system. This is of particular interest during the reentrainment to assess temporal characteristics of the reentrainment process.
8.2 Experimental setup

Animals were used as described on page 58. Three groups of 10 rats were housed individually. They were adapted to a 12:12 LD cycle (lights on from 10.00 h until 22.00 h) for a period of two weeks. Then the cycle was 8 h phase advanced by shortening the day period (day 0). In the three groups, a microdialysis probe was implanted as described on page 59, on day 1, 4 and 9 after the phase shift respectively. On the day following the implantation (day 2, 5 and 10), microdialysis started and melatonin was measured for at least 20 h, as described on page 63.

To describe both the circadian time point of increase and decrease of melatonin levels in a quantitative way, two phase markers, $IT_{50}$ and $DT_{50}$ were calculated as described on page 70.

8.3 Results

■ Control situation

In previous studies the nocturnal melatonin production has been investigated (chapter 7). It appeared to be a pronounced rhythm, in which melatonin production started to increase about two hours after lights off, reaching a plateau value two hours later, which was 15-fold higher than normal basal levels. The $IT_{50}$, reported for this control curve was $2.9 \pm 0.5$ h. The high level of melatonin production remained constant throughout the night and started to decline rapidly about one hour before lights on, resulting in a $DT_{50}$ of $-1.0 \pm 0.2$ h.

![Figure 8.1](image-url) Melatonin profile two days after an 8 h phase advance. Melatonin is expressed as percentage of average daytime level and is presented as the mean ± S.E.M. ($n = 7$).
Figure 8.2 Melatonin profile five days after an 8 h phase advance. The upper panel (A) shows the animals that were not rhythmic in their melatonin release (n=4). The lower panel (B) shows the rhythmic animals (n=4). Melatonin is expressed as percentage of average daytime level and is presented as the mean ± S.E.M.
**Two days after the phase shift**

In Fig. 8.1 the effects of an eight hour phase advance of the LD cycle are shown after two days. No increase in melatonin production was seen throughout the experiment, which lasted for 20 h. Also in the remaining 4 hours of the 24 h cycle, no changes in melatonin production were detected (data not shown). The average absolute output of melatonin was $18.5 \pm 6.1$ fmol/sample (20 min). The lack of rhythmicity made it impossible to calculate $IT_{50}$ and $DT_{50}$ values.

**Five days after the phase shift**

On day five some animals did not show any sign of recovery from the phase shift. Four animals lacked any rhythmicity in their pineal melatonin production (Fig.8.2a). However, four other animals did show rhythmicity (Fig.8.2b). The calculated $IT_{50}$ value was $2.5 \pm 0.3$ h, which was comparable to the control situation. At $t= 4.3$ h, the maximum melatonin production was reached at a level of $1560 \pm 148 \%$. From that moment on a gradual decrease in melatonin output was recorded, reaching basal levels again at $t= 9.0$ h ($159 \pm 92 \%$), which was 3 hours before lights on. Since the decline was gradual and showed variations between individuals, the $DT_{50}$ values showed a substantial variation. The average $DT_{50}$ value was $-4.4 \pm 0.7$ h, which was a significant phase advance compared to the control situation. The average basal level in absolute amounts was $18.2 \pm 11.3$ fmol/sample (20 min).

Interestingly, the amplitude at the onset of melatonin production was similar to control conditions, with rather small standard errors. This indicated a fast reentrainment of onset and made reappearance of melatonin production as a gradual process unlikely. Support for this on/off characteristic of onset of melatonin production comes from one animal.

**Figure 8.3** Melatonin profile ten days after an 8 h phase advance. Melatonin is expressed as percentage of average daytime level and is presented as the mean ± S.E.M. (n = 5).
that was measured on day 4 and 6 after the shift (data not shown). On day 4, the animal was not rhythmic while on day 6 a rhythm was recorded similar to the average rhythm on day 5. The offset of melatonin production was accompanied by large variances in amplitude. This indicated a slow reentrainment of offset with gradual changes.

**Ten days after the phase shift**

A fully restored rhythmicity was achieved in all animals ten days after the phase shift, (Fig. 8.3). Melatonin levels started to increase after about 1 h and reached a plateau at $t = 3$ h. Levels remained elevated until $t = 10.7$ h. Then they dropped down within 40 minutes to basal. The $\text{IT}_{50}$ ($1.9 \pm 0.1$ h) showed a tendency to be somewhat smaller, but the difference compared to the control $\text{IT}_{50}$ was not significant. The $\text{DT}_{50}$ value was $-0.9 \pm 0.2$ h, exactly in the range of the control situation. Absolute output of melatonin at basal level was $26.0 \pm 11.2$ fmol/sample (20 min).

A bar graph representing an overview of the phase markers $\text{IT}_{50}$ and $\text{DT}_{50}$ under the various circumstances is given in Fig. 8.4.

![Bar graph representing the phase markers $\text{IT}_{50}$ and $\text{DT}_{50}$ under control conditions (Ctrl.) and 2, 5 and 10 days after the phase shift. Control values are taken from chapter 7, page 137. On day 2, there was no rhythmic, therefore the bar is set at 0. On day 5 only the rhythmic animals are represented. Data are presented as mean ± S.E.M. Asterisks (*) indicate significant difference (P < 0.05) from control.](image-url)

8. Eight hour phase advance reveals new characteristics of underlying pacemaker
8.4 Discussion

The 8 h phase advance in the LD cycle caused complete disappearance of rhythmicity in melatonin release for at least two days in all animals, and for at least five days in half of the rats. Such arrhythmicity could be due to arrhythmicity of the underlying pacemaker, or to desynchronisation of elements in a more complex pacemaker system. Locomotor activity in similar situations, normally maintains its rhythmicity and gradually adjusts to the new LD cycle via phase delays. Also the sleep/wake cycle in terms of brain activity remains rhythmic, although the amplitude is damped. These observations suggest that there is still a rhythmically active underlying pacemaker and makes a desynchronisation of elements within that pacemaker the most likely explanations.

On day 5, partial recovery of rhythmicity was achieved in two ways. First, about half of the animals were rhythmic, the other half were not. This is probably due to variations in the endogenous biological clock system among individuals, which can be substantial. Second, while onset of melatonin production was fully reentrained, its offset had phase shifted over ± 12 h, more than the phase shift in the LD cycle. Such an intermediate state between arrhythmicity and rhythmicity could reveal important information on the mechanisms responsible for the observed processes.

- One oscillator

Assuming that the underlying pacemaker consists of one oscillator, based on activity measurements both onset and offset of melatonin production would phase delay to the new LD cycle. Damping of the oscillator and direct masking effects of light could well explain the lack of rhythmicity on day 2. However, on day 5 the situation would become more complicated. The offset of melatonin production would keep experiencing the direct masking effects of light until entrained to the new LD cycle. During the transient period, it may be questionable whether pacemaker induced stimulation of melatonin production at any time during the circadian cycle is possible when the onset is masked. Even if this would be possible, production would start right after lights off and result in an IT_{50} value of ± 1 h. This is in contrast to the IT_{50} value actually measured that indicate a normal entrained onset of melatonin production. These considerations make the hypothesis of one oscillator regulating melatonin production unlikely.

- Two oscillators

Pittendrigh and Daan introduced the hypothesis of a pacemaker consisting of two coupled oscillators. Their proposal of an evening oscillator (E) and a morning oscillator (M) was based on the splitting of activity rhythms in hamsters under constant conditions. Later, Illnerova and Vanecek explained the different sensitivity of onset and offset of N-acetyltransferase activity towards light pulses with this model of two coupled oscillators. During the past years, the possible existence of more than one oscillator has frequently been proposed. Pinealectomy appeared to uncouple two oscillators and therefore increase the number of ultradian components in constant light (LL). Exogenous melatonin showed to be efficient in coupling the desynchronized oscillators.
A unilateral lesion of the SCN in hamsters resulted in abolishment of splitting, which could be evidence for an anatomical localisation of one oscillator in each SCN. Recently, Shinohara et al. demonstrated different periods for arginine vasopressin and vasointestinal peptide rhythms in SCN cultures in vitro, indicating the presence of different functional oscillators.

Figure 8.5 A schematic representation of the effects of an 8h phase advance on both E and M (left panel) and the resulting melatonin profile (right panel). Grey areas represent the dark period. Under normal LD conditions, E controls the evening rise in melatonin and M controls the morning decline. After an 8h phase advance in the LD cycle, initially the melatonin production is actively suppressed by the light, resulting in a flat profile on day 2. Directly after the phase shift, E starts to phase advance (---) to the new LD cycle. It takes approximately 5 days for E to reach the new position, which is a distance of 8h. In the mean time, M starts to phase delay to the new position (- - -), which is at a distance of 16h and takes about twice as long. During these couple of days after the shift, the inverse relation between E and M during the dark period, together with the active suppression of light, cause the melatonin profile to remain flat. On day 5, E has entrained the onset of melatonin, while M is at sufficient distance to allow melatonin to be produced. It takes until day 10 before both onset and offset are entrained and the coupling between E and M are re-established.

A unilateral lesion of the SCN in hamsters resulted in abolishment of splitting, which could be evidence for an anatomical localisation of one oscillator in each SCN. Recently, Shinohara et al. demonstrated different periods for arginine vasopressin and vasointestinal peptide rhythms in SCN cultures in vitro, indicating the presence of different functional oscillators.
It is generally believed that melatonin acts as an internal “Zeitgeber” and synchronizer. Under conditions of continuously low melatonin production, as is the case in the present study, that synchronization fails and the two oscillators may phase delay and/or advance independently from each other. This principle is the basis for a model that describes the results from the present study.

**New pacemaker characteristics, a two oscillator model**

Under normal conditions, the melatonin profile is maintained by an evening oscillator, which we will refer to as E, according to the model of Pittendrigh and Daan, and a morning oscillator, indicated by M. After a phase advance of the LD cycle by 8 h, both E and M will gradually move to the new position, however in opposite directions (Fig. 8.5). E phase advances to the new situation while M apparently phase delays. The first day after the shift, it is obvious that the melatonin levels do not increase, because the light directly suppresses melatonin production during most of the former night period. However, also during the following days, melatonin levels will not show rhythmicity, because E and M have either an inverse phase relationship, or are too closely coupled. After 5 days, E has reached the correct position, while M is still phase delaying to the new onset of light. This results in a melatonin profile which is entrained to the onset of darkness, but not to the offset. After 10 days, both E and M have reached their correct positions. This is reflected in a normal shape of the melatonin profile. The tendency of E to be somewhat phase advanced to control conditions could be in favour of the phase advancing property of E. Furthermore this could indicate that the coupling between E and M is not fully restored, a process which might take longer than 10 days. Assuming that E and M phase shift with about the same speed, it takes M twice the time compared to E to arrive, because the phase shift for M is 16 h, while E only shifts 8 h. This time relationship correlates with the fact that E is entrained within 5 days, while M is entrained in 10 days.

In this model, it is assumed that E phase advances and M phase delays. Although this may indicate that advancing or delaying are properties of the oscillators, in terms of their individual tau (τ) period, this need not be the case, because the experiments are performed in an LD cycle. Illnerova et al.\textsuperscript{132} reported the effect of an 8 h phase advance on N-acetyltransferase activity. Similarly, they reported lack of rhythmicity the first days after the shift. Although their time-resolution was only about 2 h, close examination of the data suggests that upon reappearance of the N-acetyltransferase activity M was entrained earlier than E. In contrast with our studies, they applied the phase shift by shortening one dark period, while in the present study one light period was compressed. When such a difference in procedure could cause E and M to react in the opposite way, i.e. E by phase delaying and M by phase advancing, it would explain the data reported.\textsuperscript{32}

During the transition period from one LD cycle to the other, circadian rhythms of various parameters may either follow E or M in their rhythm, or become perturbed. Melatonin apparently gets perturbed, but locomotor activity rhythm appears to follow one oscillator. Redman and Armstrong\textsuperscript{75} have shown that melatonin can alter the direction of re-entrainment after a phase shift. They showed that following an 8 h advance by compressing one light period, rats normally respond by a phase delay to the new LD cycle. In terms of our model, rats followed M. Melatonin administration on the new dark
onset, however, appeared to reverse the direction of re-entrainment and caused the locomotor activity rhythm to phase advance to the new LD cycle. In the present model this would mean that activity follows E instead of M. Apparently activity rhythms can be maintained by one oscillator, while the melatonin profile needs both oscillators. In the actograms following an 8h phase advance reported by Redman et al., there are indications that the locomotor activity rhythm is indeed enforced by one oscillator, because the active period is shortened during the transient state. The lack of correlation between re-entrainment speed of locomotor activity and 6-sulphatoxy-melatonin excretion as reported by Kennaway could also be a consequence of this dissimilarity in responsiveness to both oscillators. Further speculations on the difference in nature between E and M may lead to the conclusion that E is melatonin sensitive, while M is light sensitive. This is supported by the fact that entrainment of circadian rhythms in constant darkness is possible by either melatonin at the onset of the subjective night or light at the onset of the subjective day. The description of two families of phase-response curves in the golden hamster could be in support of this.

In conclusion, it is emphasized that the rhythmic melatonin production bears detailed information about the underlying pacemaker(s). Variations in its pronounced rhythm can reveal changes of individual oscillators. These details can best be studied using a high sampling frequency and on-line melatonin assay as is the case with in vivo microdialysis in conscious rats.
Eight hour phase advance reveals new characteristics of underlying pacemaker