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Integration

NATURAL ENTRAINMENT

Entrainment is the key functional property of circadian rhythms. Research on circadian systems has primarily focused on self-sustainment of circadian rhythmicity in DD, although this is only a derived property of a mechanism evolved under entrainment (Roenneberg et al. 2003a). Circadian rhythms are entrained by both instantaneous phase shifts and velocity changes of the circadian pacemaker in response to light. The direction and magnitude of both phase shifts and velocity changes are phase dependent. They may or may not eventually turn out to be part of a single, temporally decaying response, with the instantaneous phase shift just being the first cycle part of the longer-term response.

Recent studies from our lab have shown that both the instantaneous shift and the longer term τ response play a functional role (Hut et al. 1999; Beersma et al. 1999; Daan 2000). The optimal ratio of these responses may well vary between diurnal animals (exposed to erratic light signals, such as in humans; see chapter 2) and nocturnal animals (often shielded from the light in daytime, *e.g.*, by burrowing behaviour). The long-term changes in τ may well be the product of a mechanism integrating erratic light signals over time, canceling out all the minute by minute variations to which diurnal animals in particular are exposed (Hut et al. 1999). This would allow the system to listen much less to behaviour-induced (burying!) and other changes in light intensity. Whether a form of feedback exists between behaviour and light input for the circadian system remains to be tested (ultimately in a 'yoked control' experiment). The ability to integrate light information will in general prevent perturbations of the circadian clock by fluctuations in light intensity. The behaviour of humans in modern society induces large variations in the light perceived by their circadian systems. Yet they stay accurately entrained, and this may also be attributable to integration of light information. An entrainment simulation study on actually perceived light shows that the accuracy of human entrainment may well benefit from τ responses (see Chapter 2).

In order to test whether an imposed instead of a self-induced dark pulse is able to disturb the circadian pattern in behaviour, we exploited the solar eclipse on August 11, 1999 above a population of *susliks* or European ground squirrels (*Spermophilus citellus*) in a natural environment near Vienna, Austria (see chapter 3). This eclipse caused a sudden reduction in light intensity by over two log units. As expected, it induced no detectable changes in the circadian activity pattern of the ground squirrels. This observation, made possible by a unique natural experiment, gives credence to the interpretation that the slow average changes in light intensity in the afternoon long before sunset are the essential stimuli for entrainment in the diurnal ground squirrel. It is around this time of day that the ground squirrels completely withdraw underground, away from the light (Hut et al. 1999). Since they don't observe sunset, they can not be entrained by it. However, they might be entrained by the behaviour-induced

sudden darkness, if the behaviour, *i.e.*, retreating into their burrow, itself were induced by a light reduction. The eclipse observations show it is not.

For nocturnal mammals, the gradient of light intensity does not seem to be the key signal for entrainment. Since early on, the contention implicit in the PRC theory has been that circadian systems distinguish between dawn and dusk by their own phase at which the light signal is falling. Pittendrigh (1981) has made this explicit by postulating that the complete photoperiod in a zeitgeber could be replaced by two brief and identical light pulses at the times of prior dawn and dusk. He showed most convincingly for *Drosophila pseudoobscura* rhythms that indeed their entrainment is predictable in great detail from the assumptions of simple phase resetting in response to light pulses. The whole field has accepted this view on entrainment without questioning. Yet the fact that brief rectangular pulses yield a predictable response does not preclude that the same system would respond very differently to pulses characterized by a slowly increasing light intensity - a “dawn pulse” - from its response to a slowly decreasing light pulse - a “dusk pulse”.

I have tested precisely this question by exposing mice to a zeitgeber composed of two alternating dawn and dusk pulses 12 hours apart (chapter 4). Indeed the mice did not distinguish between these pulses, at least not in a general sense. They could be entrained with their subjective night, indicated by activity, either in the interval from the dusk to the dawn pulse, or in the interval from the dawn to the dusk pulse. Yet it is important to note that it seems to be the photons and the subjective time at which they hit the system that are crucial for entrainment, not whether there is a gradual increase or decrease.

It has been proposed that the long-term velocity responses, observed as ‘after effects’ in the circadian period τ , emerge from changing phase relationships between two component oscillators E and M (Pittendrigh and Daan 1976c). These changes would subserve the function of adjusting the seasonal program to daylength, such as documented most elegantly in the European ground squirrel at two different latitudes by observation in the field near Vienna (Everts et al. 2004) and by recording from light-sensitive radiocollars (Hut et al. 1999) reporting when the animals were above ground in large outdoor pens in the Netherlands (Figure 11.1). Indeed after-effects of photoperiod on τ have been published (7 different mammal and bird species in Pittendrigh and Daan 1976a table 3) and in mice (*Mus musculus*, Possidente et al. 1995). If τ changes are indeed the result of a different phase relationship between these oscillators, one might expect that there are differences in the extent of phase relationship changes between diurnal and nocturnal mammals, since there appear to be systematic differences in the size of their τ response (Daan 2000).

In view of the importance of the substructure of the pacemaker for functional adjustment of behavioural programs to season, as well as for their basic function in entrainment I embarked on a detailed evaluation of this substructure in part 2. This eventually became the main part of the thesis.

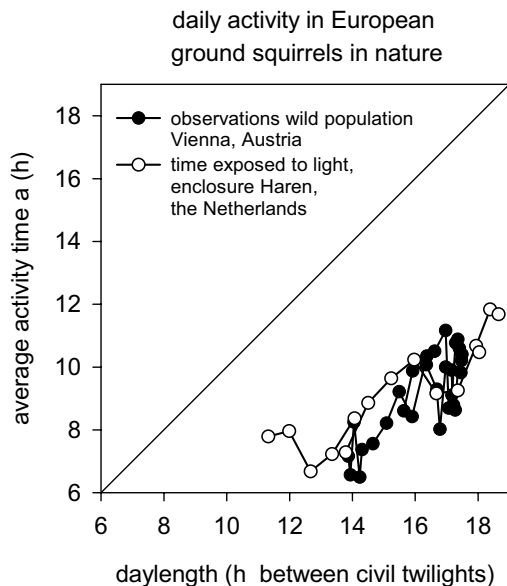


Figure 11.1

THE DUAL OSCILLATOR MODEL

Specific observations in circadian behaviour, in particular splitting of the rhythm into two different components and the compression of activity time (α) led to the formulation of the dual (EM) oscillator theory (Pittendrigh and Daan 1976c). According to this theory, the central circadian pacemaker consists of two mutually coupled oscillators, E and M. The evening oscillator (E) runs fast in darkness and is slowed down by light, the morning oscillator (M) runs slower in darkness and is accelerated by light. By these properties, E would lock on to dusk and M to dawn. However, the oscillators are also coupled to each other, and thereby subject to a force towards maintaining the same phase relationship. The resulting phase angle difference between the two oscillators (ψ_{EM}) regulates the duration of the activity time α and thus the flexible adjustment of activity to daylength. In addition, ψ_{EM} would determine the intrinsic period length of the pacemaker. A gradual change in ψ_{EM} would explain transient after-effects. The EM hypothesis would yield testable predictions for a pacemaker that has either a functional E or M oscillator. A pacemaker with only the E oscillator intact would have a shorter τ in darkness, and mainly be decelerated by light. In constant conditions with increasing light intensity, the period length should lengthen more than an intact pacemaker. A pacemaker with only a functional M

oscillator, on the other hand, should have a longer τ in darkness and be accelerated by light. In constant conditions with increasing light intensity, the circadian period should gradually shorten.

As long as a possible structure or location for E or M components is unknown, these predictions can not properly be tested experimentally. If present, the components should be sought in the mammalian *suprachiasmatic nuclei* since these nuclei retain information encoding prior daylength (e.g. Sumová and Ilnerová 1998; Jagota et al. 2000). E and M are unlikely to be involved in the phenomenon of splitting, although splitting inspired the original theory. Splitting with coupling of two components in 180° antiphase requires functional identity rather than differentiation of the components (Daan and Berde 1978b), as indeed observed in the response of split components towards light pulses (Meijer et al. 1990). Splitting has now been demonstrated to involve separation of the left and right SCN (de la Iglesia et al. 2000) (see chapter 10).

The recent unraveling of molecular genetic feedback loops involved in the generation of circadian rhythmicity, and the possibility to modify core oscillator genes in mice (van der Horst et al. 1999; Albrecht et al. 2001) led to an attempt to identify genetic components with the E-M concept (Daan et al. 2001). This hypothesis yielded a number of specific testable predictions for mice with disabled genetic core clock elements.

The first signs revealed by the phenotype of mice with mutations in clock genes were indicative for a duality in the oscillating group of clock genes. Mice mutant for one of the *Per* genes seemed to lose the ability to either phase advance or delay (Albrecht et al. 2001), and knocking out one of the *Cry* genes induced either a significant increase or decrease in the free running period (van der Horst et al. 1999). In addition, mice without functional *Per1* and *Per2* were completely arrhythmic with only masking behavior in an LD cycle (Bae et al. 2001), and so were mice without *mCry1* and *mCry2* (van der Horst et al. 1999).

Test of the model in mutant mice

Phase shifts and cFos expression

In order to investigate whether the specific properties in resetting found by Albrecht et al (2001) were not due to differences in phase angle during entrainment, and to obtain a complete picture of the phase resetting capabilities of the mutant mice, we measured a full PRC in both *Per* and both *Cry* mutants (Chapter 6). In contrast to the data on phase shifts to light pulses directly after entrainment from *Per* mutant mice (Albrecht et al. 2001), all genotypes tested (*mPer1^{Brdm1}*, *mPer2^{Brdm1}*, *mCry1^{-/-}*, *mCry2^{-/-}*, wildtype) were able to respond with both phase advances and phase delays when exposed to light pulses in freerun, albeit with clear quantitative differentiation. The most prominent difference from wildtype mice was observed in *Per2* mutants. This genotype has a PRC that is elevated in both the advance and delay region (InT 18 – 10 h) compared to wildtype mice. While these differences may not be impressive compared

to the absolute differences in phase shifts found by Albrecht et al. (2001), one should realize that a slight elevation of a PRC implies drastic changes in the way an organism responds to different light conditions. If the response to light of an intact circadian system is tuned to induce an appropriate, phase dependent phase shift, a small but structural elevation or lowering of the PRC will directly have an impact on entrainment properties.

The *cFos* induction in the SCN of *mPer* mutant mice, did not show a differential response to light in the subjective evening or morning (Chapter 7). In *mPer2^{Brdm1}* mice, light induced responses were severely reduced in both entrained and freerun conditions. If the presence of *mPer2* protein is directly responsible for a phase delay (Muñoz et al. 2005) these observations tally with the elevation of the *mPer2^{Brdm1}* PRC. In the *mPer1^{Brdm1}* in freerun no significant differences with wildtype mice are observed, neither in *cFos* expression nor in phase shifting. In entrained conditions, in contrast, there is reduced *cFos* sensitivity in *mPer1^{Brdm1}* mutants in the subjective evening, without accompanying reduction in phase shift. Clearly there was no proportional relationship between *cFos* expression and the extent of the phase shift.

The results for phase resetting in the *Cry* mutant mice are less conclusive. Both *mCry1^{-/-}* and *mCry2^{-/-}* did show phase advances and phase delays. Significant differences were restricted to the delay section of the PRC (InT 18 – 02) where *mCry1* mutant mice showed larger delays compared to *mCry* mutant mice. The *cFos* response to light pulses in the *mCry* mutant mice in entrained conditions showed that *mCry1^{-/-}* had suppressed *cFos* in the morning, *mCry2^{-/-}* in the evening. Although seemingly consistent with the EM model, we should realize that *cFos* expression does not predict phase shifts. Furthermore, there was considerable variation between the *cFos* responses in entrained and freerunning conditions. Thus the *cFos* response presumably reflects differences in the gating of the light input to the pacemaker rather than the internal machinery of that pacemaker.

τ Changes in LL

A powerful test of predictions derived from the EM hypothesis is the assessment of the circadian phenotype in constant light with increasing intensity. It was predicted originally that animals lacking a functional morning oscillator component (*Per1* or *Cry1*) should lengthen their τ more than wildtypes, and that animals lacking a functional evening component (*Per2* or *Cry2*) should shorten τ in LL. This was first tested by Steinlechner et al (2002a) who found indeed that these predictions were upheld for *mPer1* and *mPer2* mutants. Shortening τ with increasing light intensity is a remarkable phenotype, not shown by any wildtype mammal species, and in accordance with the prediction for the circadian response of an organism carrying only an intact M oscillator. In order to duplicate these important findings, and to apply the same test to the *mCry1* and *mCry2* mutant mice, we repeated the LL experiment for *mPer1^{Brdm1}*, *mPer2^{Brdm1}*, *mCry1^{-/-}*, *mCry2^{-/-}* and corresponding wildtype mice. In our setup

mPer2^{Brdm1} mice decreased, and *mPer1^{Brdm1}* mice increased period length in LL, fully confirming the results of Steinlecher et al (2002a). In order to elucidate the effects of LL on the molecular system, Muñoz et al (2005) measured transcription and protein levels of mCRY1, mCRY2, *mPer1*, *mPer2* in LL. After 50 days of constant light, mPER2 (protein) levels in the SCN of wildtype mice were constantly elevated. The authors suggested that this could be a molecular explanation for Aschoff's rule if these elevated *mPer2* levels induce persistent phase delays. In *mPer2^{Brdm1}* mice, these quantities of *mPer2* protein are not present, and therefore cannot induce these permanent delaying effects. Long term light failed to induce permanent transcription of *mPer1* or permanent high *mPer1* levels. The τ shortening effect might therefore not be attributable to the contribution of *mPer1* to the molecular clock system.

The circadian activity pattern in *mCry* mutant mice in LL was clearly different, both *mCry1^{-/-}* and *mCry2^{-/-}* increased in period length with increasing light intensity. Hence, although the DD period length of both genotypes indicates a differential function for the *Cry* genes, the LL phenotype does not indicate a function of either *mCry* gene in an E or M like oscillator.

Rhythmicity

The collection of phase responses in DD for the assessment of its PRC was complicated by reduced circadian rhythmicity in locomotor activity in all mutant mice. Of all genotypes, *mPer2^{Brdm1}* mice were most affected and suffered frequently from complete arrhythmicity. When placed in LL, however, *mPer2^{Brdm1}* mice not only retain circadian rhythmicity (Steinlechner et al. 2002a), but spontaneously regain rhythmicity when arrhythmic in locomotor activity by prior DD without intermediate entrainment (Chapter 9). Circadian rhythmicity of behaviour in *mPer1* seems to be inversely proportional to *mPer2^{Brdm1}*: *mPer1^{Brdm1}* spontaneously regained rhythmicity with decreasing light intensity after having been made arrhythmic by prior LL. The latter finding does not tally with the observations by Steinlechner et al. 2002a) who reported persistent circadian rhythmicity for *mPer2^{Brdm1}* mice in LL.

A possible explanation for the improved rhythmicity in LL in *mPer2^{Brdm1}* mice could be the absence of constantly elevated levels of mPER2 in the SCN. These may be responsible for a progressive instability of the circadian rhythm. In wildtype mice that are kept for 50 days in LL, *mPer2* is transcribed rhythmically but mPER2 levels are constantly high (Muñoz et al. 2005). Comparable, but less distinct results for expression patterns and protein levels for *mPer2* were found by Sudo et al (2003) in mice that were exposed to LL for 7 days. The *mClock* mutant is another genotype that retains and even regains circadian rhythmicity in LL, as we reported in Chapter 5 (Spoelstra et al. 2002). In these mice the SCN *mPer2* transcription rhythm is severely blunted (Jin et al. 1999). *mPer2* and *mClock* mutant mice may agree in not being exposed to high *mPer2* protein levels in LL. Additional experiments are necessary in order to reveal whether these elevated *mPer2* levels indeed lead to (behavioural) arrhythmicity.

ψ and α changes with skeleton photoperiods.

Since the EM hypothesis intends to explain the ability of organisms to adapt their activity pattern to changing day length, a logical step is to subject circadian mutants to different photoperiods. If the onset of activity is delayed by the E component and the offset is advanced by the M component in a nocturnal mammal, a system lacking either component will not be able to compress α . In LD cycles with subjective night lengths longer than 6 hours, activity is normally restricted to the dark phase of the photoperiod by masking. In order to avoid this problem, we have used a skeleton photoperiod to gradually compress α , thereby allowing activity in the subjective day (Chapter 8). Another advantage of a skeleton photoperiod that gradually compresses the subjective night is the possibility to record the minimum length of the subjective night before the circadian system 'decides' to replace the (compressed) subjective night for the (long) subjective day. When exposed to this protocol, wildtype mice indeed do compress their nocturnal activity before they break lose of the subjective night and reentrain to the preceding subjective day. *mCry1*^{-/-} shows least compression of its nocturnal activity and is tied to a light pulse in its subjective night. *mCry1*^{-/-} mice entrain to a light pulse in the subjective morning, compress their activity with the shortening of the subjective night and eventually reentrain to the previous subjective day by delaying their activity rhythm. Activity rhythms of both *mPer1*^{Brdm1} and *mPer2*^{Brdm1} eventually became too unstable as soon as the light regime was switched to a skeleton photoperiod. This experiment has therefore to be repeated with *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mice with an additional mutation in the *mCry1* and *mCry2* gene, respectively (see perspective). So, at least one mutant shows reduced α compression, but additional work is necessary.

Are knockouts useful ?

An important question is whether circadian mutants form a suitable model for testing circadian properties such as the presence or absence of E or M components. A mutation in a specific gene will affect all cells in the body where it is normally expressed. Therefore the circadian phenotype of a clock mutant may not only reflect the changed action in the master circadian pacemaker. All peripheral tissues tested appear to contain the capacity for circadian rhythmicity (Yoo et al. 2004). There is no solid evidence for effects from these peripheral clocks on the SCN. A strong peripheral clock like the food entrainable oscillator appears to have no influence on the SCN (Aschoff et al. 1982; Damiola et al. 2000; Stokkan et al. 2001). Since we can not a priori exclude such effects, however, we can not be completely sure that a circadian phenotype represents the properties of the SCN.

Sujino et al (2003) have carried out experiments in which they studied the circadian phenotype of (arrhythmic) host mice that received SCN grafts from embryonic tissue from different genotypes. This is essentially the same as the work earlier done for tau mutant hamsters by Ralph et al (1990). Circadian rhythmicity in DD in *Clock* mutant

mice which have a weak circadian pacemaker (Vitaterna et al. 1994) or in *mCry1^{-/-}mCry2^{-/-}* double knockout mice lacking both central and peripheral oscillators (van der Horst et al. 1999; Albus et al. 2002) can be rescued by mouse fetal SCN tissue. The circadian period in locomotor activity in DD of SCN-lesioned mice (wildtype or heterozygous *Clock* mutant) that received fetal SCN grafts matched the free running period length of the donor mice (Sujino et al. 2003). Hence, the period length of peripheral oscillators had no effect on the period length of the master pacemaker. The presence of robust peripheral oscillators in the host mice is apparently not necessary, since homozygous clock mutant mice with SCN grafts from wild type mice display a clear free running rhythm. The rhythm in DD of SCN lesioned double knockout *mCry1^{-/-}mCry2^{-/-}* mice without functional peripheral oscillators (Yagita et al. 2001) with SCN grafts from *mCry2^{-/-}* single knockout or wild type mice shows that the presence of functional peripheral oscillators is not required at all (Sujino et al. 2003).

SCN grafting experiments can not provide insight in the effects of mutations or defects in clock genes on the modulation of light before it reaches the SCN. The retina has its own circadian oscillator - at least in Syrian hamsters (Tosini and Menaker 1996) - that can in principle be affected by a mutation or deletion of one or more clock genes. Although there is no evidence that retinal clocks are driven by endogenous photoreceptor oscillators (Green and Besharse 2004), the input of Zeitgeber signals might be clock controlled (Morrow et al. 2003; Geier et al. 2005). At the tissue level, some evidence is available that SCN circadian rhythms are affected by the absence of intact eyes (Yamazaki et al. 2002) or functional rod photoreceptors (Lupi et al. 1999). However, data on pupil reflexes in *mCry1^{-/-}* and *mCry2^{-/-}* mice show little differences in pupil constriction compared to wild type mice (Van Gelder et al. 2003). Witkovsky et al (2003) studied the expression of *mPer1* in specific cell types in the murine retina in *Per1::GFP* mice and observed no *mPer1* expression in retinal ganglions cells projecting to the SCN.

Taken together, the current state of affairs is that despite the recently growing insight that endogenous circadian rhythmicity is found in many tissues and to a large extent independent of the presence of the SCN, the SCN still appears to be the master clock controlling and synchronizing much of the rest of the body. It further plays a major role in maintaining entrainment with the lightdark cycle, and in taking care of the seasonal changes in the circadian program. At present, there is no evidence that a circadian activity pattern in an organism with a reduced functionality of a core clock gene reflects functional changes in tissues other than the SCN.

Protocols: free-running or entrainment?

According to the EM concept described in the original hypothesis (Pittendrigh and Daan 1976c), differences in phase responses in freerun and entrainment are inevitable due to a different internal phase relationship between the two oscillators. In circadian mutants, these differences could be much more prominent if E or M is not fully

functional since this will likely induce different external phase angle differences with the Zeitgeber. That differences are present in the state of the pacemaker between entrained and freerun conditions has been predicted by the original EM hypothesis itself (Pittendrigh and Daan 1976c) and has become clear by the behavioural responses (Chapter 6) and SCN *cFos* expression (Chapter 7) to light exposure in these two conditions.

The circadian system has evolved in entrained conditions. DD is not a natural situation and the properties of the circadian system in DD may reflect derived aspects of the mechanism that has its true function in entrainment. For a realistic functional insight in the pacemaker, assessments in entrainment are therefore more effective. Phase shifts as measured after entrainment (the “Aschoff type II” protocol) face other serious practical problems that arise from the masking effects in an LD cycle. It can be difficult to assess the circadian phase if the actual onset of activity is masked by anticipation of lights-off.

Reinterpretation: dual components of a single oscillator

In a comprehensive argumentation, Roenneberg and Mellow (2003) have made clear that the circadian system may consist of several feedback loops controlled by specific and individual genes which in turn may interact with each other at different levels. In this broad view of the putative mechanism of rhythm generation the two components primarily responsible for locking on to dawn and dusk may well exist without each acting as a fully self-sustained separate oscillator. A duality in the function of a subset of genes may well have a specific E or M like function within a single group of interacting genes forming a single circadian oscillator. In this more general formulation, *Per1/Cry1* might act as a Morning component of the system, and *Per2/Cry2* as an evening component of the system, even if neither is a separate self-sustained oscillator. In Henrik Oster's experiments, a rhythm is still present in the combined *Per1/Cry1* knockout (Oster et al. 2003a) and in the combined *Per2/Cry2* knockout (Oster et al. 2002). Hence both separately have oscillatory capacity, and may be termed oscillators. Whether we speak of oscillators or merely of components, I conclude that the predictions from the model concerning PRC, LL and photoperiod so far have virtually all been upheld for the genes *Per1* and *Per2*, but not for the genes *cry1* and *cry2*. Thus it appears that *per1* truly behaves like part of a morning component in the oscillatory machinery, while *Per2* behaves like part of an evening component. There is no evidence to support the contention that *cry1* and *cry2* specifically tie up with either of the PER proteins, or otherwise specifically represent M or E components.

Spatial differentiation ?

In *Drosophila*, recent evidence demonstrates the presence of two oscillators, one regulating activity around the transition from dark to light (dawn) and one regulating activity around the transition from light to dark (dusk). These two oscillators are

located in the ventral (M oscillator) and dorsal (E oscillator) lateral neurons. Stoleru et al (2004) eliminated the functionality of both groups separately of neurons by local activation of a previously inserted cell-death gene. Elimination of the ventral lateral neurons abolished the anticipatory activity preceding lights-on, and elimination of the dorsal lateral neurons removed anticipatory activity preceding lights-off. In *dPer* mutant flies, rescue of expression in ventral lateral neurons restores the anticipation of lights-on, and rescue of expression in both ventral and dorsal lateral neurons restores anticipation to both lights-on and lights-off (Grima et al. 2004). In *cry^b ss¹* flies, *Drosophila* circadian rhythms dissociate in a fast and a slow component in LL. PER expression in the dorsal lateral neurons correlates with the slow period component, and PER expression in the ventral lateral neurons correlates with the fast component (Yoshii et al. 2004). Thus, both oscillators are clearly present in *Drosophila*, and are spatially separated in different groups of neurons. Stoleru et al (2004) also provided evidence that the morning and evening oscillators in *Drosophila* employ different genes: PDF and CRY, respectively.

If, in the mammalian circadian system, specific genes are differentially affected by light perception, these genes may induce spatial differences in the activity of pacemaker cells within the SCN. Clear differences exist between expression patterns and output factors between the ventrolateral SCN (core) and dorsomedial SCN (shell; e.g. Yan and Silver 2002; de la Iglesia et al. 2004; Hamada et al. 2004). There are no indication for a possible role of SCN subregions in tracking dusk and dawn. The two peaks in multi-unit activity (MUA) that follow dusk and dawn in horizontal slices in hamster (Jagota et al. 2000) are observed in single derivations in (horizontal) SCN slices. Possible E and M like properties in circadian behavior of SCN subregions might only be found if slices are studied that are prepared in a different orientation or if differences are studied in consecutive slices from the dorsal towards the caudal part of the SCN. As the *Drosophila* situations shows, a duality in the function of specific genes does not exclude a spatial organization of possible E and M oscillators.

PERSPECTIVE

At the end of this thesis I wish to list some of the major questions looming ahead in the functional analysis of rodent circadian pacemakers in relation to the E/M system.

(a) The first is the question of **functionality**. The exploitation of an Evening - Morning system may have clear consequences both for the flexible adjustment of the endogenous daily program to the external daylength, but also for the measurement of the time of year in seasonal reproduction (Pittendrigh and Daan 1976c). One might expect that strictly seasonal animals require a tighter locking on of two components to dawn and dusk, respectively than year-round breeders. Thus, a comparative approach

to the study of functional oscillator structure will be very important in the future. In this context recent findings in a strictly seasonal species, the Djungarian hamster, which even changes coat colour in response to daylength (Kuhlmann et al. 2003), are of particular interest. Steinlechner et al (2002b) exposed Djungarian hamsters (*Phodopus sungorus*), entrained to a 16:8 light/dark cycle to light pulses in the subjective night. Light pulses in the beginning of the subjective night induced α compression by a delay of the onset of activity (measured by body temperature) and an advance of the offset. A light pulse in the end of the subjective night induced α compression by an advance in the offset only. When the latter light pulse was followed by a light pulse early in the subjective night, profound arrhythmicity could be induced that lasted up to several months. Since this arrhythmicity is observed in body temperature, melatonin rhythm and activity it is likely that the central pacemaker has become arrhythmic itself. It would be highly interesting to combine Steinlechner's (2002b) experimental setup with electrophysiological measurements as done by Jagota (2000) to assess whether indeed ψ_{EM} is changed to a totally dysfunctional distance such that arrhythmicity ensues. It may be true that the more an organism is dependent on seasonal timing, the better it would be to have a weak internal coupling of both oscillators in order to keep sufficiently track of changing day length. A laboratory species such as *Mus musculus* possibly has lost much of its seasonal adjustment through unavoidable artificial selection against short day suppression of reproduction. The strong focus on genomic analysis on such species may well hamper progress in our insight. There is a strong need for comparative approaches in different animal species. The assessment of the capacity of different species to adapt their behavior to different day length will clarify whether this function is actually preserved. Especially such insights are required for the ancestor of our lab mice, the domestic house mouse (*Mus musculus*).

b) The ability of α **compression** and expansion - although crucial for the functional interpretation of a dual pacemaking system - in mice mutant for circadian clock genes is unfortunately not clear yet for the *mPer* mutant mice (chapter 8). We faced the drawback that *mPer1* or *mPer2* single mutants even when rhythmic have a very scattered circadian rhythm in locomotor behaviour, without clearly discernible and measureable activity time. Therefore we need to measure α compression in these mutants using a different protocol. The use of the double mutant *mPer-mCry* mice that show the same phenotype in phase resetting as the single *mPer* mutant mice (Oster et al. 2002; Oster et al. 2003a) in these experiments may turn out to be useful, since the *mPer2^{Brdm1}mCry2^{-/-}* mice have a much more stable circadian rhythm than *mPer2^{Brdm1}mCry2^{+/+}* (Oster et al. 2002).

d) Finally, the advent of **Genomics** now allows a wholly new series of questions to be asked. We have only embarked on a very preliminary attempt towards integration of genomic manipulations and formal circadian analysis. The new possibilities are bewildering. One issue is the potential for whole genome analysis of circadian timing

of gene transcription. By the use of DNA chip arrays, Panda et al. (2002a) have shown that many genes undergo strong circadian oscillations in transcription. Of the genes, transcribed in the SCN a majority has peak expression in LD 12:12 either around External Time 16 (ExT16) or ExT04. Their expression thus appears to anticipate lights-off (ExT 18) or lights-on (ExT 6). Whether the transcription of these genes is phase locked to dawn and dusk under naturally changing photoperiodic regimes remains to be seen. Those genes that will, will be strong candidates for further leads towards understanding the adaptive physiology in the face of change in daylength.